# Wnt signaling in human colonocytes: roles for the tumor suppressor APC in β-catenin cytoplasmic retention and destruction complex localization

By

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Taybor William Parker

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**Wnt signaling in human colonocytes: roles for the tumor suppressor APC in β-catenin cytoplasmic retention and destruction complex localization**

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**Abstract**

The Wnt signaling pathway is remarkably conserved across all metazoans. This pathway coordinates a large variety of processes involved in development, establishment of cell polarity, maintenance of tissue homeostasis, and promotion of cellular proliferation. Intriguingly, components of the Wnt pathway are frequently altered in diseases such as cancer and result in aberrant Wnt activation. In the case of colorectal cancer (CRC), mutation to the Wnt antagonist *Adenomatous Polyposis Coli* (*APC*) occurs in over 80% of human CRCs resulting in expression of a truncated form of APC. The APC protein is a component of the cytoplasmic β-catenin destruction complex which functions in the absence of Wnt ligands to phosphorylate β-catenin, marking it for ubiquitination and proteasomal degradation. However, Wnt ligand engagement with cell surface receptors inhibits the β-catenin destruction complex through an incompletely understood mechanism. Functions of APC in normal destruction complex activity and in Wnt signal transduction have remained elusive. To better understand how Wnt-induced destruction complex inhibition occurs and whether APC has a role, I utilized Wnt3a-Dynabeads to study the localization of the destruction complex in response to Wnt. In this work, I found that the β-catenin destruction complex re-orients following Wnt exposure toward a localized Wnt cue in colon epithelial cells of normal and malignant origin, and that this process occurs in an APC-dependent fashion.

Mutation of *APC* has been theorized to act in a "just-right" manner, in which specific regions of the APC protein are retained in order to allow a specific level of β-catenin activity that is optimal for tumor growth. My data also provides support that less frequently occurring β-catenin mutations may work in a "just-right" manner as well. I find that a mutation thought to "stabilize" the downstream Wnt pathway effector, β-catenin, does not interfere with the ability of

cells to respond to Wnt and find that APC loss results in further accumulation and nuclear translocation of this stabilized β-catenin.

Kinetic analysis of the destruction complex response to Wnt reveals that components of the complex can traffic to the Wnt-receptors immediately, suggesting that this trafficking is an initial Wnt response. I also find that APC loss impairs cells ability to accumulate β-catenin in response to Wnt and that β-catenin degradation is compromised in these cells.

The mechanisms by which APC functions in the destruction complex to regulate β-catenin are not completely understood, nor are the reasons why certain regions of APC are commonly lost or retained due to truncation in CRC. In this work, I find that full-length APC is required for Wnt-induced complex localization and propose that regions found within the frequently lost C-terminus of APC mediate destruction complex trafficking and inhibition. These findings provide key mechanistic details to better understand and therapeutically target the Wnt signaling pathway.

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# Chapter 1

# Introduction

## Wnt Signaling: Discovery, Development, and Disease

### *The Wnt Signaling Pathway*

Multicellular organisms rely on many signaling pathways and gene expression programs to correctly develop, form various tissues, regenerate tissues upon injury, and maintain homeostasis at the adult stage. The Wnt signaling pathway is heavily conserved across metazoans and is essential for cellular proliferation, polarity, developmental cell-fate determination, tissue homeostasis, body plan organization, and spatial-patterning of developing animals [1,2]. Nearly 40 years since the discovery of the first Wnt, many of the key components, downstream effects, and regulatory mechanisms of the pathway have been identified. However, some questions have yet to be answered and further investigation into the mechanistic underpinnings of the pathway could provide additional insights into disease progression, aid in disease diagnosis, and likely open new avenues for therapeutic targeting.

Wnt glycoproteins are the key signaling molecules in the Wnt pathway. There are 19 known members of the Wnt family [3]. These proteins are around 40 kDa, cysteine-rich, and are secreted due to several post-translational modifications [4]. Wnt proteins undergo N-linked glycosylation and lipid modifications involved in either the secretion of Wnt or the binding of Wnt to its receptor [5–7].

Wnt signaling, often termed Wnt/β-catenin signaling for its downstream effector, relies on the precise regulation of β-catenin levels to control pathway activity. The canonical pathway, which will be the primary discussion of this work, is typically in an "OFF" state unless a Wnt ligand is present (Fig. 1.1A). In the OFF-state, a cytoplasmic "β-catenin destruction complex" regulates the level of β-catenin by sequestering, phosphorylating, and targeting β-catenin for

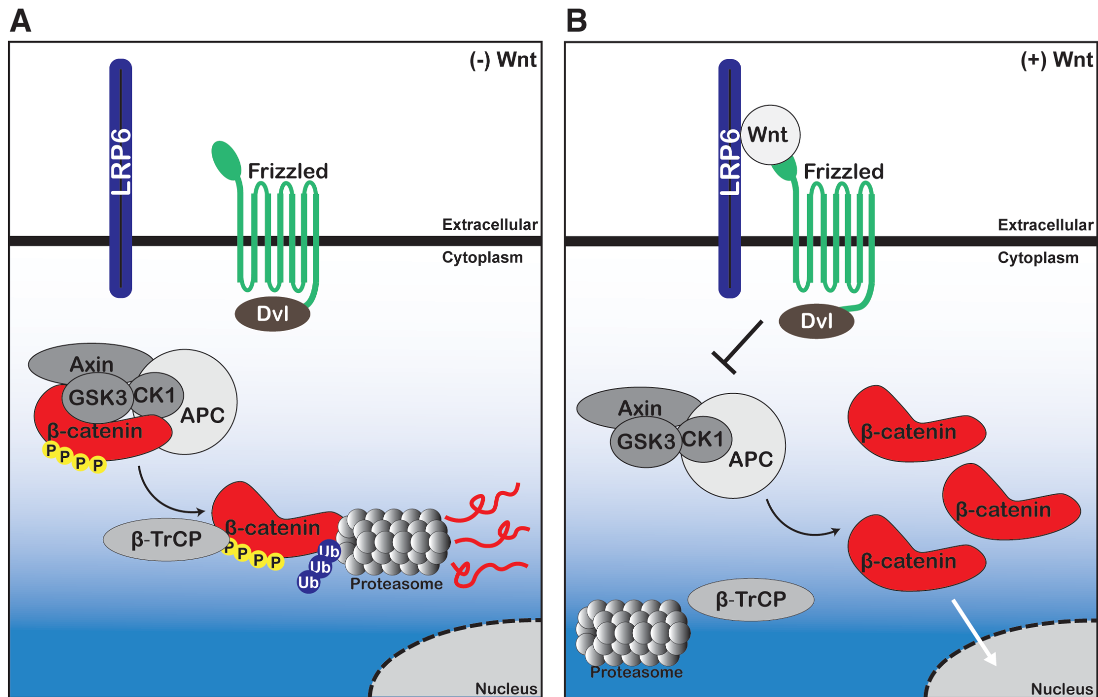



**Figure 1.1** *The Wnt Signaling Pathway.* (A) In the absence of a Wnt ligand, the Wnt signaling pathway is in an OFF-state. A cytoplasmic β-catenin destruction complex composed of APC, Axin, CK1-α, and GSK-3β binds and phosphorylates β-catenin. Phosphorylated β-catenin is ubiquitinated by β-TrCP resulting in its proteasomal degradation. (B) Binding of a Wnt ligand to the Frizzled and LRP5/6 co-receptors results in inhibition of the β-catenin destruction complex allowing β-catenin to accumulate and translocate to the nucleus where it is a co-transcription factor for Wnt target genes.

ubiquitination by β-TrCP which ultimately leads to proteasomal degradation [8–10]. The destruction complex is composed of the core components APC, Axin, CK1-α, and GSK-3β, however it is speculated that other proteins are also involved [11]. The APC (Adenomatous Polyposis Coli) protein is a large, multidomain protein with no known enzymatic activity. APC contains multiple protein binding sites and is thought to act as a scaffold for the complex. APC and its roles in Wnt signal regulation will be the focus of this dissertation and will be described in more detail later in this chapter.

Upon Wnt ligand binding to the Frizzled (FZD) and low-density lipoprotein 5/6 (LRP5/6) co-receptors, a heterotrimeric "signalosome" is formed and results in inactivation of the β-catenin destruction complex through a mechanism involving an adaptor protein, Dishevelled (Dvl) [12,13]. The β-catenin protein, when complexed in the nucleus with TCF/LEF, is a transcriptional coactivator of Wnt target genes [14,15]. Wnt presentation and destruction complex inhibition allow β-catenin to accumulate and translocate to the nucleus where it activates the Wnt-transcriptional program (Fig. 1.1B). While the basic mechanism and downstream effects of Wnt signaling have been well characterized, the molecular events leading to destruction complex inhibition are not completely understood. Further, determination of the exact roles of APC in normal destruction complex function have been difficult to elucidate and are a focus of this dissertation.

### *An int-roduction to Wnt: Discovery and naming of Wnt*

Prior to any knowledge of the Wnt signaling pathway and its crucial roles across many areas of biology, scientists had discovered that different laboratory mice had high instances of mammary tumors. Ultimately, it was found that the tumor phenotype was passed from mother to

offspring by a retrovirus contained in the breast milk, termed Mouse Mammary Tumor Virus (MMTV). Over 40 years passed before Roel Nusse and Harold Varmus initiated a screen utilizing MMTV to identify new genes which may confer a tumorigenic growth advantage when activated by MMTV retroviral insertion [16]. They identified the proviral MMTV in multiple mammary tumors isolated from different mice inserted near the same gene which they termed *int1*, for integration site 1 [16].

For several years following the discovery of *int1*, the function of the int1 protein remained unknown due to a lack of established tools. Clues to the function of int1 were provided by the protein coding sequence. The *int1* gene coded for a 370 amino acid protein that interestingly had an N-terminal region composed of primarily hydrophobic amino acids [17]. This finding supplied the initial clue that int1 may have some interaction with the cell membrane, however the overall function of this protein was largely unknown. Ultimately, the cloning of the int1 homolog in *Drosophila* provided the initial clues of potential function of the int1 protein.

Following the cloning and mapping of the *Drosophila* int1 gene, it was discovered that int1 was the homolog of a *Drosophila* gene named *Wingless* (*Wg*), identified by Sharma and Chopra for its phenotype of absent wings in *Wingless* mutant flies [18,19]. Interestingly, *Wg* was already known to be heavily involved in developmental processes in *Drosophila*, along with a host of other genes known to regulate embryonic development [20]. As several unrelated genes were also becoming identified by MMTV proviral insertion, the field was quickly becoming cluttered with other *int-* genes. In an effort to deconvolute the *int1*/*Wg* field, several of the early researchers in the field agreed upon a new nomenclature.  Paying homage to the originally identified *int1* and the *Drosophila Wingless*, these genes would be termed *Wnt* [21]. We now know that vertebrates have 19 different *Wnt* family members, and that the Wnt pathway is highly

conserved across all metazoans (reviewed in [1]). Initial evidence demonstrating the connection of *int1* to evolutionarily conserved developmental pathways came when McMahon and Moon found that injection of *int-1* RNA into *Xenopus laevis* embryos resulted in dual axis formation [22]. Subsequent studies shed light on the role of the Wnt pathway in human cancers and disease.

***The Wnt pathway in Development and Disease***

Early findings in various model organisms such as mice, *Drosophila* and *Xenopus laevis* revealed that the Wnt signaling pathway was evolutionarily conserved. Wnt signaling is crucial to the development of many organs and systems, including the brain, eye, spinal cord, bone, cartilage, skin, lung, teeth, mammary gland, gastrointestinal organs, heart, liver, kidney, pancreas and the hematopoietic and reproductive systems [1,23]. Not only is the Wnt pathway implicated in developmental programs, but also in maintenance and homeostasis of the adult intestine, hematopoiesis, and the hair and skin [24–27]. It comes as no surprise that a signaling pathway heavily involved in cell proliferation and cell-fate decisions is often deregulated in diseases such as cancers, fibrosis, metabolic disease, and neurodegenerative disorders [28–35]. Heavily involved in tissue regeneration, Wnt signaling is implicated in the repair of lung and liver damage [36–38]. Wnt signaling is also crucial for cellular processes such as cell migration, maintenance of genome stability, and apoptosis in various tissue and cell types [39–45].

***Therapeutic targeting of the Wnt pathway***

The Wnt pathway is crucial to early embryonic development and tissue homeostasis throughout the lifespan. Given the enormous range of functional involvement of the Wnt pathway in developmental and homeostatic processes, it is essential that the pathway is highly

regulated. The involvement of Wnt signaling in disease has resulted in a significant investment in the development of therapeutic approaches targeting this pathway (Fig. 1.2). Complicating matters, there are 19 different Wnt proteins and multiple co-receptors that can act in different combinations to elicit a Wnt response [46]. Further, the β-catenin-dependent pathway is referred to as the "canonical" Wnt pathway, however another "non-canonical" pathway exists that is involved in maintenance of planar cell polarity and therefore referred to as the Wnt/PCP pathway [47]. For these reasons, therapeutic targeting efforts toward the Wnt pathway have been problematic. The complexity of the pathway involving many protein interactions and a known role in normal homeostasis and stem cell maintenance means that precise modulation of the pathway must occur in such a way so as to affect the diseased cells and tissues with minimal harm to the rest of the body. To achieve such targeting, the mechanistic details of Wnt signaling must be understood in great detail.

Despite the inherent risks of targeting the Wnt pathway, the reward of successful therapeutic development could be significant. The ancient story of the "Sword of Damocles" is commonly used as a metaphor for Wnt pathway targeting [48]. The story goes that Damocles was jealous of the lifestyle of his king, Dionysius. Dionysius offered to trade places but had a very large sword suspended only by a single hair of a horse tail placed above the throne to represent the constant danger and fear that came with being king. The same goes for the Wnt pathway--with any targeting approach, whether the pathway is activated or inhibited, there exists inherent danger that the side effects involving normal tissue homeostasis and stem cell maintenance could be critically impaired.

Many groups have attempted to target the Wnt pathway through the use of small molecules, peptides, blocking antibodies, and the use of combination therapy with conventional

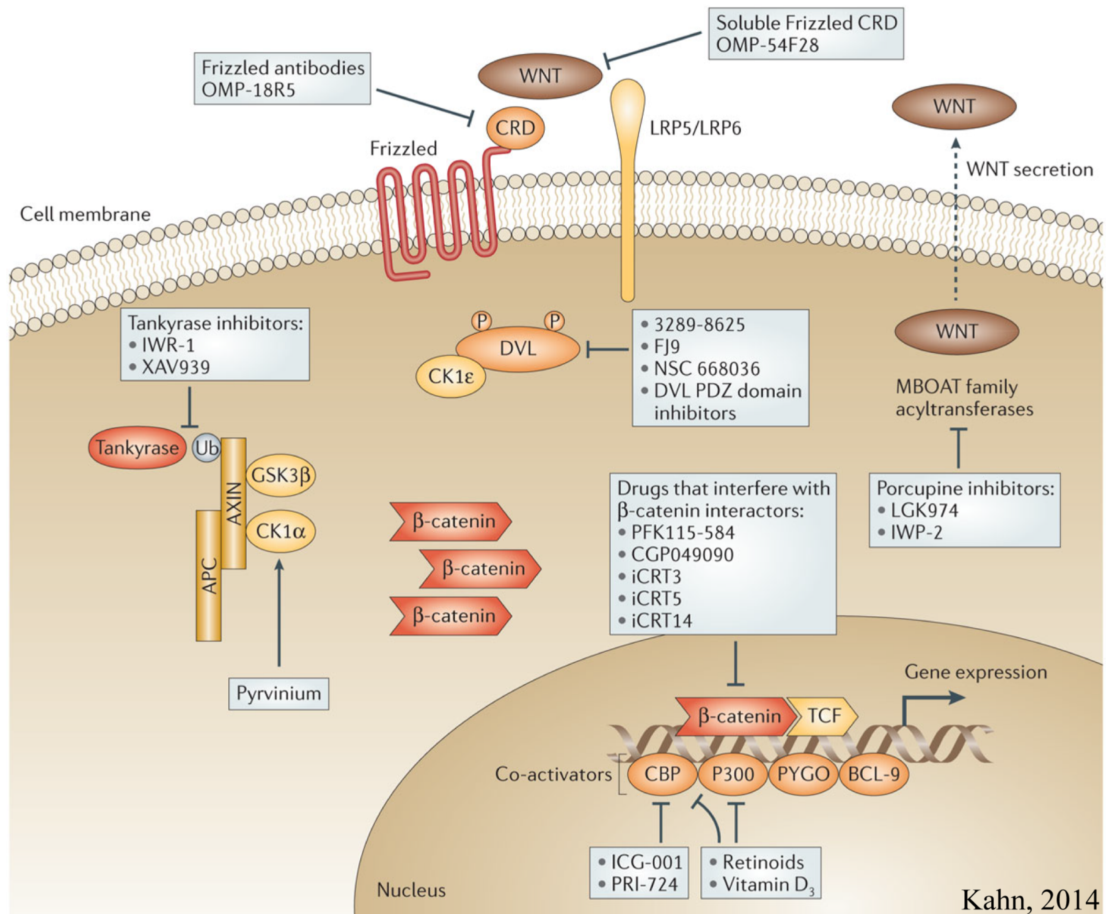



**Figure 1.2** *Therapeutic targeting of the Wnt pathway.* Identified inhibitors of various steps along the Wnt pathway. Graph from Kahn, *Nature Reviews,* 2014.

chemotherapeutics [28]. As summarized in Figure 1.2, many inhibitors to various components of the pathway have been identified. These include Frizzled blocking antibodies (OMP-18R5), a soluble Wnt-sponge containing the Frizzled cysteine rich domain (OMP-54F28), inhibitors of the adaptor protein Disheveled, inhibitors of the Wnt secretion protein Porcupine, and inhibitors of Tankyrase, an enzyme which promotes Axin degradation [49–55]. While there are additional therapeutics that have been identified (reviewed in [28,48]), the overwhelming complexity of the pathway has made effective targeting difficult. Even the decision of whether Wnt antagonists or agonists should be used depends on the type of cancer since specific tissue types may need increased Wnt activity rather than an inhibition of aberrant activation [48]. These reasons, paired with holes in our understanding of the mechanistic steps of Wnt signal transduction, make it glaringly clear that further details are needed of tissue-specific Wnt-activation mechanisms and how these are perturbed in diseases such as CRC.

## Colorectal Cancer and the Identification of APC

### *Global and U.S. Cancer Burden*

Both globally and in the United States, cancer is an enormous burden on human life. In the U.S. alone, over 1.8 million new diagnoses and over 600,000 deaths are expected to occur in 2020 [56]. Behind only heart-disease, cancer is the second-leading cause of death in the United States. Despite the decreasing rates of cancer-related mortalities, data such as this highlight the importance of novel prevention and treatment strategies as well as a continued commitment to better understanding the mechanistic underpinnings of various cancers.

### *Colorectal Cancer*

Colorectal cancer (CRC) is the second most common cause of cancer-related death in the United States [56,57]. While genetic and molecular underpinnings of colorectal cancer are incredibly important, and are the focus of this work, it is estimated that over one-half of cases could potentially be preventable through decreased smoking, increased physical activity and body-weight management, and implementation of healthier diets [58]. Projections estimate that ~148,000 new CRC cases will be diagnosed in 2020 with an estimated 53,000 deaths in the U.S. [57]. If diagnosed at an early stage, CRC survival rates are high with over a 90% 5-year survival in patients with localized CRC. However, the 5-year survival rate in patients with distant-stage disease drops to 14% [57]. These data highlight the incredible importance of early-screening and detection, but also highlight that more effort needs to be placed on understanding the molecular and genetic events involved in the initiation and progression of CRC to develop and implement more effective treatment interventions for patients presenting with late-stage disease.

### *Discovery of Adenomatous Polyposis Coli*

Patients with Familial Adenomatous Polyposis (FAP), an inherited form of CRC, commonly present with hundreds to thousands of colon polyps and develop CRC by age 39 [59]. FAP is the second-most common inherited CRC syndrome and originates through germline mutations in the *Adenomatous Polyposis Coli (APC)* gene. While FAP only accounts for ~1% of all CRC cases, the sporadic mutation of the *APC* gene is involved in the majority of CRC cases [60–62]. Further, it was the study of FAP that led to the identification of the *APC* gene and ultimately connected it to the Wnt signaling pathway. Unless intervention is taken, FAP will invariably result in the development of CRC. Colorectal screening by endoscopic examination

begins in FAP patients as early as 10-years old. Once there is an extensive tumor burden, patients typically undergo the surgical removal of the colon (colectomy) as a prophylactic procedure [61]. Further, FAP patients are at an increased risk of cancer development in other tissues such as thyroid cancer and medulloblastoma.

It was not until the early 1990's that genetic evidence for the basis of FAP was elucidated. Researchers previously showed that FAP was inherited in an autosomal-dominant fashion and suspected that the gene responsible for FAP was on chromosome 5q21, based on the identification of a deletion in an FAP patient [63]. This identification, paired with subsequent work, suggested that the gene responsible for FAP was highly involved in intestinal polyposis and that it may be involved in many sporadic cases of intestinal tumor development as well. In the early 1990's, two groups utilized positional cloning to clone the adenomatous polyposis coli gene, and aptly named it *Adenomatous Polyposis Coli (APC)* [64,65]. The lab of Ray White at the University of Utah identified two deletions in the *APC* region of the DNA, one smaller (~100kb) and the other larger (~260kb). The genomic sites of these deletions corresponded to three candidate genes (DP1, SRP19, and DP2.5) that may have been responsible for adenomatous polyposis coli [66]. Using the identification of intron-exon boundaries, single-strand conformation polymorphism analysis, and family inheritance studies, Ray White's group identified DP2.5 as the *APC* gene [64].

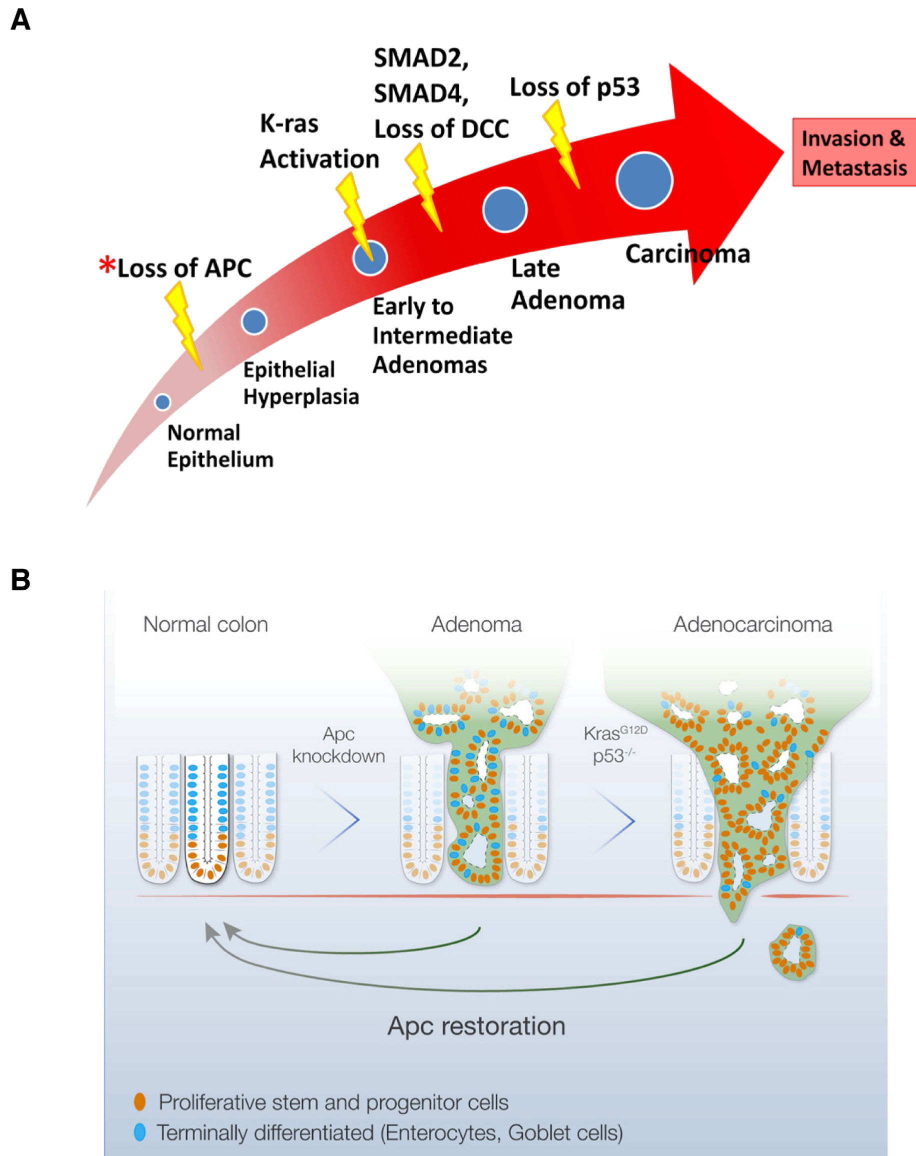
***Susceptibility and Formation of Colorectal Cancer initiates with APC Mutation***

Cancer is a disease that results from the accumulation of genetic mutations or epigenetic alterations of gene expression. Nearly half of the population will form a benign colorectal tumor, called a polyp, by the age of 70. However only a subset of those individuals will develop malignant cancer. In CRC, roughly 25% of cases have a family history of the disease, while the

vast majority of cases occur sporadically [67]. It is known that family history increases the risk and onset of colorectal cancer due to the presence of existing mutations that increase the likelihood of biallelic loss of an essential tumor suppressor gene. Typically, CRC is thought to be a disease of the elderly, and screening is only suggested beginning at 45 years-of-age [68]. A general series of genetic changes occurs during the progression from normal epithelium, to adenoma formation, to metastasis [69]. The initiating event leading to colorectal polyp formation in most human CRCs is through germline or somatic mutation of the tumor suppressor *APC* [60,62].

Adenomatous polyposis coli is mutated in ~80% of human colorectal cancers [70]. APC acts as a classic tumor suppressor and displays a recessive phenotype-- both alleles must be affected prior to tumorigenic capability [71]. Mutation to *APC* is considered to be the initiating event in colorectal tumorigenesis and in the adenoma to carcinoma sequence [62,69,70,72]. Even prior to the discovery of the *APC* gene, a genetic model was proposed by Fearon and Vogelstein in which the mutation or loss of the FAP causing gene on chromosome 5q was the initiating step from normal to hyperplastic epithelium, followed by a series of activating or loss-of-function mutations to KRAS, p53, and others to lead to carcinoma (Fig. 1.3) [73]. While this is the general series of mutations, it seems that *APC* mutation occurs in almost all cases, but the path toward malignancy will result from the acquisition of different genetic alterations depending on the case [74]. In support of the Vogelstein model of colorectal tumorigenesis, Dow et al. recently demonstrated that APC re-introduction could restore intestinal crypt homeostasis in mouse CRCs harboring KRAS and p53 mutations (Fig. 1.3B) [75].

The importance of *APC* in colorectal cancer was well-known given its prevalence of mutation, however at this time the function of the encoded protein was not understood. Despite the lack of knowledge of a functional role for APC, the early studies of FAP and the mapping




Dow et al., Cell, 2015

**Figure 1.3** *Genetic mutations leading to intestinal adenoma formation and mestastasis.* (A) The model proposed by Fearon and Vogelstein, *Cell*, 1990 describing the series of genetic mutations that transform normal epithelium to carcinoma is initiated by loss of APC. Schematic from E. Spears in the Neufeld lab and adapted from Fearon and Vogelstein, 1990. (B) APC restoration in a mouse model of adenocarcinoma returns intestinal epithelium to a normal state. Schematic from Dow et al., *Cell*, 2015.

and identifying of the *APC* gene ultimately resulted in the connection of CRC to the Wnt signaling pathway. The historical events leading to the discovery of Wnt signaling and its connection to colorectal cancer and APC will be discussed in the next section.

## APC and its roles in Wnt regulation

### *The APC/Wnt connection*

While the exact function of APC is still debated, the work of several groups in the 1990's uncovered that APC plays a prominent role in Wnt signaling. These early clues began with the development of the $Apc^{Min/+}$ mouse in the laboratory of Bill Dove. Ethylnitrosourea (ENU) treatment of C57BL/6J mice induced germline mutations that included one within the *APC* gene that was associated with multiple intestinal tumors [76,77]. $APC^{Min/+}$ mice develop multiple adenomas, ranging from 10's to 100's along the small intestine.

Shortly after the development of the Min mouse, the APC protein was found to interact with α- and β-catenin, through immunoprecipitation, nucleotide sequence analysis and peptide mapping [78,79]. At the time, the catenins were known to be involved in cell adhesion, through interaction with E-cadherin, and through studies identifying the *Drosophila* homolog *Armadillo* as a segment polarity gene [80–82]. Subsequent studies using mouse and fly genetics, cell culture, and Xenopus, reviewed in [83,84] led to the identification of the core Wnt pathway, including an essential role for APC in the regulation of β-catenin binding and stability.

### *APC Structure and Function*

The *APC* gene encodes a 2,843 amino acid multi-domain protein that is approximately 312 kDa [64]. Nearly all of the protein coding sequence is contained within the last of the 15 exons of the *APC* gene [64,69]. APC mutations most commonly occur within the "mutation cluster region" (amino acids 1286-1513) and lead to protein truncation (Fig. 1.4). These mutations are far enough into exon 15 that nonsense-mediated decay of the resulting transcript is typically avoided, resulting in the expression of a truncated APC protein [85,86].

The full-length APC protein contains oligomerization domains, armadillo repeats, four 15-amino acid repeats, seven 20-amino acid repeats, SAMP motifs, nuclear localization signals (NLS), and a basic region involved in cytoskeletal interactions (Fig. 1.4A). The many domains of APC enable the interaction with multiple proteins, implicating APC in cellular processes such as differentiation, adhesion, polarity establishment, migration, and apoptosis [87]. The most well-established role for APC is as a negative regulator of the Wnt signaling pathway as part of a cytoplasmic β-catenin destruction complex with Axin, GSK-3β, and CK1-α [11,78,79,88,89]. In particular, the 15 and 20 amino acid repeats are involved in β-catenin binding and degradation [79,90]. Consequently, mutation of either *APC, Axin,* or *CTNNB1* (β-catenin) is frequently observed in many cancers, including colorectal cancer [4,70,91].

### *APC mutation commonly leads to the expression of a truncated protein product*

Mutations in *APC* do not commonly result in complete loss of the protein, but rather, result in premature stop codons and expression of a stable, truncated protein [86,92]. As seen by the lollipop plot generated using cBioPortal, the majority of *APC* mutations occur in the MCR, a region near the middle of the protein (Fig. 1.4B) [86]. These mutations lead to the expression of a

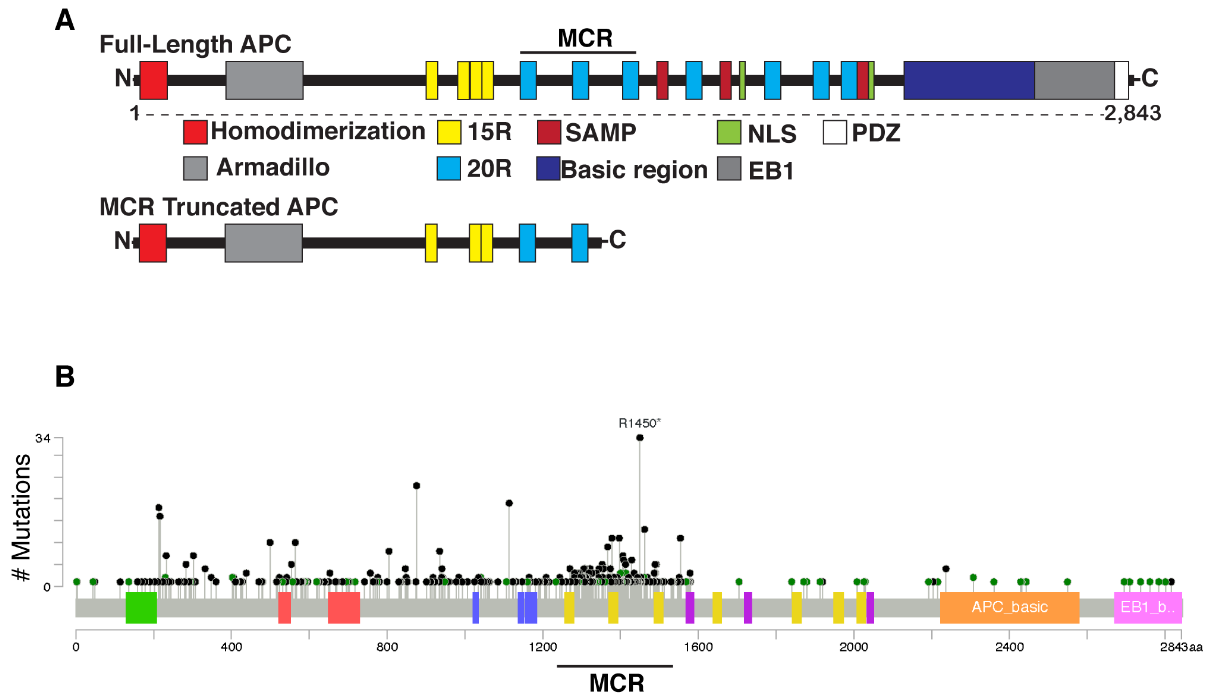



**Figure 1.4** *Structure and Mutational landscape of APC in human CRC*. (A) The protein domains of full-length APC and the effect of mutations within the MCR. The APC protein harbors many domains involved in homodimerization, β-catenin binding (15R, 20R), Axin binding (SAMP), nuclear localization (NLS), and cytoskeletal interactions (basic, EB1). (B) Lollipop plot showing distribution and types of mutations in APC across human CRC. Data from The Cancer Genome Atlas and lollipop plot generated using cBioPortal. The dark circles indicate truncating mutations and the light circles indicate missense mutations.

truncated protein which is lacking several important domains believed to be involved in normal functions of APC.

Mutations in the MCR result in truncated proteins that lack the SAMP motifs (Axin binding), NLS sequences, the C-terminal cytoskeletal interacting basic region, and a variable number of the 20-amino acid repeats involved in β-catenin binding [86,93–96]. Of note, mutations that occur outside of the MCR, either more N-terminal resulting in complete loss of the β-catenin binding sites, or more C-terminal resulting in the retention of the 15 and 20-amino acid repeats, commonly result in an attenuated phenotype [94,97]. It is possible that mutations which completely stabilize β-catenin do not support cell viability and that some level of β-catenin regulation is necessary for optimal tumorigenic capability. By analyzing somatic point mutations of *APC* in FAP patients to assess loss-of-heterozygosity of *APC*, Albuquerque et al. suggested that *APC* mutations are selected based on their ability to maintain some ability to downregulate β-catenin signaling in a "just-right" manner [93]. Perhaps, in addition to the ability to downregulate β-catenin, the loss of C-terminal regions involved in nuclear localization, Axin binding, and cytoskeletal interactions may be important for the tumorigenic capacity of cancer cells.

***APC and the β-catenin destruction complex***

In addition to the ability to bind β-catenin through its 15 and 20-amino acid repeats, APC contains three Ser-Ala-Met-Pro repeats (SAMP repeats) interspersed between the 20 amino acid repeats which mediate Axin binding [98]. Many studies have demonstrated that the β-catenin destruction complex is composed of the core components: APC, Axin1/2, GSK-3β, and CK1-α (reviewed in [11]). Both Axin and APC are considered to be scaffolding proteins for the complex, raising the question of why APC truncations appear so frequently in CRC while Axin mutations

are rare. The destruction complex binds β-catenin through interactions mediated by APC and Axin, and CK1-α phosphorylates Ser45 of β-catenin which primes Thr41, Ser37, and Ser33 for sequential phosphorylation by GSK-3β [10]. Phosphorylation at Ser33 and Ser37 create a WD40-like recognition site for β-TrCP, the subunit of the E3 ubiquitin ligase $SCF^{\beta\text{-}TrCP}$ responsible for substrate recognition, resulting in β-catenin ubiquitination and proteasomal degradation [8–10,99–101].

Several questions exist regarding the role of APC in β-catenin destruction: 1) Is APC the primary scaffold of the destruction complex? 2) Is APC involved in promotion of β-catenin phosphorylation, and if so, how? 3) Does APC maintain association with the β-catenin destruction complex following Wnt stimulation? 4) Does APC regulate β-catenin ubiquitination or its release to the proteasome? Answers to questions such as these will provide a much more detailed understanding of the Wnt pathway, but also will provide a deeper knowledge of APC functions which may aid in efforts toward therapeutic targeting the Wnt pathway. Further, it is not well understood how destruction complex composition, function, and cellular localization is altered upon Wnt signaling. These topics are a major focus of the work in subsequent chapters.

Axin is thought of by some to be the primary scaffold of the destruction complex given that it contains binding sites for all members of the complex (reviewed in [11]) and is considered to be the rate-limiting factor for complex assembly [102]. Several studies support the notion that Axin is the central scaffold, rather than APC [88,103,104]. The in vitro phosphorylation of β-catenin by GSK-3β and CK1-α is enhanced by the addition of Axin [103]. However, conflicting reports support that either APC or Axin may be the rate-limiting factor of destruction complex assembly [102,105]. Several caveats exist – Lee et al. primarily utilize mathematical modeling and experiments in *Xenopus* egg extract while Kitazawa et al. utilize mammalian cell systems, mass-spectrometry,

and the assumption that the complex associates in a 1:1:1:1 ratio of APC:Axin:CK1α:GSK3β. It is unlikely that the destruction complex actually assembles in a 1:1:1:1 ratio, given that both Axin and APC contain multiple β-catenin binding sites. Recent unpublished findings using NMR and chemical-crosslinking by Aaron Rudeen in the Neufeld lab support the notion that multiple β-catenin proteins can be bound by a single APC.

Within the context of the destruction complex, several studies have implicated APC in the enhancement of GSK-3β-mediated phosphorylation, the transfer of β-catenin to the proteasome, and in the enhancement of β-catenin ubiquitination. In support of APC involvement in β-catenin ubiquitination, a β-catenin inhibitory domain (CID) was identified between the second and third 20-amino acid repeats on APC [90]. Subsequent work in the laboratory of Mark Peifer found that the APC CID can interact with Axin, and that this interaction can be regulated by GSK-3β, and allows the access of the E3-ligase to phosphorylated β-catenin [106]. Further, Li et al. identified that β-catenin is ubiquitinated within an Axin1-immunoprecipitated complex in cells treated with the proteasome inhibitor. Upon Wnt stimulation, this β-catenin was still phosphorylated but ubiquitination was decreased and β-TrCP no longer associated with Axin1 [89]. Confirming these results, Azzolin et al. demonstrate similar findings and suggest that the removal of β-TrCP from the complex may be dependent on YAP/TAZ, integral components of the Hippo signaling pathway [107]. An earlier study by Su et al. found that full-length APC was able to protect β-catenin from the protein phosphatase PP2A which removes the regulatory phosphates at the β-catenin N-terminus responsible for its β-TrCP recognition. Cells expressing truncated forms of APC were susceptible to β-catenin dephosphorylation, and thus β-catenin was able to evade β-TrCP-mediated degradation [108]. Taken together, these data suggest that APC plays a key role in the ubiquitination of β-TrCP and identify a unique function that is lost upon

APC truncation. In addition, APC is thought to exert β-catenin regulatory roles involved in nuclear export and may have cytoskeletal functions involved in the Wnt response.

***Nuclear APC***

The best-known role for APC is as a component of the cytoplasmic β-catenin destruction complex. However, just six years after the identification of the *APC* gene, the protein was found to shuttle between the nucleus and the cytoplasm by Neufeld and White [96]. This study utilized multiple APC antibodies, cell fractionation, and immunofluorescence, leaving little doubt that APC had a nuclear function. Subsequent studies by the Neufeld lab identified two nuclear localization signals in the C-terminal region of the protein that is commonly lost in truncated APC found in CRC [109]. These nuclear localization sequences are present in human, mouse, rat, frog, and fly, indicating an evolutionarily conserved mechanism for nuclear APC [109]. Two studies identified nuclear export signals within APC and found that truncated APC localized to the nucleus in cells treated with Leptomycin B, an inhibitor of Crm1-mediated nuclear export [110,111].

Given these findings and others, it was proposed that APC may regulate nuclear β-catenin outside of destruction complex function since β-catenin acts as a transcription factor in the nucleus. This regulation could occur in several ways including the enhancement of nuclear export of β-catenin, sequestration of β-catenin in the nucleus, or a repression of the transcriptional activity of β-catenin [112]. These hypotheses are supported by findings that APC interaction with nuclear β-catenin might provide access to a transcriptional corepressor CtBP or the E3 ligase β-TrCP, sequester β-catenin from its transcriptional coactivator LEF-1/TCF, or facilitate its nuclear export into the cytoplasm for destruction complex-mediated degradation

[111,113–116]. Since these studies were conducted in cell culture, they might not precisely recapitulate what occurs *in vivo* in the mammalian intestine. To assess the roles of nuclear APC in intestinal homeostasis, a mouse model was generated by the Neufeld lab that had compromised nuclear APC by knock-in mutations which inactivate both Apc NLS sites [117]. These mice have elevated Wnt/β-catenin signaling, increased proliferation of the intestinal epithelial cells and also generate more and larger intestinal adenomas when combined with the $Apc^{Min}$ allele [117]. These studies highlight the multifaceted roles of APC within and outside of "canonical" β-catenin destruction complex function.

## Proximal events in Wnt signal transduction leading to destruction complex inhibition

### *Overview*

The basic concept of Wnt signal transduction is that Wnt ligand binding to the Frizzled and LRP5/6 coreceptors results in inhibition of the β-catenin destruction complex through a process mediated by the adaptor protein Dvl. This process ultimately results in stabilization and accumulation of β-catenin, allowing it to translocate to the nucleus where it participates as a transcriptional cofactor. Many studies have elucidated the downstream effects on gene expression of activation of the Wnt pathway and an up-to-date list of Wnt target genes is maintained by Roel Nusse at Stanford (https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). While the downstream effects of Wnt pathway activation are well characterized, the specific upstream molecular events that transduce a Wnt signal and inhibit the β-catenin destruction complex are not. In this section, the details of the initial events involved in

Wnt transduction and several proposed models of β-catenin destruction complex inhibition will be discussed.

### *The Frizzled/LRP/Wnt Interaction*

The initiating event in Wnt signal transduction involves the binding of a secreted Wnt ligand to the Frizzled and LRP5/6 co-receptors to form a "signalosome." Wnt proteins are lipid modified, and a palmitoleic acid attached to a conserved cysteine is responsible for the binding of Wnt to the extracellular domain of Frizzled [7,118]. Frizzled receptors are seven-pass transmembrane proteins belonging to the G-protein coupled receptor family and have an extracellular N-terminal cysteine rich domain (CRD) that can interact with Wnt [119,120]. Structural study of the Frizzled/Wnt interaction revealed that the palmitoleic acid on Wnt interacts with the Fzd CRD [118] and can possibly mediate Fzd dimerization through interaction with two Fzd CRDs [121,122]. The low-density lipoprotein receptors (LRP5/6) also serve as Wnt receptors and can activate downstream Wnt signaling [13]. In HeLa cells, aggregates of LRP6 are formed and colocalize with Fzd8 upon Wnt stimulation, suggesting that a ternary complex composed of Wnt/Fzd/LRP is an initial step of Wnt signal transduction [12]. Further, Fzd/LRP heterodimers were shown to form in the presence of Wnt [123,124].

### *Intracellular events at the Fzd/LRP co-receptors following Wnt stimulation*

After Wnt binding to its co-receptors LRP5/6 and Frizzled, several events take place which ultimately result in inhibition of the β-catenin destruction complex and accumulation of β-catenin. How this inhibition takes place is not well understood and is one of the largest questions that remains in the field of Wnt signaling. Immediately following Wnt ligand binding to its co-

receptors, two key events appear to happen at the cell membrane: 1) the adaptor protein Dvl polymerizes and dimerizes through its DIX and DEP domains to mediate signalosome formation, and 2) the cytoplasmic tail of the LRP6 receptor is phosphorylated, creating a docking site for additional protein interactions.

Disheveled proteins consist of three domains that mediate a multitude of protein interactions [125]. The DIX domain mediates Dvl polymerization through interaction with the DIX domain of another Dvl protein, the DEP domain mediates binding to Fzd as well as Dvl dimerization, and the PDZ domain is an interaction surface for multiple proteins including CK1, Fzd, and GBP/Frat (an inhibitor of GSK-3β) [125,126]. Dvl polymerization is essential for activation of the Wnt pathway [127]. When overexpressed in mammalian cells, Dvl immuno-precipitates with itself as determined using HA- and Flag- tagged Dvl. Overexpressed Dvl forms large puncta and causes activation of a Wnt pathway reporter. Upon mutation of the DIX domain, this punctate appearance is lost and Wnt activation is decreased. Further, electron microscopy revealed that purified DIX domains polymerize to form long protofilaments whereas mutant DIX domains are defective in protofilament formation [127]. The DEP domain of Dvl is responsible for both Fzd binding and for mediating Dvl dimerization through a biochemical domain-swapping event with nearby Dvl [128–130].

It has been proposed that the DEP dimerization and DIX polymerization occur in a concentration-dependent manner and are both required for Dvl to assemble functional signalosomes [128,131]. Interestingly, Petersen et al. demonstrate using live-cell imaging that Fzd homodimerizes [132]. These data, paired with the findings from Nile et al. and DeBruine et al. that one Wnt ligand can bind two Fzd CRDs, suggests that a single Wnt ligand binding event could result in the clustering of four Dvl proteins and potentially act as a platform for further Dvl

polymerization [121,122,131].Dvl can bind Axin through a DIX-DIX interaction [133] and Dvl knockdown in Wnt-treated cells decreases the amount of phosphorylated LRP6, suggesting that Dvl promotes LRP6 phosphorylation [12].

Phosphorylation of the cytoplasmic tail of LRP5/6 appears to be an early event in Wnt signal transduction, regulated in-part by Dvl [12]. Tamai et al. identified the presence of five conserved PPP(S/T)P motifs in the intracellular domain of LRP5/6 [134]. The injection of LRP6 mRNA missing 3/5 of the motifs had no effect on *Xenopus* embryo axis duplication, and the transfection of a similar DNA construct had no effect on the TOPflash Wnt reporter assay. However, individual mutation of the remaining two motifs resulted in decreased axis duplication and decreased TOPflash activity. These motifs are phosphorylated in a Wnt-dependent fashion and immunoprecipitated with the destruction complex member Axin only when phosphorylated [134]. Further, Zeng et al. and Davidson et al. demonstrated that LRP6 is phosphorylated by the kinases GSK-3β and CK1 [135,136]. These findings led to a model in which Wnt-induced phosphorylation of LRP6 creates a docking site for Axin, allowing recruitment of components of the destruction complex. Later work suggested that Dvl is responsible for the initial Axin recruitment through their respective DIX domains and that Axin brings GSK-3β in proximity with the cytoplasmic tail of LRP6 resulting in LRP6 phosphorylation and Axin docking [137]. Multiple studies suggest that phosphorylated LRP6 can inhibit GSK-3β activity [137–139]. This inhibition of GSK-3β may create a signal transduction mechanism that brings the cellular kinases to the Wnt receptors and simultaneously inhibits them, allowing inhibition of a specific cellular pool of GSK-3β and resulting in decreased phosphorylation of β-catenin.

Adding to the complexity of Wnt receptor signaling, endocytosis of the Wnt receptors may occur in some but not all cell types and APC may function to prevent this process from

occurring in the absence of Wnt. Both LRP6 and Dvl can interact with the clathrin adaptor protein, AP-2, involved in clathrin-mediated endocytosis [125,140]. These interactions may result in recruitment of the Fzd receptor through LRP6 and Dvl interactions. Further, APC was recently shown to prevent clathrin-mediated endocytosis in the absence of a Wnt ligand and APC immunoprecipitation in cells treated with or without Wnt resulted in association with Clathrin and AP-2 [141]. Receptor endocytosis may not be involved in all cell types. Rim et al. recently demonstrated that inhibition of endocytosis had no effect on Wnt signaling in mouse embryonic stem cells [142].

***Inhibition of the β-catenin destruction complex following Wnt stimulation***

The findings presented in the previous section were incorporated into a model where Axin and GSK-3β recruitment to the Wnt receptors is a result of β-catenin destruction complex disassembly, imparting negative feedback to the β-catenin destruction complex function [143]. In addition to the model in which Axin/GSK3 are sequestered at the membrane following Wnt binding, several other models of β-catenin destruction complex inhibition, which are not mutually exclusive, have been proposed: 1) Axin degradation decreases available destruction complexes, 2) APC and/or Axin dissociate from GSK-3β or β-catenin, 3) Wnt-induced dephosphorylation of β-catenin, 4) disassembly of the β-catenin destruction complex, or 5) complete localization of the β-catenin destruction complex to the receptors resulting in inability to phosphorylate or degrade β-catenin. Currently, the exact mechanism by which the destruction complex is inhibited is not resolved, nor is the functional role of APC in β-catenin degradation.

The degradation of Axin is supported by several studies and may even be enhanced by APC. Examination of Axin levels in the wing discs of Apc1/2 mutant flies demonstrated that

Axin levels are increased compared to wild-type flies, suggesting that *Drosophila* Apc normally functions to negatively regulate Axin levels [144]. Multiple studies using cultured cells suggest that Wnt signaling induces a translocation of Axin to the membrane and subsequent Axin degradation [102,145,146]. However, these studies rely on overexpression and may not be representative of the endogenous complex during Wnt stimulation. For example, Mao et al. demonstrated that Wnt treatment results in Axin presence within membrane fractions and that overexpression of an LRP5 domain can cause degradation of Axin. However, this study did not examine the effect of Wnt treatment on Axin levels but proposed a model in which Wnt induces Axin destabilization by stimulating LRP5 interaction with Axin [145]. In support of the Axin degradation model, Huang et al. identified a small-molecule inhibitor of the poly-ADP-ribosylating enzymes tankyrase 1 and 2, XAV939, through a TOPflash luciferase reporter screen in Wnt-treated HEK293 cells (human embryonic kidney). Inhibition of tankyrase by XAV939 treatment stabilized Axin while overexpression of tankyrase caused Axin degradation [53].

Wnt stimulation may also impair destruction complex assembly and β-catenin association. Both Axin and APC can be phosphorylated, enhancing their affinity for β-catenin and GSK-3β [11]. However, dephosphorylation by the protein phosphatases PP1 and PP2A can occur which may promote destruction complex disassembly by weakening the affinity for β-catenin [147,148]. Further, Wnt-stimulated Axin/Dvl interaction may disrupt the composition of the destruction complex. Liu et al. demonstrated that GSK-3β failed to immunoprecipitate with Axin or Axin2 in Wnt-stimulated L929 cells [149].

Most of the studies above utilized exogenous expression of proteins involved in the pathway, potentially interfering with signalosome and destruction complex stoichiometry. A study from Hans Clevers' lab in 2012 examined the dynamics of the endogenous β-catenin

destruction complex and Wnt-induced signalosome assembly. Using Axin1 immunoprecipitation, Li et al. demonstrated that the endogenous β-catenin destruction complex maintains association upon Wnt signaling and interacts with LRP6 [89]. Their data support that Axin1 is degraded upon Wnt stimulation. However, this degradation occurs after 4 hours of Wnt stimulation whereas β-catenin accumulation and transcription of Wnt target genes occurs within 30 minutes to 1 hour. These data suggest that an initial Wnt response does not involve Axin degradation but may involve interaction of the complex with the Wnt receptors. Further, Li et al. demonstrated that β-catenin associates with the complex in the presence or absence of Wnt and showed that β-catenin continues to be phosphorylated within this complex [89]. These data suggest that the β-catenin destruction complex is localized toward the Wnt receptors following Wnt signal presentation and that the initial events leading to Wnt pathway activation do not involve dissociation of the destruction complex or release of β-catenin, but rather a saturation of destruction complexes by β-catenin which allows it to accumulate. It is possible, however, that Axin1 is involved in multiple protein complexes in the cell, and that this is not an entirely accurate view of what occurs at the level of the Wnt receptors.

## Summary

The Wnt signaling pathway is highly conserved across multicellular organisms, involved in developmental and homeostasis signaling pathways, and as such is often deregulated in many diseases. The tumor suppressor *APC* is a key negative regulator of the Wnt signaling pathway and is mutated in over 80% of human colorectal cancers. However, the functions of APC in normal β-catenin destruction complex function and β-catenin regulation are not well understood.

Further, the downstream effects and transcriptional outputs of Wnt signaling are well characterized, but the proximal events leading to inhibition of the β-catenin destruction complex are lacking in mechanistic information. The Wnt pathway has enormous potential for therapeutic targeting, but without a better understanding of its mechanistic details this will likely prove to be an arduous process. In this dissertation, I explore the roles of APC in destruction complex function and localization upon Wnt ligand presentation in human colorectal epithelial cells from both a normal and cancerous origin. I also analyze the effects of "stabilizing" β-catenin mutations on β-catenin regulation and nuclear localization. Lastly, the kinetics of the Wnt response are assayed in multiple cell lines to explore how different mutations within the Wnt pathway affect the Wnt response. This work sheds light onto the underlying mechanisms of Wnt signaling and identifies new functions of APC that may assist in therapeutic targeting of this pathway.

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# Chapter 2

## APC controls Wnt-induced β-catenin destruction complex recruitment in human colonocytes

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## Abstract

Wnt/β-catenin signaling is essential for intestinal homeostasis and is aberrantly activated in most colorectal cancers (CRC) through mutation of the tumor suppressor *Adenomatous Polyposis Coli* (*APC*). APC is an essential component of a cytoplasmic protein complex that targets β-catenin for destruction. Following Wnt ligand presentation, this complex is inhibited. However, a role for APC in this inhibition has not been shown. Here, we utilized Wnt3a-beads to locally activate Wnt co-receptors. In response, the endogenous β-catenin destruction complex reorientated toward the local Wnt cue in CRC cells with full-length APC, but not if APC was truncated or depleted. Non-transformed human colon epithelial cells displayed similar Wnt-induced destruction complex localization which appeared to be dependent on APC and less so on Axin. Our results expand the current model of Wnt/β-catenin signaling such that in response to Wnt, the β-catenin destruction complex: 1) maintains composition and binding to β-catenin, 2) moves toward the plasma membrane, and 3) requires full-length APC for this relocalization.

## Introduction

The Wnt signaling pathway is essential for cell proliferation, cell polarity, developmental cell-fate determination, and tissue homeostasis [1]. As a result, deregulation of Wnt signaling is often associated with cancer and other diseases [2,3]. Notably, over 90% of colorectal cancers (CRC) have mutations that activate the Wnt pathway with over 80% containing mutations in the Wnt antagonist *Adenomatous Polyposis Coli* (*APC*) [4]. Human *APC* mutations commonly arise in a central region of the open reading frame, referred to as the "mutation cluster region" (MCR, Fig. 2.1A), resulting in a truncated protein product [5]. APC truncation results in loss of multiple β-catenin binding sites (20R), Axin interaction sites (SAMP), nuclear localization sequences, and

a C-terminal basic region which mediates cytoskeletal interactions (Fig 2.1A). Germline or sporadic *APC* mutations in colon stem cells lead to polyp formation and are considered initiating events in colorectal tumorigenesis [6,7]. In the context of Wnt signaling, it is well established that APC acts as a scaffold in the β-catenin destruction complex. Given that other proteins involved in this complex could be altered to activate Wnt signaling, it is curious that *APC* mutations predominate in CRCs. One potential explanation for this is that APC performs additional critical functions both inside and outside the context of Wnt signaling.

The canonical Wnt pathway serves to regulate the level of transcriptional coactivator β-catenin [8,9]. To prevent aberrant transcriptional activation in the absence of Wnt, a cytoplasmic β-catenin destruction complex composed of APC, Axin, CK1-α, and GSK-3β maintains low levels of β-catenin through sequestration, phosphorylation, and β-TrCP-mediated ubiquitination, leading to proteasomal degradation [10–12]. However, if a Wnt ligand binds the co-receptors Frizzled (FZD) and low-density lipoprotein receptor 5/6 (LRP5/6) to form the heterotrimeric "signalosome," the β-catenin destruction complex is inhibited through an incompletely resolved mechanism [13,14]. While the downstream effects of Wnt signaling have been extensively analyzed, the specific molecular events which cause destruction complex inhibition are not well understood. Further, the exact roles of APC in normal destruction complex function have remained elusive, and it has not been determined whether APC is also involved in promoting the transduction of a Wnt signal.

Wnt ligands are required for intestinal stem cell (ISC) self-renewal and crypt homeostasis [15–17]. Traditionally, these intestinal Wnt ligands have been thought to diffuse from the crypt base toward the luminal surface to form a signaling gradient. However, Wnt was recently shown to predominantly engage with FZD receptors on the ISCs that were immediately adjacent to Wnt-

secreting cells and not those that were further removed from the Wnt source. These findings are inconsistent with a Wnt diffusion model, but instead support a model whereby Wnt is initially bound to receptors following secretion and forms a gradient through receptor turnover and cell division [18]. Therefore, intestinal Wnt signaling may convey positional information within the crypt and direct intracellular protein localization based on the location of the Wnt source. In the current study, we will examine the effect of a local, immobilized Wnt signal on colon epithelial cells.

Several challenges have historically limited our understanding of Wnt signaling dynamics as it relates to intestinal homeostasis in the normal and cancerous state. Due to the widespread use of the $Apc^{Min/+}$ mouse model, much focus has been directed to the small intestine rather than the colon, the site of most tumor-initiating *APC* mutations in humans [19]. Further, very little is known about Wnt signaling in colon epithelial cells that are from non-malignant origin, as most studies utilize only cultured CRC cell lines. Finally, prior research has mostly relied on overexpression of specific Wnt pathway components or cells treated with soluble Wnt in the media, limiting the ability to elucidate endogenous β-catenin destruction complex dynamics in response to a local Wnt signal (reviewed in [3]).

Here, we examine the response of endogenous β-catenin destruction complex components to a locally presented Wnt signal in human colon epithelial cells of both malignant and non-malignant origin. We demonstrate for the first time that a localized Wnt source can recruit the signalosome and β-catenin destruction complex in colon epithelial cells and find that this Wnt-induced recruitment requires full-length APC. Our work identifies a novel role for APC in the regulation of destruction complex movement toward the membrane following Wnt exposure.

## Results

### ***Wild-type APC, but not truncated mutant APC is recruited toward local Wnt3a in human colon cancer cells***

Since *APC* is mutated in >80% of colorectal cancers and is a major scaffolding protein for the β-catenin destruction complex, we first asked whether APC redistributes toward a localized Wnt3a signal in a panel of human CRC cell lines. Three colon cancer cell lines, each with a different Wnt pathway status, were used: RKO (intact Wnt signaling pathway), HCT116βm (WT APC, stabilized β-catenin due to Ser45 deletion) [20], and DLD1 cells (APC truncation at amino acid 1452, WT β-catenin, see Fig. 2.1A). Previous studies relied on Wnt addition to cell culture media, thus limiting the ability to examine responses to a localized stimulus. To address this issue and examine endogenous protein response to a locally applied Wnt source – we treated cells with Wnt3a-conjugated or Unloaded-beads.

Cells treated for 12-14 hours with Wnt- or Unloaded-beads were fixed and stained for APC (Fig. 2.1B). Protein localization was scored as: (A) toward the bead, (B) away from the bead, or (C) no obvious protein polarization (Fig. 1C). Scoring results were validated with line-scan analysis. Full-length APC localized toward the Wnt3a-bead in 76% of RKO (Fig. 2.1D) and 80% of HCT116βm cells (Fig. 2.1E). In contrast, truncated APC localized toward the Wnt-bead in only 53% of DLD-1 cells (Fig. 2.1F). In all cases, the majority of cells treated with Unloaded-beads displayed random distribution of APC, suggesting that physical contact with an Unloaded-bead is not sufficient to re-orient APC. These data demonstrate that Wnt exposure induces APC re-localization toward the Wnt source in multiple human CRC lines. Furthermore, this process appears to be compromised in cells carrying truncated APC but remains functional in cells with

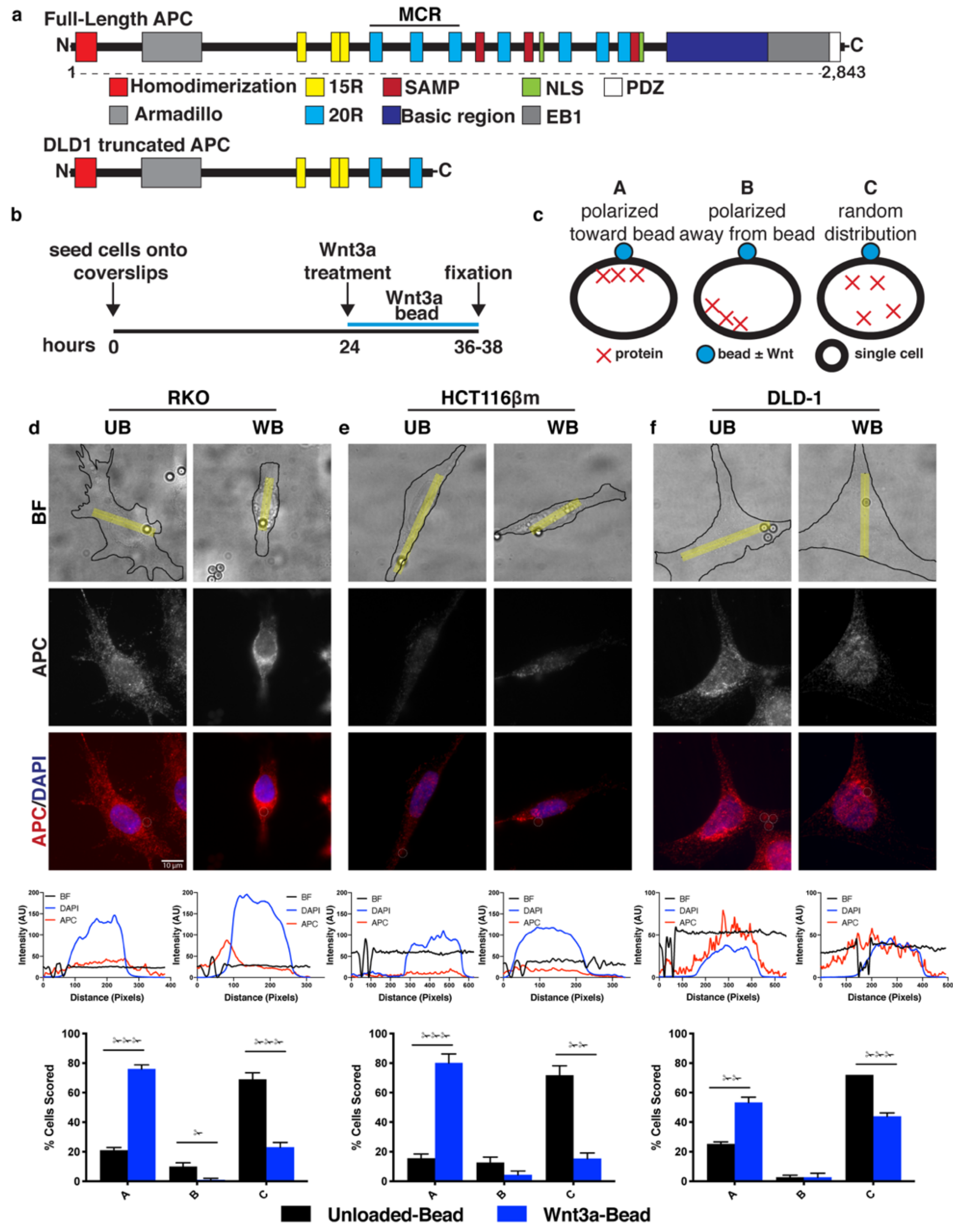



**Figure 2.1** *APC orientation in response to localized Wnt-3a in a panel of colorectal cancer cell lines.* (A) Full-length APC and its interaction domains. Truncated APC found in DLD1 cells results in loss of the C-terminal half of the protein. (B) Schematic of experimental design. One day after seeding onto fibronectin-coated coverslips, cells were treated with Wnt3a-beads for 12-14 hours, then fixed and processed for immunofluorescence microscopy. (C) Scoring criteria for protein localization. (D) Representative images of APC localization in RKO cells treated with Unloaded-bead (UB) or Wnt3a-bead (WB). Representative line scan of bright-field (BF, yellow region analyzed) demonstrates increased APC signal intensity near the WB but not the UB. For line scan graphs, intensity in arbitrary units (AU) of bead = black; DAPI = blue; APC = red. Below line scan graphs, results from three independent experiments were averaged and graphed with error bars representing SEM. For each experiment 25 cells were scored per condition. Statistical analysis by t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (E and F) Representative images, line scans, and scoring results for HCT116βm and DLD-1 cells, respectively. Scale bar, 10 μm.

an activated Wnt/β-catenin transcriptional program due to stabilized β-catenin. The phenotypic consequences of truncated APC have been previously postulated to act in a "just-right" signaling model, in which truncated APC retains partial β-catenin regulatory function to allow a specific level of Wnt activation [21]. Because truncated APC shows reduced ability to localize toward a Wnt source, it is possible that this partial localization is a method to obtain a precise level of Wnt-response.

***Wnt components are recruited toward localized Wnt3a in cells with an intact Wnt signaling pathway***

Since APC is a key scaffold for the β-catenin destruction complex, we reasoned that additional members of the destruction complex and signalosome may also localize near a Wnt-bead. RKO cells with an intact Wnt signaling pathway were treated with beads and then stained for the scaffolding protein Axin1, kinases CK1-α and GSK-3β, the Wnt receptor FZD7, the ubiquitin ligase β-TrCP, and β-catenin. FZD7 was chosen for its defined role in gastrointestinal homeostasis [22–24].

Distinct Axin1 puncta were observed near the Wnt-bead (Fig. 2.2A). While RKO cells contain low levels of β-catenin due to an intact Wnt pathway, they do have visible β-catenin staining which can be scored. Treatment with Unloaded-beads failed to induce relocalization of β-catenin (Fig. 2.2B). However, in cells treated with a Wnt-bead, β-catenin levels increase and β-catenin localized toward the bead (Fig. 2.2B). Of note, the ubiquitin ligase β-TrCP also localized toward the bead (Fig. 2.2F). These findings suggest that upon Wnt/FZD binding, the cytoplasmic destruction complex relocates to the membrane and is able to recruit β-TrCP but falls short of targeting β-catenin for destruction. This result is consistent with a model proposed by Li et al.

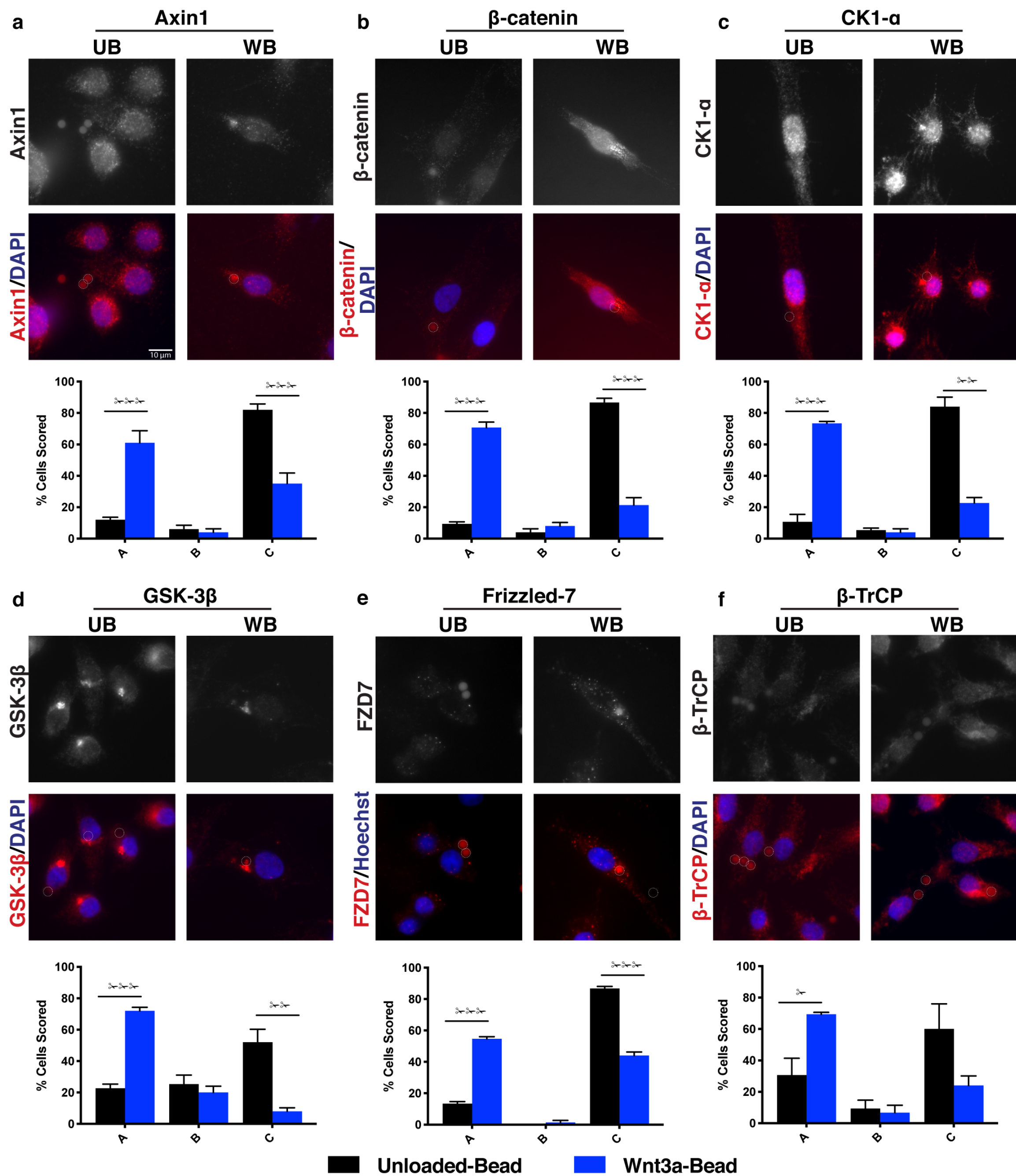



**Figure 2.2** *Wnt signaling pathway components localize toward a Wnt-bead.* Representative images of RKO cells (CRC line with intact Wnt signaling pathway) treated with UB or WB as in Fig. 1A and stained for (A) Axin1, (B) β-catenin, (C) CK1-α, (D) GSK-3β, (E) Frizzled-7, or (F) β-TrCP. Below representative images are bar graphs of the scoring quantification. Data averaged from three independent experiments (n=25 cells per condition). Error bars, SEM; Statistical analysis by t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Representative line scans provided in supplemental figure 1. Scale bar, 10 μm.

whereby the destruction complex saturates with β-catenin, leaving newly synthesized β-catenin to control downstream Wnt target gene expression following Wnt stimulation [25]. The kinases CK1-α and GSK-3β were also detected near the Wnt-bead (Fig. 2.2C, D), as was the Wnt receptor FZD7 (Fig. 2.2E).

To test for a physical interaction between the Wnt-coated beads and destruction complex components, the beads were used to "pull down" Wnt and associated proteins from lysates of Wnt-bead-treated cells. APC and β-catenin each associated more with Wnt-beads than with Unloaded-beads (Fig. 2.3). Together, these data demonstrate that signalosome and destruction complex proteins concentrate near a local Wnt3a cue and do not fully disassemble in response to ~12-hour exposure to a Wnt3a bead. Furthermore, these data support a model in which β-catenin remains associated with the destruction complex following Wnt stimulation.

***Stabilized β-catenin does not impact Wnt component localization in response to Wnt3a***

In colorectal cancer, mutations in the APC and β-catenin encoding genes appear to be mutually exclusive. Though most CRCs have *APC* mutations, about half of the small subset of colorectal cancers with wild-type *APC* harbor mutations that activate β-catenin [26]. These mutations typically result in loss of specific β-catenin residues whose phosphorylation is critical for ubiquitin conjugation and proteasome-mediated destruction. To determine whether such "stabilizing" β-catenin mutations would compromise orientation of Wnt signaling components toward a localized Wnt3a, we examined HCT116βm cells. Unlike the parental HCT116 cells, which express both wild-type and mutant β-catenin, HCT116βm possess only one mutant β-catenin allele encoding a Ser45 deletion [20]. Ser45 phosphorylation by CK1-α primes the successive phosphorylation of Thr41, Ser37, and Ser33 by GSK-3β, a prerequisite for

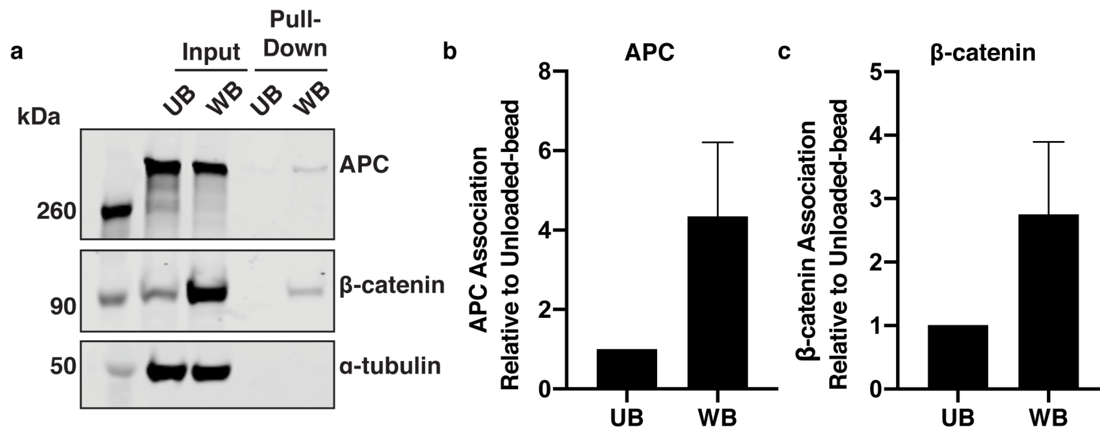



**Figure 2.3** *Wnt-beads pull-down APC and β-catenin.* (A) RKO cells treated with Unloaded-beads or Wnt-beads were lysed and proteins pulled-down with the beads. APC and β-catenin were detected in the Wnt-bead pull-down but not the Unloaded-bead pull-down. Images are from the same gel/membrane cut between 50 and 70 kDa and probed for APC and β-catenin (above 70 kDa) or α-tubulin (below 50 kDa). Multi-channel imaging was performed for APC and β-catenin through IRDye infrared secondary antibodies to allow detection on the same membrane. (B-C) Quantification of APC and β-catenin protein pulled-down by Wnt-beads compared to Unloaded-beads from five independent experiments. Protein levels of APC and β-catenin that were pulled down by Unloaded-beads or Wnt-beads were divided by the respective input protein levels and normalized to the Unloaded-bead to demonstrate fold change of APC (B) and β-catenin (C). Error bars, SEM.

recognition and ubiquitination by β-TrCP [10–12]. Note that APC localized toward the Wnt-bead in both RKO and HCT116βm cells (Fig. 2.1D, E).

As seen in RKO cells, Axin1 localized in distinct puncta near the Wnt-bead in HCT116βm cells (Fig. 2.4A). Despite expressing only stabilized β-catenin, HCT116βm cells exhibited both CK1-α and GSK-3β localized toward the Wnt-bead (Fig. 2.4C, D). This finding demonstrates that CK1-α recruitment to the destruction complex is not dependent on Ser45 of β-catenin, nor is GSK-3β recruitment dependent on Ser45 phosphorylation. FZD7 was also detected near the Wnt-bead (Fig. 2.4E). Notably, β-catenin also localized near the Wnt-bead (Fig. 2.4B), suggesting that the destruction complex maintains association with stabilized β-catenin, even in the absence of degradation. On the other hand, β-TrCP failed to localize toward the Wnt-bead in HCT116βm cells (Fig. 2.4F), demonstrating that β-catenin Ser45 is necessary for β-TrCP association with the destruction complex. Therefore, β-TrCP does not appear to be an inherent member of the destruction complex, but rather, is recruited following β-catenin phosphorylation. Together, these results demonstrate that an activated Wnt pathway through β-catenin stabilization is not sufficient to block orientation of core destruction complex proteins toward localized Wnt. β-TrCP failed to localize toward the Wnt cue, consistent with CK1-α phosphorylation of β-catenin as a prerequisite for β-TrCP recruitment.

***Cells lacking full-length APC are compromised for Wnt component localization toward Wnt***

Both HCT116βm and DLD1 cells possess an activated Wnt/β-catenin transcriptional program, induced by mutation of β-*catenin* or *APC*, respectively. As described, with the exception of β-TrCP, Wnt pathway components remained able to orient toward a localized Wnt source in HCT116βm cells (Fig. 2.4). DLD1 cells have a mutation in the mutation cluster region

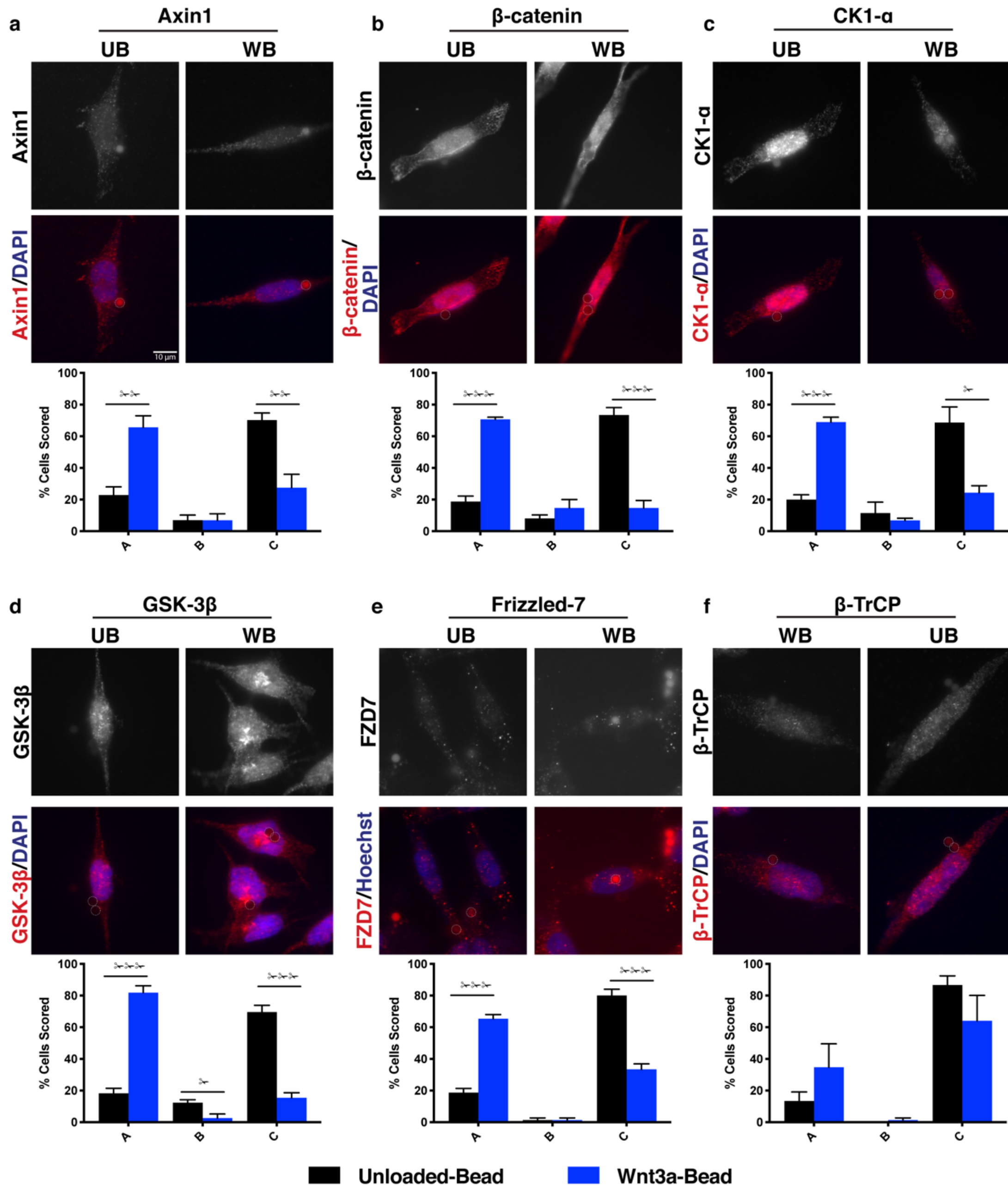



**Figure 2.4** *Activated Wnt signaling through stabilized β-catenin does not alter Wnt-mediated signaling pathway component localization.* Representative images of HCT116βm cells (CRC line with single allele encoding β-catenin that cannot be phosphorylated by CK1-α) treated with UB or WB as in Fig. 1A. Phosphorylation by CK1-α is reported to be a prerequisite for phosphorylation by GSK-3β and subsequent ubiquitin-mediated degradation. Representative images and scoring shown for (A) Axin1, (B) β-catenin, (C) CK1-α, (D) GSK-3β, (E) Frizzled-7, and (F) β-TrCP. Data averaged from three independent experiments (n=25 cells per condition). Error bars, SEM; Statistical analysis by t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Representative line scans provided in supplemental figure 2. Scale bar, 10 µm.

that leads to a truncated APC protein lacking the Axin-binding SAMP motifs and most of the β-catenin-binding 20aa repeats (Fig. 2.1A). However, this truncated APC was reported to co-precipitate with Axin1, suggesting that it maintains its scaffolding properties [25]. Given that truncated APC appeared compromised for orientation toward a Wnt-bead in DLD1 cells (Fig. 2.1F), we asked whether other β-catenin destruction complex members also displayed diminished localization toward a Wnt3a source.

If APC only serves as a destruction complex scaffold, then we would expect normal destruction complex orientation in DLD1 cells. Instead, we found that Axin1 localization to the Wnt3a-bead was detected in only 30% of DLD-1 cells (Fig. 2.5A), compared to 61% of RKO cells (Fig. 2.2A) and was not significantly different than localization to the Unloaded-bead. GSK-3β and β-TrCP were also compromised for Wnt-bead orientation in DLD1 cells (Fig. 2.5D, F). Of all proteins analyzed in DLD1 cells, only CK1-α and FZD7 were localized toward the Wnt-bead more than the Unloaded-bead (Fig. 2.5C, E). However, even this Wnt-bead localization was observed much less frequently in DLD1 cells than in RKO or HCT116βm cells, which contain full-length APC. Subcellular β-catenin reorientation could not be assessed by visual inspection of DLD1 cells due to overall high protein levels. However, line scan analyses demonstrated that β-catenin also failed to relocate in response to a Wnt-bead (Fig. 2.S3).

Together, these results indicate that full-length APC is necessary for optimal destruction complex orientation in response to a Wnt cue. Given that truncated APC co-precipitates with Axin1 and the destruction complex in DLD1 cells [25], our results also suggest that APC mediates destruction complex localization by a mechanism independent of, or in addition to, its role as a scaffold for the complex. Truncated APC in DLD1 cells lacks domains that bind dynein [27], kinesin [28], and microtubules [27–30]. These interactions potentially contribute to movement of the

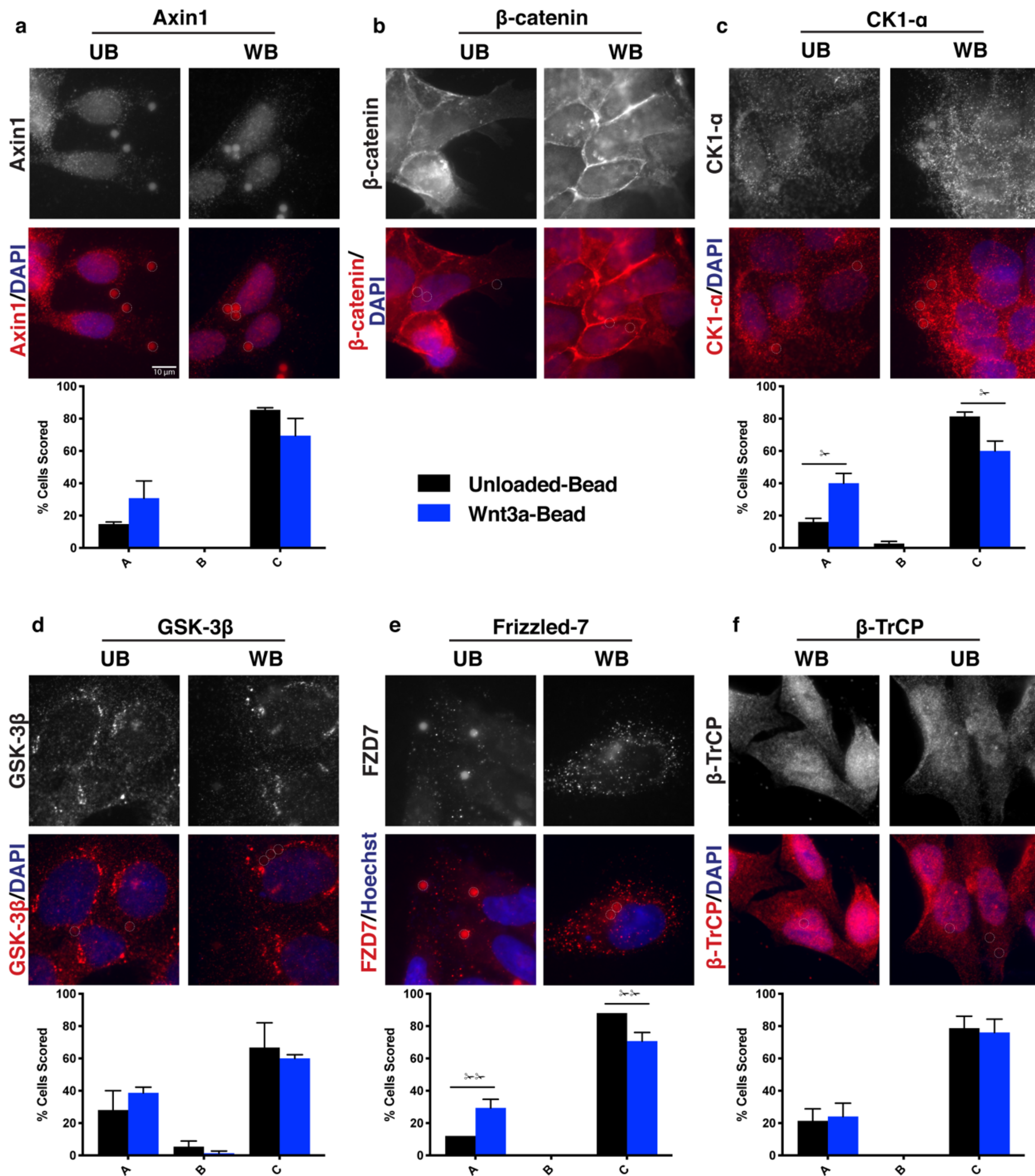



**Figure 2.5** *CRC cells with truncated APC display diminished association of Wnt signaling pathway components toward localized Wnt.* Representative images of DLD-1 cells (CRC line a expressing only truncated APC (amino acids 1-1472)) treated with UB or WB. Representative images and scoring shown for (A) Axin1, (B) β-catenin, (C) CK1-α, (D) GSK-3β, (E) Frizzled-7, and (F) β-TrCP. Data averaged from three independent experiments (n=25 cells per condition). Error bars, SEM; Statistical analysis by t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Representative line scans provided in supplemental figure 3. Scale bar, 10 µm.

destruction complex toward the Wnt source. However, because localization of FZD7 and CK1-α toward the Wnt-bead was decreased, but still present, it appears that truncated APC retains some ability to localize the complex or that alternative mechanisms may be able to localize these proteins in the absence of full-length APC.

***APC loss impairs Wnt-induced destruction complex reorientation***

Given that the β-catenin destruction complex localized toward a Wnt-bead in CRC cell lines with wild-type APC but was impaired in cells with truncated APC, we hypothesized that destruction complex localization requires full-length APC. To test the APC-dependence of Wnt-induced destruction complex localization, we utilized RKO-$APC^{KO}$ cells, generated through CRISPR/Cas9 by Lee and colleagues [31]. Immunofluorescent microscopy revealed reduced APC signal in RKO-$APC^{KO}$ cells compared to the parental RKO cell line (Fig. 2.6A). While parental RKO cells displayed Wnt-induced localization of β-catenin in 70% of cells scored (Fig. 2.2B), this localization was seen in only 25% of the RKO-$APC^{KO}$ cells (Fig. 2.6B, E). Further, Axin1 and GSK-3β failed to relocalize toward a Wnt-bead in the RKO-$APC^{KO}$ cells (Fig. 2.6C, F & 2.6D, G). These data demonstrate that Wnt-induced destruction complex localization in CRC cells is dependent on APC.

***Wnt triggers destruction complex reorientation in normal human colonic epithelial cells in an APC-dependent manner***

In normal colon tissue, adult stem cells facilitate the continual regeneration of the epithelial lining and rely on Wnt ligand for maintenance of the stem cell niche. Having observed Wnt-mediated re-localization of the β-catenin destruction complex in human colon cancer cell

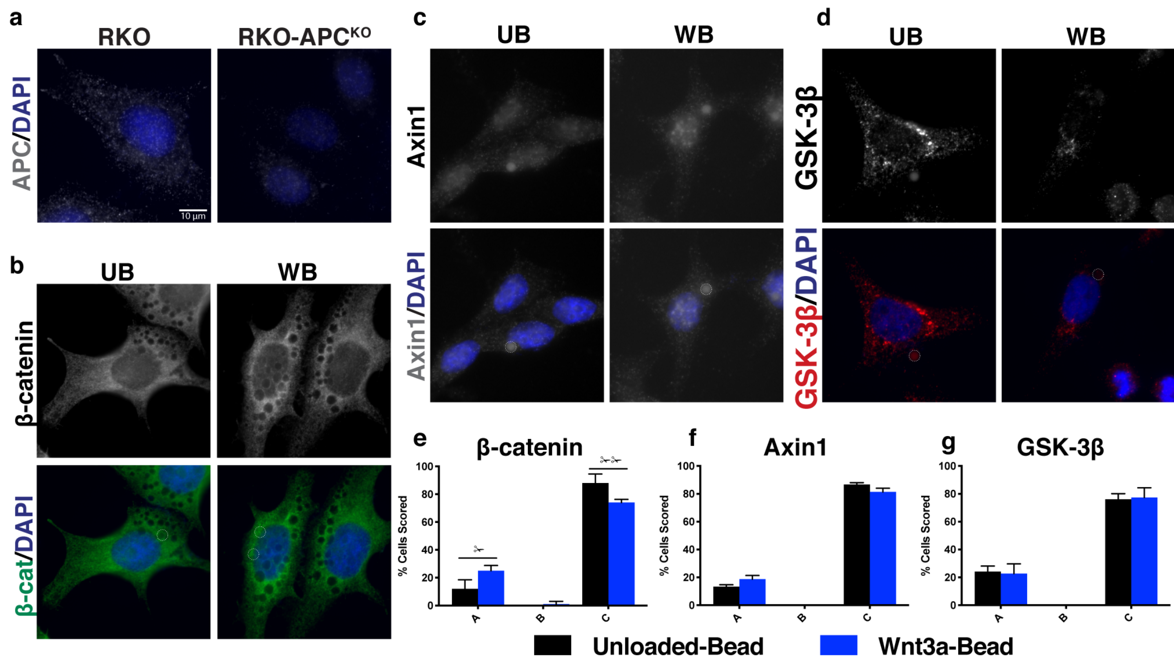



**Figure 2.6** *APC knockout in CRC cells impairs Wnt-induced β-catenin destruction complex localization.* (A) APC staining in RKO and RKO-APC$^{KO}$ cells. (B-D) Representative images of RKO-APC$^{KO}$ cells treated with UB or WB. Representative images and scoring shown for (B,E) β-catenin, (C,F) Axin1, and (D,G) GSK-3β. Data averaged from three independent experiments (n=25 cells per condition). Error bars, SEM; Statistical analysis by t-test: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Scale bar, 10µm.

lines, we wondered if normal human colon stem cells also displayed this property and if so, whether this relocalization was APC-dependent. To address this, we sought human colonic epithelial cells (HCECs) that were isolated from normal tissue and propagated in culture under near physiological conditions (low oxygen and sera). Shay and colleagues isolated HCECs from routine colonoscopy and immortalized them with expression of cyclin-dependent kinase 4 (Cdk4) and human telomerase (hTERT) [32]. These "HCEC 1CT" cells also express endogenous stem cell markers and are able to differentiate into multiple cell lineages making them a unique and valuable model of normal colon epithelial stem cells.

Using HCEC 1CT cells, we depleted APC levels with siRNA (siAPC) and then determined localization of β-catenin, Axin1 and GSK3β. APC knock-down was efficient in HCEC 1CT and resulted in increased β-catenin protein levels, as expected (Fig. 2.7A). In cells treated with scrambled siRNA (siCtl), Wnt-bead exposure resulted in re-orientation of APC, β-catenin, Axin1, and GSK-3β toward the Wnt source (Fig. 2.7B-I). Therefore, normal non-transformed human colonic epithelial cells maintain the ability to re-orient the β-catenin destruction complex toward a Wnt cue. Remarkably, APC knock-down resulted in complete loss of β-catenin, Axin1, and GSK-3β orientation toward the Wnt-bead (Fig 2.7C-E, G-I). Distinct β-catenin puncta were visualized near Wnt-beads in siCtl HCEC 1CT cells. In contrast, β-catenin levels were increased with diffuse cytoplasmic staining and prominent nuclear localization in HCEC 1CT cells depleted of APC but lacked localization toward the Wnt-bead (siAPC, Fig. 2.7C). These data are in agreement with the findings in RKO-APC$^{KO}$ cells (Fig. 2.6).

Like APC, Axin1 is a key scaffolding protein in the β-catenin destruction complex, and thus, might control proper localization of the complex. Using HCEC 1CT cells, we depleted

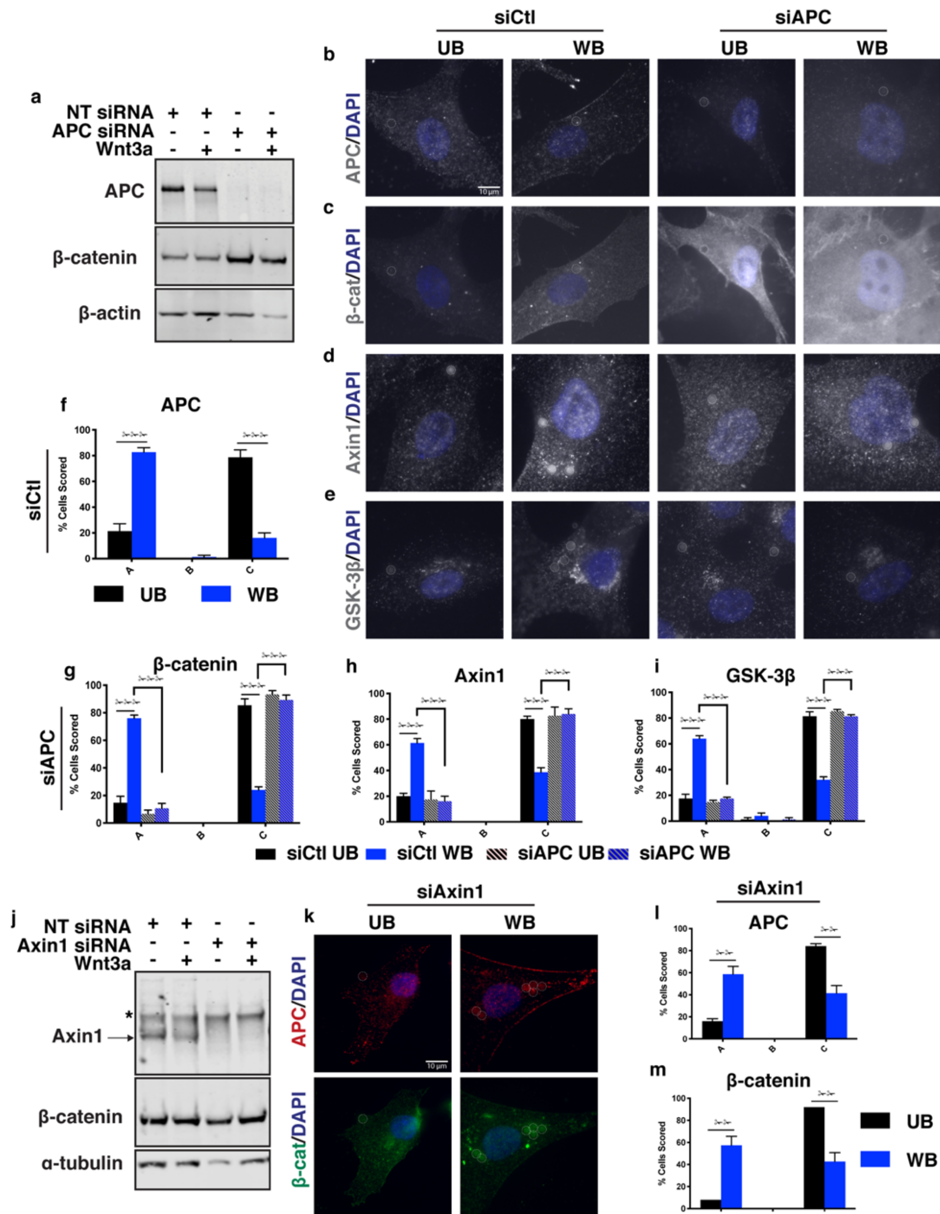



**Figure 2.7** *The β-catenin destruction complex localizes toward Wnt in normal human colonic epithelial cells in an APC-dependent manner.* Representative images and scoring analysis of HCEC 1CT cells (immortalized cells from normal human colon) treated with control siRNA (siCtl), APC siRNA (siAPC), or Axin1 siRNA (siAxin1) for 48 hours prior to UB or WB treatment. (A) APC knock-down in HCEC 1CT cells. Images are from the same gel/membrane. Membrane was cut between 50 and 70 kDa and probed for APC and β-catenin (above 70 kDa) or β-actin (below 50 kDa). Multi-channel imaging was performed for APC and β-catenin through IRDye infrared secondary antibodies so that they could be probed on the same membrane. (B, C, F, G) HCEC 1CT stained for β-catenin and APC (channels separated). (D, H) HCEC 1CT stained for Axin1. (E, I) HCEC 1CT stained for GSK-3β. (J) Axin1 knock-down in HCEC 1CT cells. Images are from the same gel/membrane cut as in (A) and probed for Axin1 and β-catenin (above 70 kDa) or α-tubulin (below 50 kDa). Asterisk indicates non-specific band. (K-M) Representative images and scoring analysis of APC and β-catenin localization in Axin1-depleted HCEC 1CT cells. Data averaged from three independent experiments (n = 25 cells per condition). Error bars, SEM: Statistical analysis by t-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Scale bar, 10 μm.

Axin1 with siRNA (siAxin1) and determined the localization of APC and β-catenin. Both APC and β-catenin still localized toward the Wnt-bead in Axin1-depleted HCEC 1CT cells (Fig. 2.7 L, M). However, this bead-induced redistribution did appear to be slightly compromised compared to that seen in siCtl HCEC 1CT cells. Given that APC-depletion resulted in complete loss of localization of destruction complex components, we conclude that APC is necessary and sufficient for Wnt-induced localization of the destruction complex, while Axin1 can enhance the localization, but is expendable.

Together, these data demonstrate that the β-catenin destruction complex retains the ability to orient toward a localized Wnt source in HCEC 1CT cells and reveal a requirement and novel function of full-length APC in destruction complex trafficking toward a Wnt cue.

**Discussion**

To investigate the role of APC in Wnt-directed destruction complex localization, we first explored signalosome and destruction complex response in commonly used CRC cell lines with varying Wnt pathway status. The data from these experiments provide novel findings that the β-catenin destruction complex re-localizes toward Wnt in colon epithelial cells and that this redistribution is impaired in DLD1 cells expressing a truncated form of APC. Truncated APC is present in the majority of human CRC, and results in loss of the C-terminal half of APC, which contains domains involved in destruction complex assembly, nuclear localization, and cytoskeletal interactions. Further, we demonstrate that APC loss ablates Wnt-induced destruction complex localization. Unlike the parental RKO cell line, Axin1, GSK-3β and β-catenin are unable to localize toward a Wnt-bead in RKO-$APC^{KO}$ cells. We extended this study to a non-transformed human colon epithelial cell line which exhibits stem cell characteristics and found

that Wnt-directed destruction complex localization also occurs in colon epithelial cells of non-malignant origin. When compared to the protein distribution in DLD1 cells, which were already limited in signalosome and destruction complex reorientation in response to localized Wnt, APC depletion in the non-transformed human colon epithelial cells completely abolished Wnt-induced destruction complex localization. Combined with a previous report that the truncated APC found in DLD1 remains associated with destruction complex protein Axin1 [25], it appears that APC utilizes its function in scaffolding as well as interactions in the C-terminus to fully traffic the destruction complex toward a Wnt cue.

Numerous models of the events following Wnt receptor activation have been proposed, however the specific molecular proceedings that occur are still debated due to the use of overexpression of Wnt pathway components, global addition of Wnt to culture media, or the wide range of cell and tissue types used. Many models propose that the destruction complex is either inactivated or partially disassembled following Wnt/co-receptor interaction [3,33–36]. However, other studies have demonstrated that some of the destruction complex may be targeted to the plasma membrane following receptor activation [25,37,38]. Recently, an elegant study exposed mouse embryonic stem cells to Wnt3a-conjugated beads, resulting in APC, β-catenin, and LRP6 localization toward a Wnt-bead and also found that the local Wnt cue could trigger asymmetric cell division [39]. To our knowledge, this is the only study examining the orientation of signalosome and destruction complex components in response to localized Wnt and was performed in pluripotent cells of nonhuman origin and limited to three endogenous proteins.

Our study builds upon the current model of Wnt signal transduction and brings to light a new role for APC. Here, we report the requirement of APC for proper Wnt-induced localization of the β-catenin destruction complex in both malignant CRC cell lines and in non-transformed

colon epithelial cells. In contrast to our findings, others have demonstrated that key members of the complex are degraded or endocytosed following prolonged Wnt exposure [25,31,40–42]. It is possible that these differences reflect more spatially restricted Wnt contact in our study compared to global Wnt exposure. It also seems likely that the bead attached to Wnt ligand in our study was so large as to prohibit endocytosis. Potentially related, recent evidence points to a role for the central region of APC in preventing clathrin-mediated endocytosis in the Wnt-off state [31]. Over time, the complexity of potential APC functions related to Wnt signaling has been gradually revealed. Previous work by our lab and others demonstrated that APC performs roles outside of its classically defined scaffolding function in the β-catenin destruction complex. For example, APC interaction with nuclear β-catenin leads to repression of Wnt target genes through several potential mechanisms: providing access to the transcriptional corepressor CtBP or the E3 ligase β-TrCP, β-catenin sequestration from transcriptional coactivator LEF-1/TCF, or facilitating β-catenin's nuclear export [43–47].

Together, our results suggest a novel mechanism of β-catenin destruction complex regulation by the APC protein (Fig. 2.8). In addition to its well-established role as a negative regulator of cytoplasmic β-catenin, we show that APC is also responsible for moving the β-catenin destruction complex to the cell membrane following Wnt exposure. We demonstrate for the first time that the endogenous β-catenin destruction complex reorients toward a localized Wnt signal in an APC-dependent manner in human colon epithelial cells of both normal and malignant origin. Finally, our data support a model whereby the destruction complex remains assembled and bound to β-catenin following Wnt ligand presentation. Because β-catenin accumulates in Wnt-treated cells, it appears that this β-catenin-bound complex is unable to effectively degrade β-catenin. Perhaps APC helps release modified β-catenin from the

destruction complex, thereby enabling its proteasomal degradation as previously proposed [25]. Another possibility is that Wnt stimulation leads to an APC-dependent membrane orientation of the destruction complex which results in complex inactivation. Upon removal of Wnt signal, this already assembled complex would be unlocked and able to process β-catenin for destruction. These two models are not mutually exclusive and might even be interdependent.

Future studies are necessary to clarify the precise mechanism of APC-regulated destruction complex trafficking to the membrane. Or study does not support an absolute requirement for Axin1 in Wnt-mediated destruction complex relocalization. Perhaps Axin2, a Wnt target, is able to compensate for Axin1 in this capacity. Axin has been postulated to localize to Wnt receptors following Wnt activation through a Dvl-dependent mechanism [48]. Based on the compromised destruction complex localization observed in DLD1 cells and APC-depleted HCEC 1CT cells, we suggest that APC is required for Axin docking to membrane-associated Dvl following Wnt exposure. In this light, our results strongly support the model proposed by Tacchelly-Benites et al., based on studies in *Drosophila*, that APC plays a major role in regulating Axin's signalosome recruitment in response to Wnt signaling by facilitating phosphorylation of Axin by GSK-3β [49]. The compromised localization observed in DLD1 cells suggests that C-terminal domains contribute to this trafficking, potentially through cytoskeletal interactions [27–30,50,51]. The mechanism by which APC moves the β-catenin destruction complex toward the signalosome is a necessary focus for future studies in order to provide insight into colon epithelial cell biology and further elucidate unknown aspects of Wnt signal transduction. Overall, our findings provide additional mechanistic details of destruction complex behavior following Wnt exposure and uncover a novel role for APC in Wnt signal transduction.

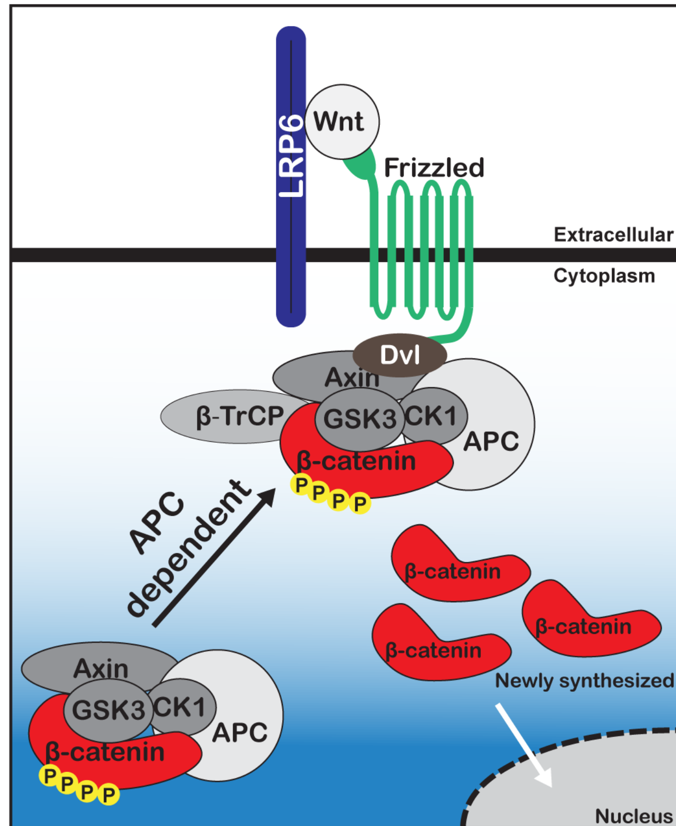
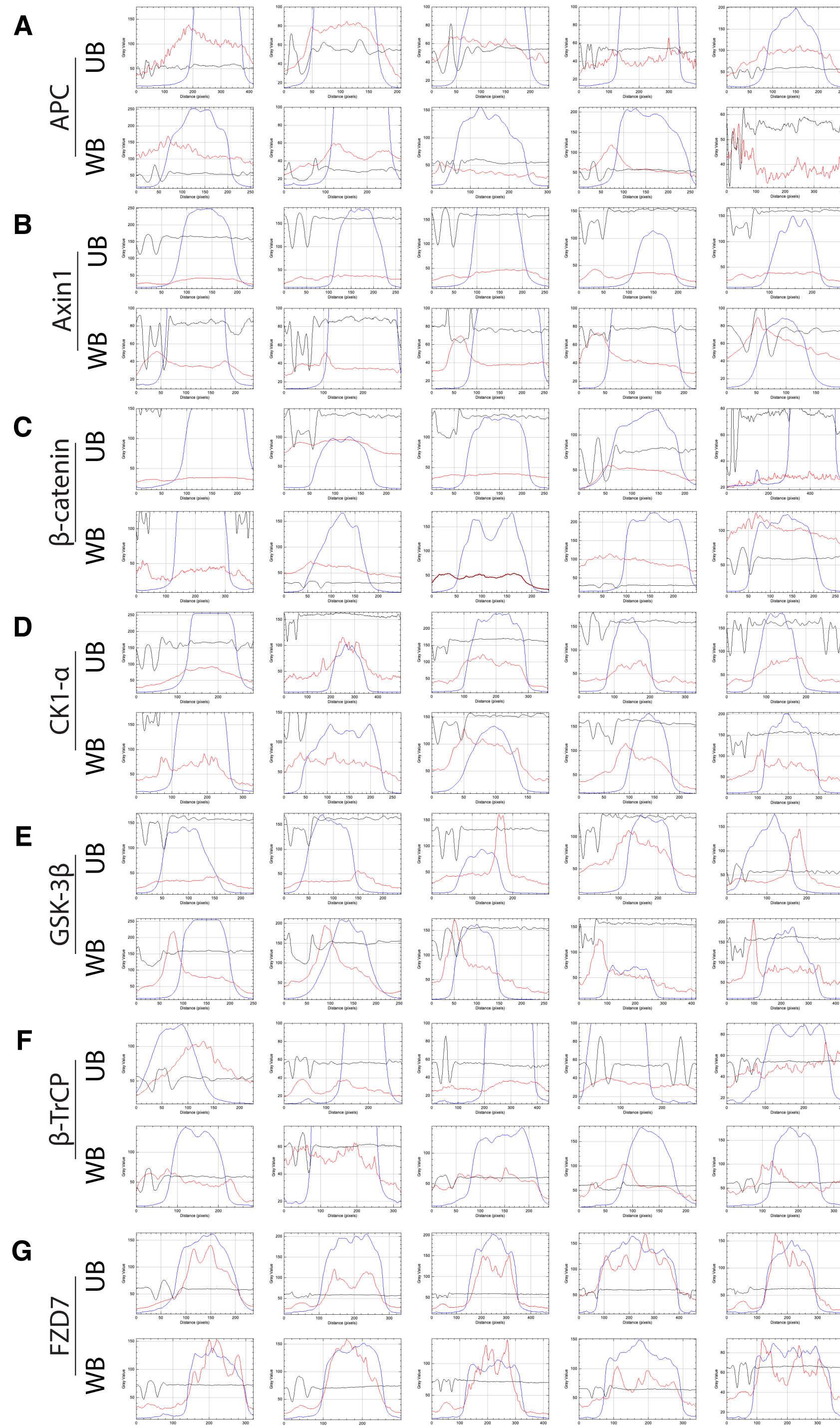
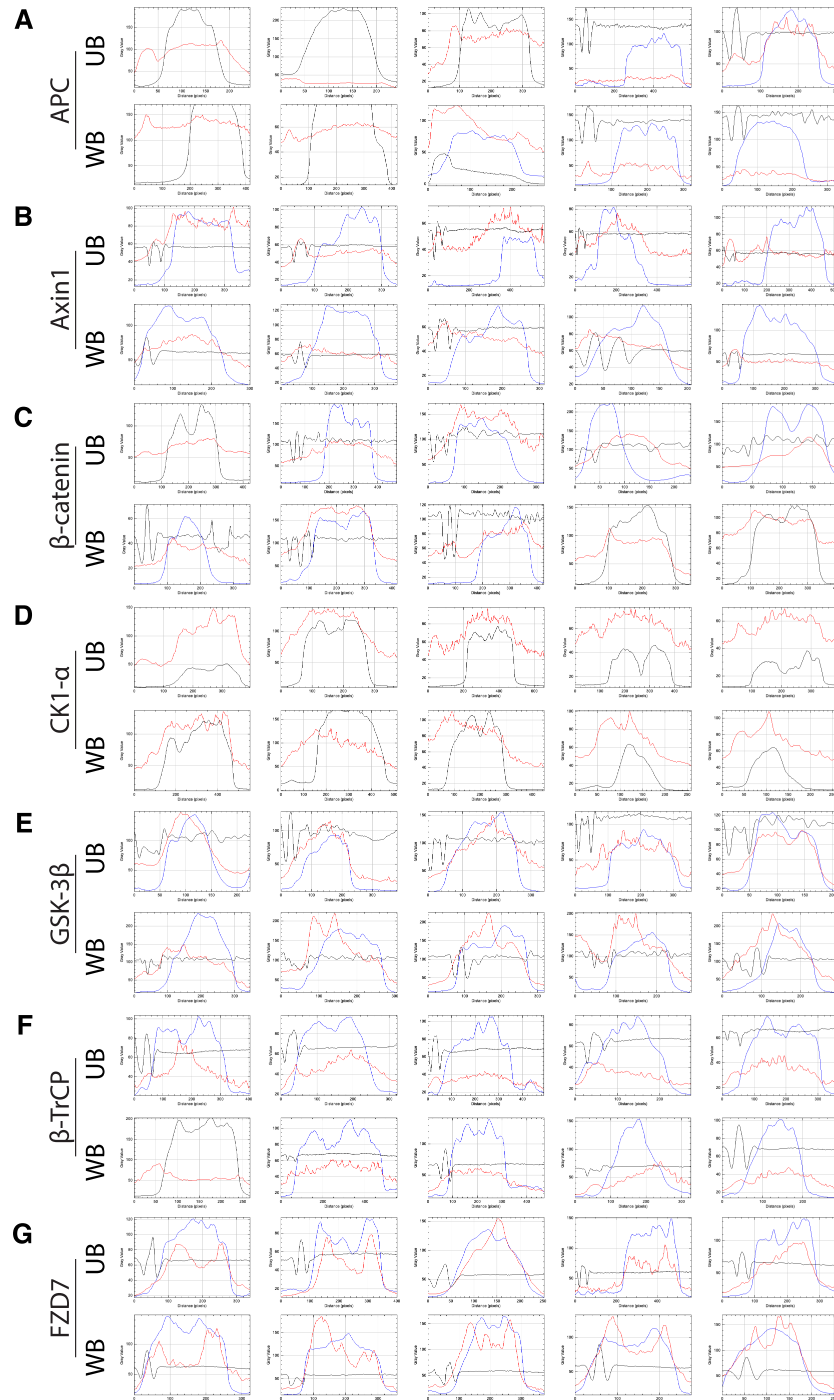


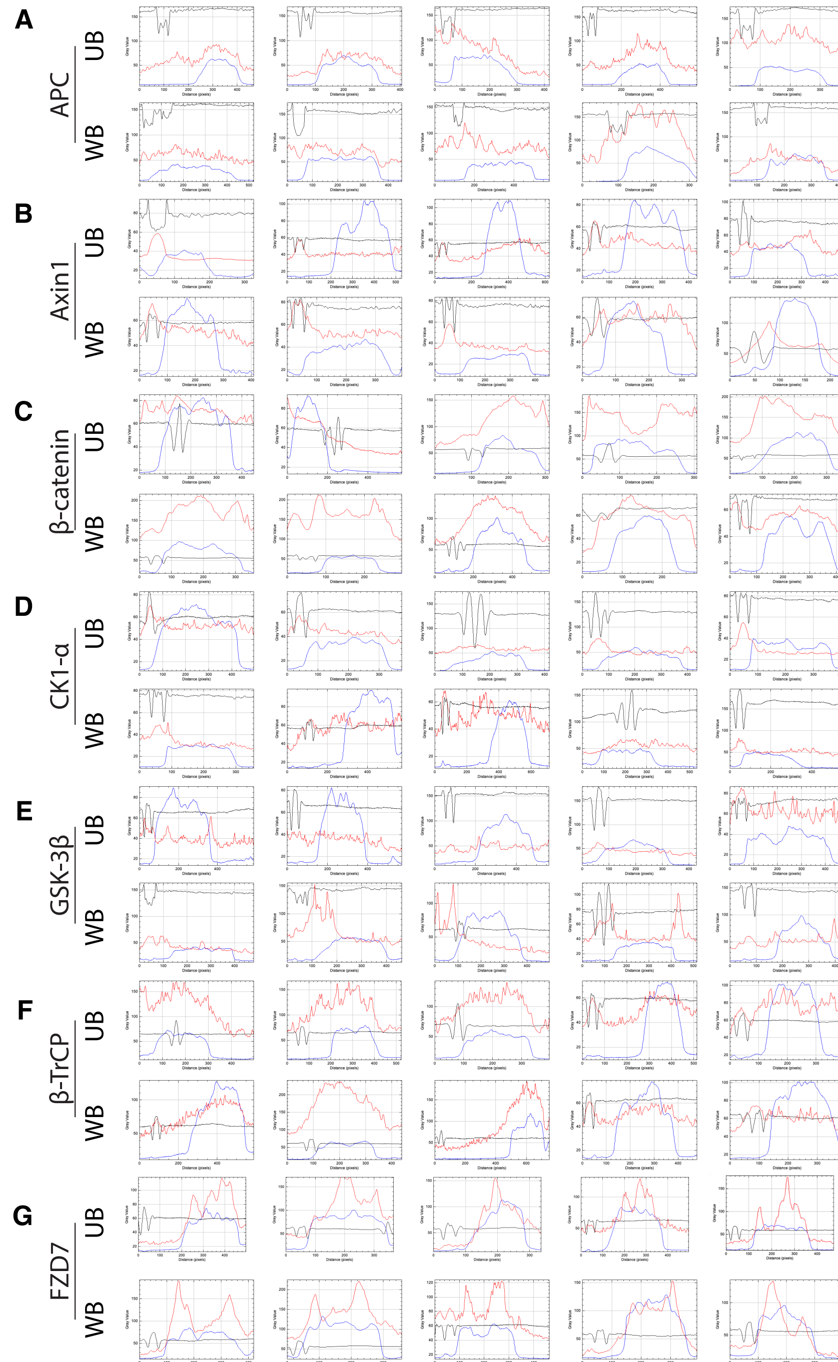

**Figure 2.8** *Proposed model of β-catenin destruction complex response to Wnt.*




**Supplemental Fig. 2.1** *Line scan analysis of signalosome and destruction components in RKO cells.* Line scans were performed on RKO cells containing an intact Wnt signaling pathway using ImageJ/FIJI (NIH). Lines were drawn beginning at the Wnt- or Unloaded-bead and run across the cell nucleus. Line colors correspond to bright-field (black), DAPI (blue), and protein (red). If brightfield image was unavailable, the black line corresponds to DAPI. Line scans correspond to A) APC, B) Axin1, C) β-catenin, D) CK1-α, E) GSK-3β, F) β-TrCP, and G) FZD7. Line scans were performed on five cells per condition.




**Supplemental Fig. 2.2** *Line scan analysis of signalosome and destruction complex in HCT116βm cells*. Line scans were performed on HCT116βm cells containing stabilized β-catenin through Ser45 deletion. Lines were drawn beginning at the Wnt- or Unloaded-bead and run across the cell nucleus. Line colors correspond to bright-field (black), DAPI (blue), and protein (red). If brightfield image was unavailable, the black line corresponds to DAPI. Line scans correspond to A) APC, B) Axin1, C) β-catenin, D) CK1-α, E) GSK-3β, F) β-TrCP, and G) FZD7. Line scans were performed on five cells per condition.




**Supplemental Fig. 2.3** *Line scan analysis of signalosome and destruction complex in DLD1 cells.* Line scans were performed on DLD1 cells which harbor truncated APC. Lines were drawn beginning at the Wnt- or Unloaded-bead and run across the cell nucleus. Line colors correspond to bright-field (black), DAPI (blue), and protein (red). If brightfield image was unavailable, the black line corresponds to DAPI. Line scans correspond to A) APC, B) Axin1, C) β-catenin, D) CK1-α, E) GSK-3β, F) β-TrCP, and G) FZD7. Line scans were performed on five cells per condition.

## Materials and Methods

### *Cell culture and treatments*

RKO, HCT116βm, DLD1, and RKO-APC$^{KO}$ cells were cultured in DMEM (with L-Glutamine and 4.5 g/L Glucose; without Sodium Pyruvate) supplemented with 10% FBS and were maintained at 37°C and 5% $CO_2$. HCEC 1CT cells were generously provided by Dr. Jerry Shay, UT-Southwestern and cultured as described previously in X-media (4:1 DMEM/Medium 199) supplemented with EGF (20ng/ml), hydrocortisone (1µg/ml), insulin (10µg/ml), apo-transferrin (2µg/ml), sodium selenite (5nM), and 2% cosmic calf serum [32]. Cells were maintained at 37°C in low-oxygen incubators to reduce stress-induced senescence from normal atmospheric oxygen tension and to better recapitulate physiological conditions, as described previously [52]. For immunofluorescence assays, cells were seeded onto fibronectin/Poly-D-Lysine coated coverglass (Neuvitro) at 25% confluence and incubated at least 24 hrs prior to treatment with 5µl beads/well for 12-14 hrs. HCEC 1CT cells were seeded at 10-15% confluence prior to siRNA transfection and Wnt-bead treatment.

For siRNA-mediated inhibition, HCEC 1CT cells were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions with 37.5nM of each siRNA targeting human APC (Smartpool siRNAs 1-3; Dharmacon), human Axin1 (Smartpool siRNA 4; Dharmacon) or nontargeting siControl siRNA (Dharmacon). Cell media was changed one day following siRNA transfection, and cells were grown 48 hrs prior to Wnt-bead or Unloaded-bead treatment.

### *Immobilization of Wnt protein*

Wnt3a was immobilized onto Dynabeads as described previously [39]. Briefly, 2.8 µm Dynabeads M-270 Carboxylic Acid (Invitrogen) were activated by NHS/EDC (Sigma, 50mg/ml each in cold 25mM MES pH 5) then washed three times with cold MES buffer. Wnt immobilization was performed by diluting 0.5µg of purified Wnt3a protein (Peprotech, #315-20) in cold MES buffer and incubated at room temperature (RT) for 1 hr. To quench non-reactive carboxylic acid groups, beads were incubated with 50mM Tris pH 7.4 at RT for 15 min. Beads were washed twice in PBS pH 7.4 before final resuspension in 400µl PBS/0.5% BSA and stored at 4°C. Unloaded-beads were prepared in parallel by incubating 1 hr in MES without Wnt. Wnt3a activity following bead immobilization was verified using a TOPflash luciferase reporter assay [53].

***Immunofluorescence***

Cells were briefly rinsed in PBS prior to fixation. Cells were fixed in 4% PFA in Brinkley's Buffer 1980 (80mM PIPES pH 6.8, 1mM $MgCl_2$, 1mM EGTA) for 20 min at RT, washed two times in PBS prior to permeabilization in TBS/0.2% Triton X-100 for 5 min. Cells were washed in TBS two times prior to incubation for 1 hr at RT in blocking buffer containing TBS/0.2% Triton X-100, 1% BSA, and 3% Normal Goat Serum. Primary and secondary antibodies were incubated for 1 hr at RT. Cells were washed in TBS three times following primary and secondary antibody incubations. Coverslips were mounted and counterstained with Prolong Diamond Antifade Mountant with DAPI (Invitrogen). Staining of FZD7 was performed without cell permeabilization or use of detergent to keep the cell membrane intact. Nuclei were counterstained with 5 µg/ml Hoechst 33342 (Invitrogen) in PBS for 5-10 min prior to mounting with Prolong Antifade (Invitrogen). Antibodies were diluted in blocking buffer as follows: anti-

APC-M2 rabbit pAb (1:4,000, [54]), anti-Axin1 rabbit mAb (1:500, Cell Signaling, #2087), anti-β-catenin mouse mAb (1:250, BD Transduction Laboratories, #610154), anti-CK1α rabbit pAb (1:500, Bethyl Laboratories, #A301-991A), anti-GSK3β rabbit pAb (1:500, Bethyl Laboratories, #A302-049A), anti-β-TrCP rabbit pAb (1:500, Abcam, #71753), and anti-Frizzled-7 rabbit pAb (1:500, EMD Millipore, #06-1063), Alexa Fluor 488 and 568 conjugated secondary antibodies (1:1,000, Invitrogen). Stained cells were examined using an Axioplan microscope (Zeiss) with a X100 objective. Images of stained cells were captured using an Orca $R^2$ digital camera (Hamamatsu).

***Immunoblotting***

Cells were washed 1x in PBS prior to harvesting in pre-heated high-salt sample lysis buffer (20% glycerol, 2% SDS, 30% 10x PBS, 2.5% β-mercaptoethanol). Scraped cells were transferred to Eppendorf tubes, heated at 95°C for 1 min, pulled through an insulin syringe three times, and heated again. Samples were separated on 7.5% SDS-PAGE (Bio-Rad, TGX FastCast Acrylamide Kit) using Tris-Glycine running buffer and transferred to a nitrocellulose membrane (GE) with a 0.45-µm pore size. Antibodies were diluted in Odyssey Blocking Buffer TBS (LI-COR) as follows: anti-APC-M2 rabbit pAb (1:2,000), anti-β-catenin mouse mAb (1:1,000), anti-Axin1 goat (1:500, R&D Systems #AF3287), and anti-β-actin mouse mAb (Sigma) (1:1,000), IRDye 680LT and 800CW anti-rabbit or anti-mouse secondary antibodies (1:15,000). Immunoblots were imaged on a LI-COR Odyssey CLx imaging system.

***Wnt-Bead Pull-Down***

Cells were grown in 6-well tissue culture plates and treated with 40µl Unloaded-beads or Wnt-beads for four hours. Following bead treatment, cells were briefly washed in 1x PBS prior to lysis in 200µl lysis buffer, described previously (150mM NaCl, 30mM Tris pH 7.5, 1mM EDTA, 1% Triton X-100, 10% glycerol, 0.1mM PMSF, 0.5mM DTT, and HALT protease and phosphatase inhibitors) [25]. After addition of lysis buffer, cells were scraped into 1.5ml tubes and rotated at 4°C for 30 min. Beads were isolated using a magnet and the supernatant was transferred to a new tube. Beads were washed three times in 500µl of cold lysis buffer. Following the final wash, beads were resuspended in 40µl cold PBS and 20µl 3X SDS sample buffer. Samples were analyzed by western blot as described above.

***Line Scan Analysis***

ImageJ/FIJI (National Institutes of Health, Bethesda, MD) was used for line scan analyses to quantify protein localization in relation to a Wnt- or Unloaded-bead. Line width was set to 50, corresponding to approximately the size of a Dynabead. Lines were drawn beginning at the bead and across the cell, moving through and across the center of the nucleus. For graphical presentation in (Fig. 2.1C-E), each scan was set to zero by subtracting the lowest intensity value across the line. At least five representative cells were measured per condition. Refer to Supplemental Figures S1-S3 for line scan analysis of RKO, HCT116βm, and DLD1 cells.

***Statistical Analysis***

Blinded scoring was performed on a subset of samples using the scoring system presented in Figure 2.1A. Slides were covered and numbered prior to scoring and imaging and decoded following completion of each experiment. Experiments were performed a minimum of

three times with at least 25 cells scored per condition per experiment. T-tests (two-tailed) were performed using GraphPad Prism 8 to determine statistical significance between score frequency in Wnt-bead and Unloaded-bead groups as well as between control siRNA and APC siRNA samples in the HCEC 1CT experiments.

## References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# Chapter 3

## Oncogenic β-catenin mutations are susceptible to Wnt stimulation and regulation by APC

## Abstract

The Wnt/β-catenin signaling pathway is deregulated in nearly all colorectal cancers (CRCs), predominantly through mutation of the tumor suppressor *Adenomatous Polyposis Coli* (*APC*). *APC* mutation is thought to allow a "just-right" amount of Wnt pathway activation by fine-tuning β-catenin levels. While at a much lower frequency, mutations that result in a β-catenin that is compromised for degradation occur in a subset of human CRCs. Here we investigate whether such "stabilized" β-catenin continues to be regulated, thus allowing β-catenin levels conducive for tumor formation. We utilize cells harboring a single mutant allele encoding Ser45-deleted β-catenin (β-catΔS45) to test the effects of Wnt3a treatment or APC-depletion on β-catΔS45 regulation and activity. We find that APC and β-catΔS45 retain interaction with Wnt receptors. Unexpectedly, β-catΔS45 accumulates and activates TOPflash reporter upon Wnt treatment or APC-depletion, but only accumulates in the nucleus upon APC-loss. Finally, we find that β-catenin phosphorylation at GSK-3β sites and proteasomal degradation continue to occur in the absence of Ser45. Our results expand the current understanding of Wnt/β-catenin signaling and provide an example of a β-catenin mutation that maintains some ability to respond to Wnt which may be key to establish β-catenin activity that is "just-right" for tumorigenesis.

## Introduction

Discovered nearly 40 years ago, the Wnt signaling pathway has proven essential for many cellular functions, including proliferation, polarity, developmental cell-fate determination, and tissue homeostasis [1]. Consequently, the Wnt pathway is often deregulated in cancer and

other diseases. Normal colon tissue homeostasis is quite dependent on well-controlled Wnt signaling, as Wnt pathway components are mutated in over 90% of CRCs [2–4].

The key downstream effector molecule in the Wnt pathway is the transcription co-factor β-catenin. In the absence of Wnt signal, a cytoplasmic β-catenin destruction complex efficiently catalyzes the proteasome-mediated degradation of β-catenin. The core components of the complex include two scaffolding proteins, APC and Axin, as well as two kinases, GSK-3β and CK1-α [5]. In the absence of ligand, the complex binds and phosphorylates β-catenin, leading to β-TrCP-mediated ubiquitination and proteasomal degradation [6–8]. Binding of Wnt to membrane-bound co-receptors Frizzled and LRP5/6 results in inhibition of the β-catenin destruction complex through an incompletely resolved mechanism and subsequent β-catenin accumulation, nuclear translocation, and activation of the Wnt transcriptional program [9,10].

In canonical Wnt signaling, β-catenin destruction is initiated through a dual-kinase mechanism. First, CK1-α phosphorylates Ser45 of β-catenin (Fig. 3.1A, D) [8,11]. Next, phospho-Ser45 primes GSK-3β activity, which typically requires a phospho-Ser or threonine and the <u>S/T</u>-X-X-X-pS/pT motif [12–14]. Three sites on β-catenin are then primed and phosphorylated hierarchically, beginning with Thr41, then Ser37, and finally Ser33, as each phosphorylating event primes the next (see Fig. 3.1). Phosphorylation of Ser33 and Ser37 generates a WD40-like binding site for β-TrCP, the substrate recognition subunit of the E3 ubiquitin ligase $SCF^{\beta\text{-}TrCP}$ which ubiquitinates β-catenin and marks it for degradation by the proteasome [7,15–18]. A mutation that eliminates any one of the phosphorylation sites is thought to result in a stabilized β-catenin protein that can evade destruction and activate downstream Wnt signaling.

Mutations in the tumor suppressor *Adenomatous Polyposis Coli* (*APC*) occur early in the development of over 80% of CRCs. The vast majority of *APC* mutations lead to expression of

truncated APC protein that retains some ability to interact with and regulate β-catenin. The "just right" model rationalizes the limited range of APC truncations observed in CRCs as facilitating a precise level of β-catenin for optimal cellular proliferation—not too much or too little [19]. In addition to a scaffolding function, other APC activities have been suggested to contribute to Wnt signaling. For example, APC can interact with nuclear β-catenin, leading to repression of Wnt target genes through several proposed mechanisms: providing access to the transcriptional corepressor CtBP or E3 ligase β-TrCP, sequestration of β-catenin from the transcriptional coactivator LEF-1/TCF, or facilitating β-catenin's nuclear export [20–24]. APC can maintain interaction with β-catenin following Wnt stimulation and appears critical for trafficking the destruction complex to the Wnt receptors [25,26]. Additionally, APC has been postulated to promote β-catenin ubiquitination. APC truncation commonly found in human CRCs renders cells unable to appropriately ubiquitinate β-catenin and target it for proteasomal degradation [25,27,28]. A region of APC C-terminal to common truncations appears to be sufficient for rescue of β-catenin ubiquitination [25,28].

CRCs without *APC* mutations commonly have mutations in genes encoding other components of the Wnt pathway. Despite being the key downstream effector molecule in Wnt signaling, the β-catenin gene, *CTNNB1,* is mutated in only ~9% of CRCs lacking an *APC* mutation. It is curious why mutations which protect β-catenin from degradation are not more prevalent in CRC, as this would be a direct path to β-catenin mediated transcription. It is possible that mutations which completely stabilize β-catenin do not support cell viability. In this event, β-catenin mutations may also act in a "just-right" manner to precisely tune the amount of β-catenin for optimal levels of Wnt activation. Another, not mutually exclusive, possibility is that destroying β-catenin is not the only critical tumor suppressive role for APC, and that additional

APC-mediated processes such as cytoskeletal arrangement, β-catenin localization, and cellular orientation during cell division must be affected to initiate adenoma formation.

Mutations that eliminate the GSK-3β or CK1-α phosphorylation sites are reported to "stabilize" β-catenin, making it resistant to regulation by the destruction complex and thus resistant to regulation by Wnt signaling [5]. Cells expressing β-catenin containing a Ser45 deletion or S33Y substitution are reported to display constitutively active Wnt signaling [29]. HCT116 cells, which express two version of β-catenin, a wild-type and a Ser45-deleted, show elevated Wnt reporter activity when treated with Wnt, however, this was explained by the retained ability to regulate the wild-type β-catenin [25]. While the prevailing notion has been that oncogenic β-catenin mutations result in an abolished response to Wnt, new evidence suggests otherwise. We recently demonstrated that cells expressing only mutant β-catenin (Ser45del, termed β-catΔS45) still show redistribution of both the destruction complex and β-catΔS45 toward a localized Wnt ligand [26]. Phosphorylation at the GSK-3β sites (Ser33/Ser37/Thr41) was previously reported to occur in the absence of Ser45 [30]. These results indicate that the presence of a "stabilized" β-catenin does not render cells completely unresponsive to Wnt and raised additional questions about the mechanism underlying β-catenin regulation. Answers to this question might help explain why *APC* mutations are so dominant in CRC while mutations that alter β-catenin protein are rare.

Here, we sought to test the "just-right" model of β-catenin mutation by examining the Wnt response of HCT116 CRC cells that have been modified to harbor only one *CTNNB1* allele which encodes β-catenin with a Ser45 deletion [31]. Ser45 modifications are seen in roughly one-eighth of those CRCs with β-catenin alterations and are considered to be stabilizing (Fig. 3.1C). We demonstrate for the first time that HCT116βm cells accumulate β-catΔS45 when treated with

Wnt3a or when depleted for APC and also display increased downstream Wnt transcriptional activation. However, β-catΔS45 nuclear translocation is only elevated in cells depleted for APC and not by Wnt3a treatment alone. We also find that β-catΔS45 is phosphorylated on the S33/S37/T41 residues, albeit somewhat less than wild-type β-catenin. To our knowledge, this is the first detailing of possible "just-right" signaling resulting from β-catenin mutation, rather than *APC* mutation. Our work suggests that β-catΔS45 is regulated by the destruction complex and responds to Wnt by accumulating and becoming more active in promoting Wnt target gene transcription. Further, these results implicate additional roles for APC in β-catenin regulation beyond those as a destruction complex scaffold.

## Results

### *Phosphorylation sites important for β-catenin destruction are mutated in a subset of colorectal cancers*

Over-active Wnt signaling has been linked to many malignancies. In liver and endometrial cancer, *CTNNB1* mutations are common while *APC* mutations are rare [32]. In the majority of CRCs, mutations in *APC,* a key member of the β-catenin destruction complex, predominate. In CRCs without *APC* mutation, the Wnt pathway is often activated by other means, such as mutation of another pathway component. To assess the frequency of β-catenin mutation in CRCs, we utilized three datasets from cBioPortal (DFCI, Genentech, and TCGA) and found that β-catenin mutations occurred in 76/1225 of patients (6%), with 14 patients harboring two or more mutations (Fig. 3.1B) [33,34]. Of these β-catenin mutations, 25/91 were in the destruction complex phosphorylation sites (Fig. 3.1C). An additional 25% of the β-catenin

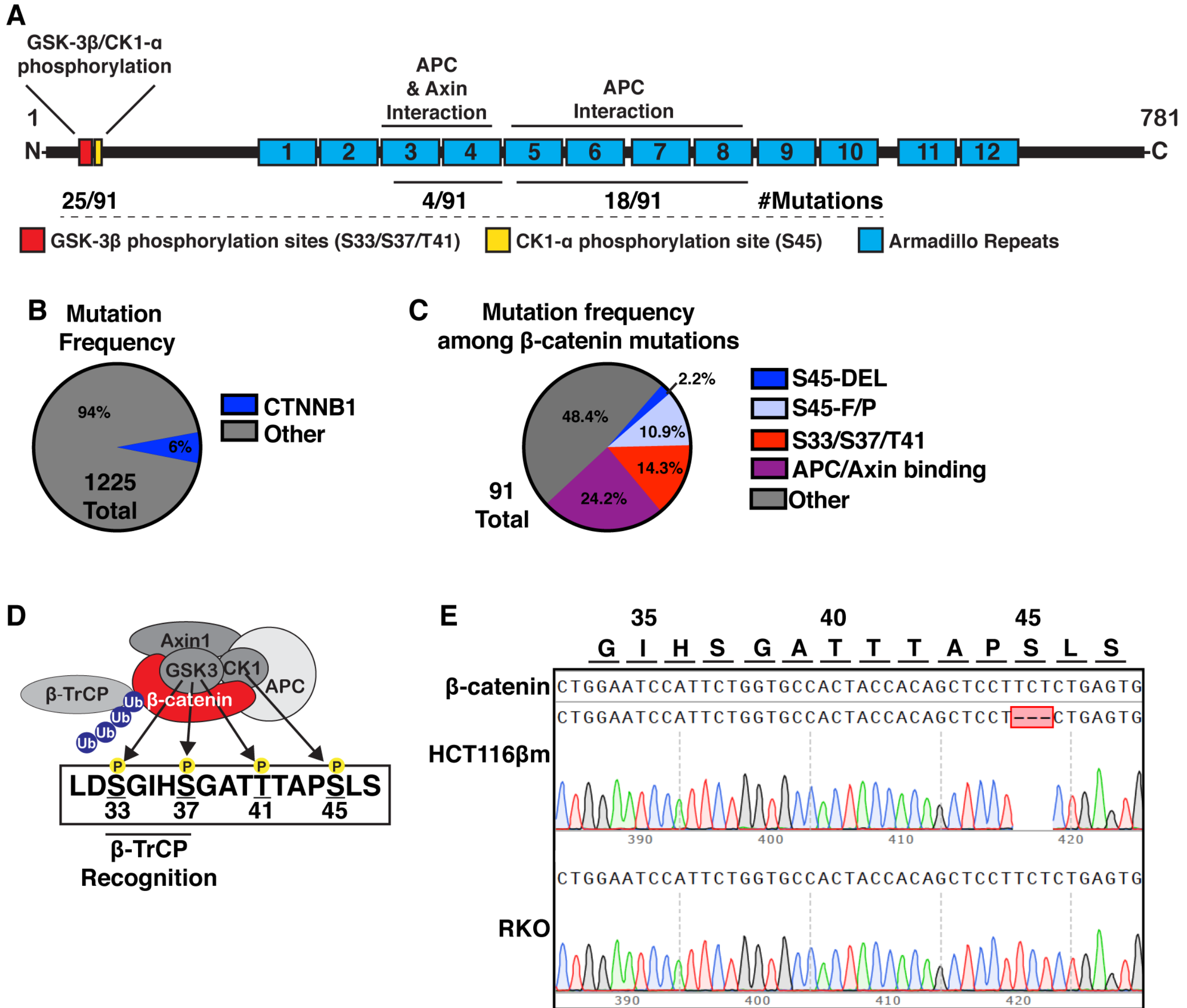


**Figure 3.1** *β-catenin phospho-regulation by the β-catenin destruction complex.* (A) Diagram of β-catenin structure, indicating the N-terminal GSK-3β phosphorylation sites (Ser33/Ser37/T41), the CK1-α phosphorylation site (Ser45), and the twelve armadillo repeats. (B) Mutation frequency of *CTNNB1* among 1,225 colorectal cancer patients using cBioPortal. (C) Mutation frequency of destruction complex phosphorylation sites among *CTNNB1* mutations. (D) Schematic of the β-catenin destruction complex composed of Axin, APC, GSK-3β, and CK1-α, which bind and phosphorylate β-catenin at Ser45, Thr41, Ser37, and Ser33. Phosphorylation at Ser33/Ser37 creates a β-TrCP recognition site. (E) Sanger sequencing and alignment of *CTNNB1* around the destruction complex phosphorylation sites in HCT116βm and RKO colon cancer cell lines.

mutations are in domains that interact with APC or the scaffolding protein Axin. Unexpectedly, in the datasets analyzed, colorectal cancers with wild-type *APC* only displayed mutant *CTNNB1* in 9% of cases (41/448). The "just-right" signaling hypothesis has been proposed as a means for the cell to regulate levels of Wnt activity through mutation of the *APC* gene. We wondered if mutations of the effector protein, β-catenin, also invoke a similar "just-right" response, allowing a specific level of Wnt regulation.

The parental HCT116 colon cancer cell line harbors one mutant and one wild-type β-catenin allele. The HCT116βm cell line, was modified to contain a single allele encoding β-catenin with a Ser-45 deletion (herein referred to as βcatΔS45) [31]. We verified that a single allele encoding β-catenin is present in these cells by performing Sanger sequencing in both HCT116βm cells and RKO cells which express only wild-type β-catenin (Fig. 3.1D). A previous report that parental HCT116 cells were Wnt-responsive attributed this finding to regulation of β-catenin protein encoded by the wild-type allele (Li et al., 2012). Another report suggests that βcatΔS45 can be phosphorylated at S33/S37/T41 [30]. Here we consider the possibility that there are mechanisms of β-catenin regulation in response to Wnt ligand that are independent of S45 phosphorylation.

***β-catΔS45 associates with a locally-applied Wnt-3a ligand***

Using immunofluorescence microscopy, we previously established that β-catΔS45 localizes toward a Wnt cue in HCT116βm cells [26]. To test for a physical interaction between Wnt and β-catΔS45, Wnt3a-conjugated beads were applied to HCT116βm cells and then used to "pull down" associated proteins from cell lysates (Fig. 3.2A). APC associated with Wnt-beads more than with the Unloaded-beads (Fig. 3.2B). β-catΔS45 also associated more with the Wnt-beads

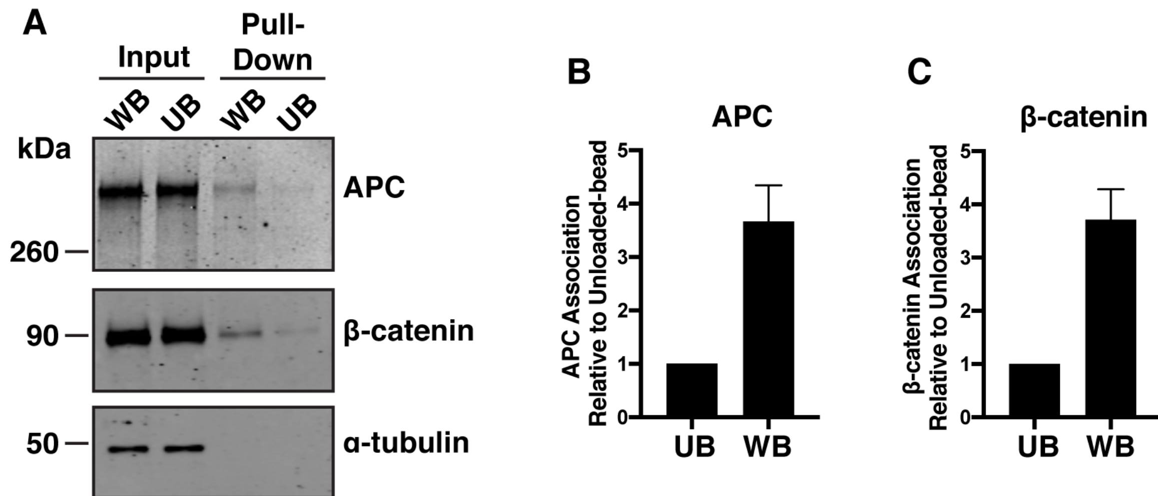


**Figure 3.2** *Wnt-beads pull-down APC and β-catΔS45.* (A) HCT116βm cells treated with Wnt-beads or Unloaded-beads were lysed and proteins were pulled-down with the beads. Both APC and β-catΔS45 were detected in the Wnt-bead pull-down but not the Unloaded-bead pull-down. (B) Quantification of APC pulled-down by Wnt-beads compared to Unloaded-beads from three independent experiments. (C) Quantification of β-catΔS45 pulled-down by Wnt-beads compared to Unloaded-beads from three independent experiments. Protein levels that were pulled-down by beads were divided by the respective input protein levels and normalized to the Unloaded-bead to demonstrate fold change. Error bars, SEM.

than with the Unloaded-beads (Fig. 3.2C). These data demonstrate that a core component of the destruction complex and a "stabilized" β-catenin both respond to a Wnt3a cue by localizing to the membrane, presumably through interactions with Frizzled and LRP5/6 co-receptors.

***Wnt3a exposure or APC-depletion increases level of β-catΔS45 protein***

We previously reported that β-catenin destruction complex localization toward a Wnt cue was APC-dependent and appeared to correlate with an increased level of β-catenin in cells harboring fully intact Wnt signaling pathways such as RKO and HCEC-1CTs (Parker and Neufeld, 2020). Whether Wnt influences β-catΔS45 protein levels has yet to be determined. The β-catΔS45 protein expressed in HCT116βm cells lacks the site of CK1-α phosphorylation and is therefore generally assumed to be compromised for phosphorylation by GSK3β and subsequent proteasome-mediated destruction. Notably, treatment with Wnt3a resulted in a 1.57-fold increase in total β-catΔS45 compared to untreated cells (Fig. 3.3A, C). This unexpected result indicates that HCT116βm cells at least partially respond to Wnt by stabilizing β-catΔS45 and therefore, β-catenin degradation can occur independent of Ser45. We efficiently depleted 90-95% of APC in HCT116βm cells with small interfering RNA (siAPC) (Fig. 3.3A, B). This APC depletion led to a 1.47-fold increase in total β-catΔS45 protein level while APC depletion combined with Wnt3a treatment led to a 1.31-fold increase (Fig. 3.3C). All of these increases were significant when compared to control-siRNA-treated cells (NT siRNA). These data indicate that cells exposed to Wnt ligand are able to further increase β-catΔS45 levels and that APC participates in β-catΔS45 destruction. Because APC depletion together with Wnt treatment did not result in β-catΔS45 protein levels greater than either condition alone, it seems likely that these two components function in the same pathway to mediate β-catΔS45 protein levels.

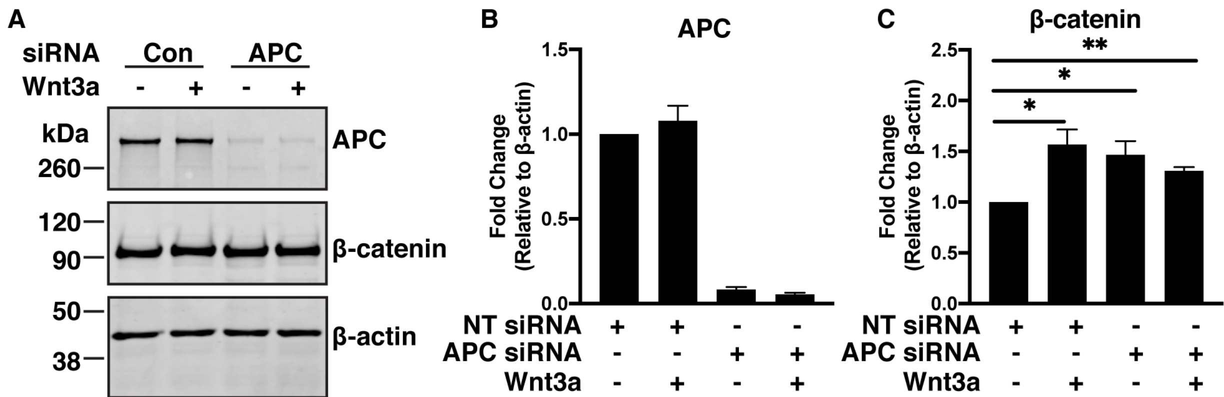


**Figure 3.3** *β-catΔS45 accumulates upon Wnt-3a treatment or APC-depletion.* (A) Western blot of HCT116βm cells transfected with control-siRNA or APC-siRNA and treated in the presence or absence of 125ng Wnt3a. (B) Quantification of APC fold-change relative to β-actin and normalized to control siRNA without Wnt. (C) Relative fold-change of β-catenin relative to β-actin and normalized to control siRNA without Wnt. Data averaged from four independent experiments. Error bars, SEM; Statistical analysis by t-test: *P<0.05; **P<0.01; ***P<0.0001.

***HCT116βm cells activate a Wnt reporter following APC-depletion or Wnt3a exposure***

In cells with an intact Wnt signaling pathway, cellular β-catenin accumulation is followed by β-catenin's nuclear translocation and interaction with the transcription co-factor TCF-4 to activate Wnt target genes. HCT116βm cells harbor an activated Wnt pathway, demonstrated through increased TOPflash compared to isogenic cells only expressing wild-type β-catenin [31]. We wondered if the increased level of cellular β-catenin following Wnt exposure or APC-depletion would result in further increases in nuclear β-catenin activity. To test this, we co-transfected HCT116βm cells with TOPflash Wnt luciferase reporter plasmid and then depleted APC with siRNA, in the presence or absence of Wnt3a. Wnt3a treatment resulted in a 3-fold increase and APC depletion led to a 2.2-fold increase in Wnt reporter activity (Fig. 3.4A). Unexpectedly, cells both depleted for APC and exposed to Wnt3a displayed a 4-fold increase in Wnt reporter activity (Fig. 3.4A). In RKO cells, which possess an intact Wnt signaling pathway, Wnt treatment resulted in an 8.2-fold increase in Wnt reporter activity (Fig. 3.4B). However, in RKO cells with CRISPR-deleted APC or in DLD1 cells which express endogenous truncated APC, Wnt3a presentation has no effect on Wnt reporter activity (Fig. 3.4B).

Combined, these data indicate that β-catΔS45 is still regulated by Wnt signaling, despite being able to evade Ser45 phosphorylation by CK1-α. Interestingly, cells with *APC* knock-out or mutant *APC* are resistant to further Wnt reporter activation, presumably due to maximal levels of pathway activation (Fig. 3.4B) [25]. Yet, HCT116βm cells containing a stabilized β-catΔS45 are responsive to APC depletion, even more responsive to Wnt addition, and show the most Wnt reporter activation when APC-depletion is combined with Wnt stimulation. This finding suggests that Wnt3a presentation and APC loss may stimulate Wnt reporter activity in an additive manner. This additive response may indicate involvement of multiple pathways.

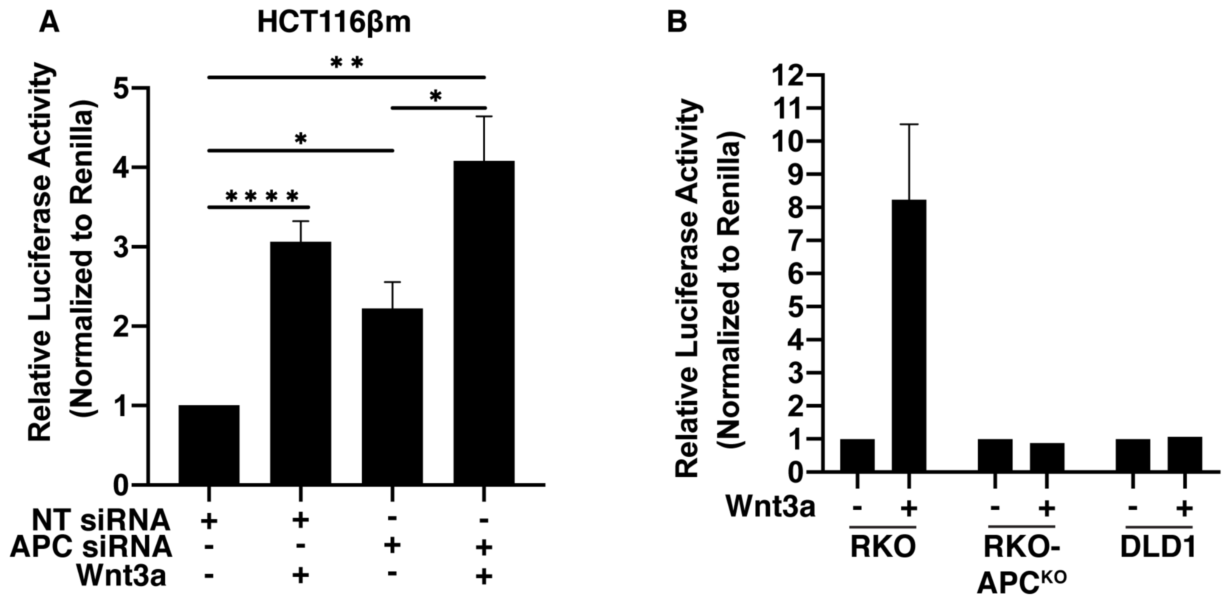


**Figure 3.4** *The Wnt transcriptional program is induced in HCT116βm upon Wnt-3a treatment or APC-depletion.* (A) HCT116βm cells were co-transfected with pTOPflash and pRenilla luciferase-reporter plasmids as well as control-siRNA or APC-siRNA, and subsequently treated with 125ng Wnt3a for 24 hours. Data averaged from seven individual experiments. Relative luciferase activity determined by the TOPflash/Renilla ratio of each group, followed by normalization to the control. Error bars, SEM; Statistical analysis by t-test: *P<0.05; **P<0.01; ***P<0.0001. (B) TOPflash-reporter assay in RKO, RKO-$APC^{KO}$, and DLD-1 colorectal cancer cell lines. Data for RKO averaged from four individual experiments, and RKO-$APC^{KO}$ and DLD1 are from one experiment. Error bars, SEM.

***Increased nuclear localization of β-catΔS45 upon APC-loss but not Wnt exposure***

APC depletion has revealed a mechanism to regulate β-catΔS45 activity that appears distinct from that of Wnt stimulation. To explain this, we turned to other potential APC functions. Notably, APC is reported to be involved in sequestration, trafficking, and nuclear shuttling of proteins as well as Wnt-induced membrane localization of the destruction complex. We therefore considered the possibility that APC aids in the sequestration or trafficking of β-catΔS45, despite the ability of β-catΔS45 to evade destruction complex mediated Ser45 phosphorylation. We found that cells treated with Wnt displayed no changes in the ratio of nuclear to cytoplasmic β-catΔS45 compared to control cells (Fig. 3.5A, B). However, upon APC-depletion, β-catΔS45 shifted into the nucleus, displaying an increased nuclear/cytoplasmic ratio and increased level of nuclear β-catenin compared to control (Fig. 3.5A-C). The ratio of nuclear to cytoplasmic β-catΔS45 seen with APC depletion did not further increase with added Wnt treatment (Fig. 3.5B). Although still more nuclear than in control cells, the percent of change in nuclear β-catΔS45 in APC-depleted cells that were also treated with Wnt was significantly less than in cells only depleted of APC (Fig. 3.5C). These data indicate that regulation of a "stabilized" β-catΔS45 is not merely through a degradation mechanism, but also may involve cytoplasmic sequestration or nuclear export, facilitated by APC.

Assessment of β-catΔS45 nuclear and cytoplasmic localization showed that Wnt treatment alone does not affect nuclear translocation. However, APC knockdown or Wnt treatment plus APC-knockdown caused a dramatic increase in nuclear β-catΔS45 compared to control. Of note, through assessment of β-catenin level (Fig. 3.3), β-catΔS45 activity (Fig. 3.4), and β-catΔS45 nuclear translocation (Fig. 3.5), we found that increases in the level and nuclear localization of β-catΔS45 did not always translate into increased activity. Using these different

data sets, we normalized the TOPflash values to the level of nuclear β-catΔS45 to estimate activity per unit of nuclear β-catΔS45. This analysis revealed that, though there was little change in nuclear β-catΔS45 levels, Wnt treatment alone resulted in a near doubling of the β-catΔS45 activity (1.92-fold) compared to untreated cells. In contrast, APC-depletion resulted in more β-catenin protein and nuclear localization but did not alter the activity per β-catΔS45 unit (1.05-fold) compared to untreated cells. The combination of APC-depletion and Wnt treatment resulted in elevated β-catenin protein and nuclear localization, similar to that observed with only APC-depletion. But the activity per β-catΔS45 unit was much higher (2.5-fold) than that of untreated cells or only APC-depleted cells.

***β-catΔS45 is phosphorylated at the GSK-3β sites and susceptible to proteasomal degradation***

Solely considering the destruction complex, it is perplexing that APC depletion or Wnt exposure would impact the protein level of a "stabilized" β-catenin. Previously, it was reported that β-catΔS45 is phosphorylated at the GSK-3β sites Ser33/Ser37/Thr41[30]. However, it was unknown whether β-catΔS45 is also degraded by the proteasome. We detected more total β-catenin and phospho-Ser33/Ser37/Thr41-β-catenin in RKO cells treated with MG132 proteasome inhibitor (Fig. 3.5D). This was expected since RKO cells have an intact β-catenin destruction complex. The ratio of p-β-catenin to total β-catenin decreased in RKO cells treated with MG132 (Fig. 3.5F). This decrease is potentially due to the large increase in total β-catenin level (~14-fold), and may reflect saturated destruction complexes unable to phosphorylate all of the accumulated β-catenin as previously proposed [25]. We also detected phospho-β-catΔS45 in HCT116βm cells, thus confirming previous reports (Fig. 3.5D) [30]. MG132 treatment resulted in increased total β-catΔS45 and phospho- β-catΔS45 levels, indicative of proteasomal degradation

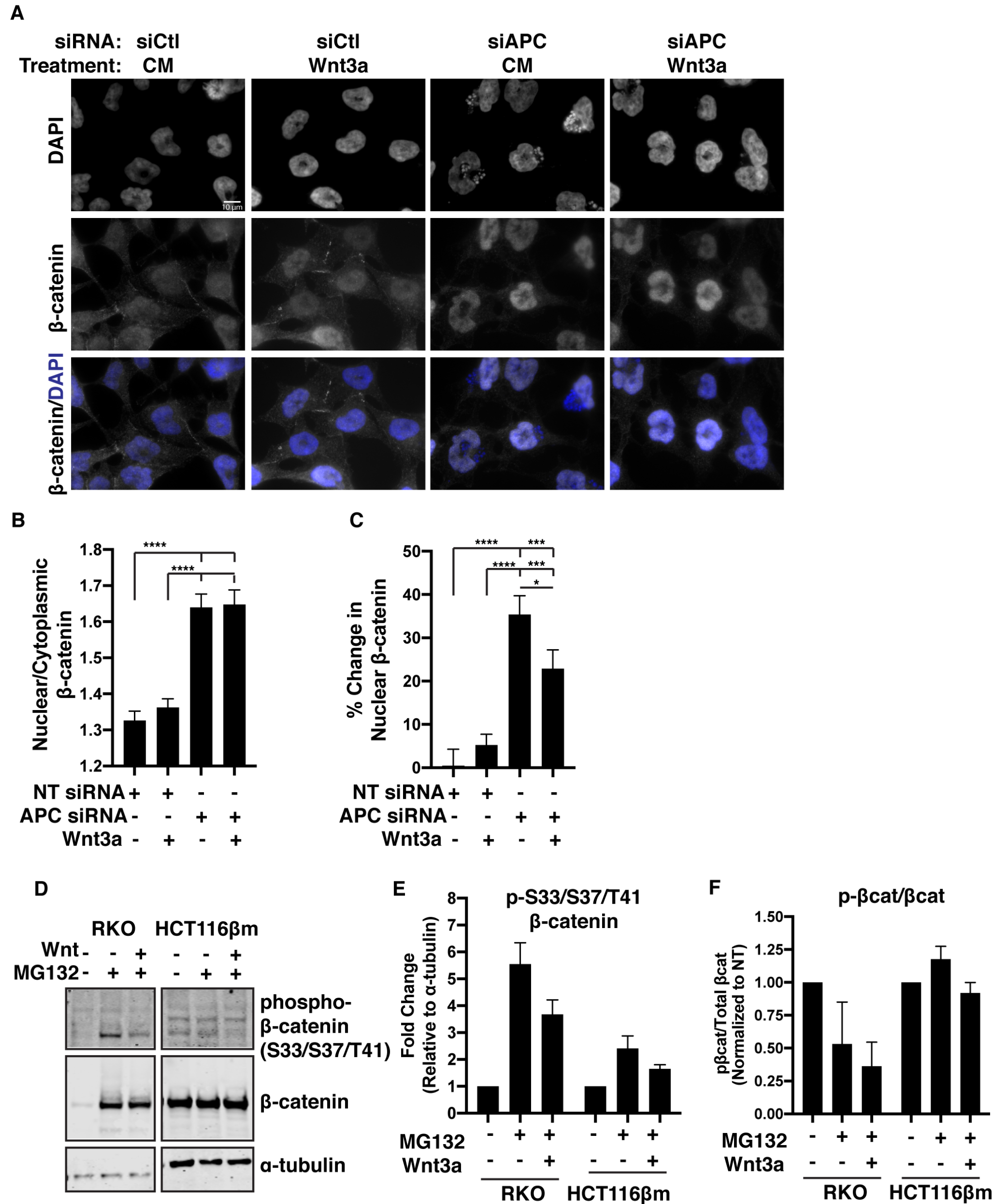


**Figure 3.5** *β-catΔS45 nuclear localization is controlled by APC and continues to be susceptible to GSK-3β phosphorylation and proteasomal degradation.* (A) HCT116βm cells transfected with control-siRNA or APC-siRNA were treated with 125ng Wnt3a and β-catenin localization was visualized by immunofluorescent detection. (B) Ratio of nuclear to cytoplasmic β-catenin quantified from immunofluorescent staining. Data representative of at least 36 individual cells per group. Error bars, SEM; Statistical analysis by t-test: *P<0.05; **P<0.01; ***P<0.0001. (C) Percent change in nuclear β-catenin. Percent of nuclear β-catenin was calculated by dividing nuclear β-catenin by total β-catenin. Using nuclear β-catenin percentage, the percent change was calculated by comparing to control. (D) RKO and HCT116βm cells were treated with 10mM MG132 in the presence or absence of 25ng/ml Wnt3a for four hours prior to lysis. Western blot utilizing anti-phospho-Ser33/Ser37/Thr41-β-catenin or anti-β-catenin antibodies. (E) Quantification of fold change of Ser33/Ser37/Thr41-phosphorylated β-catenin. Data averaged from two independent experiments. Error bars, SEM. (F) Quantification of the ratio of phosphorylated-β-catenin to total β-catenin in RKO and HCT116βm cells following treatment with MG132 +/- Wnt3a. Data averaged from two independent experiments. Error bars, SEM.

(Fig. 3.5E). However, we did not observe a decreased ratio of p-β-catΔS45/β-catΔS45 in HCT116βm cells treated with both MG132 and Wnt (Fig. 3.5F). Together, these data support a potential mechanism in which cells containing a deletion of the CK1-α phosphorylation site are able to maintain some β-catenin regulation through the potential alternative priming of GSK-3β-mediated phosphorylation and through proteasomal degradation. Of note, this ability to respond to Wnt-ligand distinguishes β-catenin-mutant colorectal cancer cells from the APC-mutant colorectal cancer cells (Fig. 3.4B).

**Discussion**

The Wnt/β-catenin pathway is typically described as signaling in a linear manner; Wnt ligand binds the Frizzled and LRP5/6 coreceptors, leading to inhibition of the β-catenin destruction complex and subsequent β-catenin stabilization, accumulation, and nuclear translocation. Mutation to a downstream component such as APC or β-catenin is thought to activate the pathway and render it unresponsive to regulation by upstream pathway components. The results presented here demonstrate that "downstream" components of the Wnt pathway can continue to be regulated by "upstream" components despite the presence of stabilizing mutations. In the context of APC mutations, a "just-right" signaling hypothesis has been proposed in which *APC* mutations occur to allow a specific level of β-catenin that supports tumorigenesis. Here, we propose that β-catenin itself may also be prone to just-right mutations that maintain some regulation of the β-catenin protein, limiting its accumulation and activity. As an initial proof of concept, we used the HCT116βm cell line which harbors a deletion of β-

catenin Ser45, to assess the Wnt/Receptor/β-catΔS45 interaction and the changes in β-catΔS45 protein levels, activity, and localization upon Wnt3a stimulation or APC loss.

In agreement with our previous immunofluorescence data [26], we demonstrate that both β-catΔS45 and APC pull-down with a Wnt-bead, indicative of interaction between Wnt ligand, receptor, β-catΔS45, and APC. Surprisingly, we find that Wnt3a treatment, APC knock-down, or treatment plus knock down, results in elevated β-catΔS45 protein levels as well as increased TOPflash Wnt reporter activity. APC-depletion results in predominantly nuclear β-catΔS45, whereas Wnt treatment alone does not change the nuclear localization of β-catΔS45. Finally, we confirm that β-catΔS45 can be phosphorylated by GSK-3β at Ser33/Ser37/T41 and find that β-catΔS45 is regulated by the proteasome.

We propose the following expansion of the current Wnt signaling mechanism to explain our findings: 1) Wnt signaling leads to a secondary effect that further activates β-catenin in the nucleus, essentially amplifying the signal. An example of this would be that LEF/TCF is a Wnt target gene [35,36]. 2) APC inhibits the activity of this Wnt-induced β-catenin inducer. 3) APC promotes cytoplasmic localization of β-catenin, perhaps through cytoplasmic sequestration or nuclear to cytoplasmic shuttling. Our results are inconsistent with a role of APC in sequestering nuclear β-catenin or facilitating nuclear import of β-catenin. 4) Wnt can also inhibit nuclear localization of β-catenin, independent of APC. Our work is in agreement with previously published results by our lab and others, demonstrating that interaction of APC with nuclear β-catenin leads to repression of Wnt target genes [20,21,23,24]. Further, it is intriguing that β-catΔS45 continues to be phosphorylated by GSK-3β and degraded by the proteasome, albeit less efficiently than wild-type β-catenin. The deletion of Ser45 may place Ser47 in close enough proximity (within 5 residues upon S45del) to T41 to act as a priming site for β-catenin

phosphorylation [37]. Suggestive of alternative mechanisms for GSK-3β phosphorylation, S33/S37/T41 phosphorylation is also observed in LS174T cells containing a S45F substitution [25,30].

Colorectal cancer is consistently promoted by Wnt signal activation. Here we provide insight into both the mechanism by which β-catenin is regulated in the Wnt-on or Wnt-off states and demonstrate that β-catenin mutations can be regulated by additional Wnt signaling. We find that APC mutant cells are unresponsive to additional Wnt signaling, whereas β-catenin mutant cells are responsive to extracellular Wnt ligand. Perhaps complete stabilization of β-catenin would result in too much β-catenin protein and activity and impair cell viability. Our finding that the Wnt/β-catenin signaling pathway does not act in a strictly linear fashion emphasizes the importance of a therapeutic strategy which targets multiple aspects of the pathway. Finally, we propose that some forms of mutant β-catenin are still susceptible to regulation and that such mutations may act to provide "just-right" signaling for optimal tumorigenic capability.

## Materials and Methods

### *Cell Culture and Treatments*

HCT116βm, RKO, DLD1, and RKO-APC$^{KO}$ cells were cultured in DMEM (with L-Glutamine and 4.5g/L Glucose; without Sodium Pyruvate) supplemented with 10% FBS and were maintained at 37°C and 5% $CO_2$. Wnt treatment was performed by adding Recombinant Wnt-3a (Peprotech #315-20) at the indicated concentration/time prior to cell lysis or immunofluorescence analysis. For siRNA-mediated inhibition, HCT116βm cells were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions

with 37.5nM of each siRNA targeting human APC (Smartpool siRNAs 1-3: Dharmacon) or non-targeting siControl siRNA (Dharmacon). Cell media was changed one day following siRNA transfection, and cells were grown 48 hrs prior to Wnt treatment. MG-132 treatment was performed by addition of 10mM MG-132 to a final concentration of 10μM in cell media for four hours.

***Analysis of CTNNB1 mutation frequency***

Mutation frequency of the *CTNNB1* gene was assessed using the cBioPortal [33,34]. Colorectal adenocarcinoma datasets from DFCI (Cell Reports 2016), Genentech (Nature 2012), and TCGA (PanCancer Atlas) were queried for the *CTNNB1* and *APC* genes [38–40]. Of the 1,287 total cases, 1,225 had mutations data and were utilized for mutation frequency and mutual exclusivity determinations.

***CTNNB1 Sanger Sequencing and Alignment***

HCT116βm and RKO cells were harvested and DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit according to manufacturer's instructions. PCR amplification was performed using the following primers: 5'-cctcctaatggcttggtgaa-3'; 5'-caggacttgggaggtatcca-3'. Following amplification, PCR products were gel-purified and sequenced by Genewiz. Trace files and sequence alignment were analyzed using SnapGene software (Insightful Science).

***Immobilization of Wnt Protein***

Wnt3a was immobilized onto Dynabeads as described previously [41]. Briefly, 2.8µm Dynabeads M-270 Carboxylic Acid (Invitrogen) were activated by NHS/EDC (Sigma, 50 mg/ml each in cold 25mM MES pH 5) then washed three times with cold MES buffer. Wnt immobilization was performed by diluting 0.5µg of purified Wnt3a protein in cold MES buffer and incubated at room temperature (RT) for 1hr. To quench non-reactive carboxylic acid groups, beads were incubated with 50mM Trish pH 7.4 at RT for 15min. Beads were washed twice in PBS pH 7.4 before final resuspension in 400µl PBS/0.5% BSA and stored at 4°C. Unloaded-beads were prepared in parallel by incubating 1hr in MES without Wnt. Wnt3a activity following bead immobilization was verified using a TOPflash luciferase reporter assay [42].

***Immunoblotting***

Cells were washed 1x in PBS prior to harvesting in pre-heated high-salt sample lysis buffer (20% glycerol, 2% SDS, 30% 10X PBS, 2.5% β-mercaptoethanol). Scraped cells were transferred to Eppendorf tubes, heated at 95°C for 1min, pulled through an insulin syringe three times, and heated again. Samples were separated on 7.5% SDS-PAGE (Bio-Rad, TGX FastCast Acrylamide Kit) using Tris-Glycine running buffer and transferred to a nitrocellulose membrane (GE) with a 0.45-µm pore size. Antibodies were diluted in Odyssey Blocking Buffer TBS (LI-COR) as follows: anti-APC-M2 Chicken pAb (1:2,000), anti-β-catenin mouse mAb (1:1,000), anti-phospho-Ser33/Ser37/Thr41-β-catenin rabbit pAb (Cell Signaling, 1:500), anti-α-tubulin DM1A mouse mAb (Santa Cruz, 1:1,000), anti-β-actin mouse mAb (Sigma, 1:1,000), and IRDye 680LT and 800CW anti-rabbit, anti-mouse, or anti-chicken secondary antibodies (1:15,0000). Immunoblots were imaged on a LI-COR Odyssey CLx imaging system.

***Wnt-Bead Pull-Down***

Cells were grown in 6-well tissue culture plates and treated with 40µl Unloaded-beads or Wnt-beads for four hours. Following bead treatment, cells were briefly washed in 1x PBS prior to lysis in 200µl lysis buffer, described previously (150mM NaCl, 30mM Tris pH 7.5, 1mM EDTA, 1% Triton X-100, 10% glycerol, 0.1mM PMSF, 0.5mM DTT, and HALT protease and phosphatase inhibitors) [25,26]. Following the addition of lysis buffer, cells were scraped into 1.5ml tubes and rotated at 4°C for 30min. Beads were isolated using a magnet and the supernatant was transferred to a new tube. Beads were washed three times in 500µl of cold lysis buffer. Following the last wash, beads were resuspended in 40µl cold PBS and 20µl 3X SDS sample buffer.

***Luciferase Reporter Assay***

Cells were seeded into 12-well plates 24 hours prior to transfection. On day of transfection, cell culture media was changed one hour prior to siRNA and plasmid transfection. Both reporter plasmids and siRNAs were transfected concurrently using Lipofectamine 3000 according to manufacturer's instructions. Human APC siRNA or control siRNA was transfected as done above. For luciferase reporters, cells were co-transfected with TOPflash (450ng) and Renilla (50ng) expression plasmids. As a control, identical wells were transfected with FOPflash (450ng) and Renilla (50ng) expression plasmids to validate that a scrambled TCF-binding sequence does not result in increased reporter activity upon treatment. Following two days of APC-depletion, cells were treated with Wnt3a for 24h and lysed using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase signal was normalized to Renilla luciferase signal, and data were normalized to control (set to 1).

***Immunofluorescence and Analysis***

Cells were briefly rinsed in PBS prior to fixation. Cells were fixed in 4% PFA in Brinkley's Buffer 1980 (80mM PIPES pH 6.8, 1mM $MgCl_2$, 1mM EGTA) for 20min at RT, washed two times in PBS prior to permeabilization in TBS/0.2% Triton X-100 for 5min. Cells were washed in TBS two times prior to incubation for 1hr at RT in blocking buffer containing TBS/0.2% Triton X-100, 1% BSA, and 3% Normal Goat Serum. Primary and secondary antibodies were incubated for 1hr at RT. Cells were washed three times in TBS following primary and secondary antibody incubations. Mounting of coverslips was performed using Prolong Diamond Antifade Mountant with DAPI (Invitrogen). Antibody was diluted in blocking buffer as follows: anti-β-catenin mouse mAb (1:250, BD Transduction Laboratories, #610154). Stained cells were examined using an Axioplan microscope (Zeiss) with a X100 objective. Images of stained cells were captured using an Orca $R^2$ digital camera (Hamamatsu).

For calculation of the nuclear and cytoplasmic distribution of β-catenin, CellProfiler software [43] was used to identify and measure the total and mean pixel intensities of β-catenin protein in cytoplasmic or nuclear compartments. Briefly, nuclei identification was first performed using the DAPI image, followed by cell identification by propagation. Cytoplasm was identified using the identified "total cells" and the identified nuclei to remove nuclei from cytoplasmic calculation. Automated pipeline creation was performed using the following as a guide: https://github.com/CellProfiler/tutorials/blob/master/Translocation/Translocation.pdf.

***Quantification and Statistical Analysis***

Fluorescent-detection and quantification of immunoblots was performed using the LI-COR Odyssey CLx imaging system and Image Studio™ Lite software (LI-COR). Graphs were

generated using Graphpad Prism (GraphPad Software, Inc.), and all statistical analyses were performed using a two-tailed, unpaired t-test in which a value of $p<0.05$ is statistically significant.

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# Chapter 4

# Dynamics of the Wnt Response

**Abstract**

The Wnt signaling pathway is essential for the maintenance of intestinal homeostasis and is frequently deregulated in human colorectal cancer (CRC) due to mutation to the tumor suppressor, *Adenomatous Polyposis Coli* (*APC*). The stem cells of the intestine are regulated by locally secreted Wnt ligands which maintain their proliferative capacity. For this reason, the appropriate balance of Wnt pathway activation is crucial to intestinal homeostasis. Regulation of the Wnt pathway occurs through strict control of β-catenin levels by a cytoplasmic β-catenin destruction complex composed of APC, Axin, CK1-α, and GSK-3β. We have previously demonstrated that Wnt stimulation relocalizes the destruction complex to the Wnt receptors in an APC-dependent manner, however we do not know the timing by which relocalization occurs. Here, we identify that downstream pathway activation occurs within two hours of Wnt treatment which is preceded by β-catenin accumulation within 1-2 hours in CRC cells. Destruction complex localization occurs within 15 minutes of Wnt exposure and we find that newly synthesized or pre-existing pools of β-catenin can associate with the membrane-localized complex. Additionally, the loss of APC impairs the ability of CRC cells to further accumulate β-catenin upon Wnt stimulus or properly degrade β-catenin. We find that APC loss does not interfere with phosphorylation of β-catenin but that proteasomal inhibition causes β-catenin accumulation. Our data support a model in which APC enhances the ubiquitination and proteasomal degradation of β-catenin and that APC is involved in the transduction of a Wnt signal by rapidly localizing the destruction complex to the membrane.

## Introduction

The Wnt/β-catenin signaling pathway is involved in the maintenance of many tissues and cellular processes and is frequently deregulated in cancer and other diseases [1–3]. Therapeutic targeting of the Wnt pathway has been challenging due to its immense complexity, tissue-specific signaling, and incompletely resolved mechanistic details [4]. Wnt signaling regulates the expression of many genes involved in cellular proliferation (for a current list of Wnt target genes: https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). In the absence of a Wnt ligand, a cytoplasmic β-catenin destruction complex composed of APC, Axin, CK1-α, and GSK-3β negatively regulates the pathway by binding and phosphorylating β-catenin, targeting it for ubiquitination mediated by β-TrCP followed by proteasomal degradation [5–7]. However, the engagement of a Wnt ligand with the co-receptors Fzd and LRP5/6 results in the formation of a heterotrimeric signalosome and ultimately leads to inhibition of the β-catenin destruction complex and activation of the Wnt transcriptional program [8–10]. The mechanisms underlying Wnt-induced inhibition of the β-catenin destruction complex are not well understood despite the frequent alteration of Wnt pathway components in human disease.

In colorectal cancer (CRC), Wnt pathway mutations are present in over 90% of cases resulting in deregulated Wnt pathway activity [11]. In over 80% of human CRC, the Wnt pathway is activated by mutation to the Wnt antagonist, *APC* [11]. Human *APC* mutations often arise in a region referred to as the "mutation cluster region" (MCR) which result in a truncated protein product [12,13]. Mutations to the MCR result in loss of β-catenin binding sites (20R), Axin-interaction sites (SAMP), nuclear localization sequences, and a basic region at the C-terminus involved in cytoskeletal interactions. Despite the majority of human CRC containing *APC*

mutations, it is not well-defined what the exact function of APC is in Wnt regulation or why *APC* mutations predominate since mutation to other components could activate Wnt signaling. Through its β-catenin binding domains and SAMP motifs, it is well-established that APC acts as a scaffold in the β-catenin destruction complex. However, another scaffold protein, Axin, is present in the complex and has binding sites for the core machinery of the β-catenin destruction complex. APC has been implicated in nuclear interactions with β-catenin to repress Wnt target gene expression, enhancement of GSK-3β activity, and in the ubiquitination of β-catenin [14–20].

Recently, we demonstrated that APC is also responsible for Wnt-induced relocalization of the β-catenin destruction complex to Wnt3a-Dynabeads [21]. This data was collected at between 4-14 hours (4h WB Pull-down, 12-14h Wnt-bead IF) after addition of Wnt-beads. However, several studies have indicated that an immediate Wnt response may occur. Live-cell imaging of HEK293 cells expressing YFP-tagged β-catenin reveals that β-catenin accumulation and nuclear localization occur within 60 minutes [22]. The same study found that YFP-β-catenin membrane localization occurs rapidly and remains constant for several hours following the initial accumulation. Further, Wnt3a treatment in NIH3T3 cells resulted in the presence of Axin in membrane fractions within 20 minutes and FRET analysis demonstrated an interaction between LRP5 and Axin just three minutes after Wnt stimulation [23]. Analysis of the endogenous β-catenin destruction complex revealed that Axin1 interacts with the LRP6 receptor within 30 minutes of Wnt stimulation [24]. These data, obtained in multiple cell types, indicate that an initial response to Wnt stimulation may involve rapid translocation of the destruction complex in order to accumulate β-catenin. Given our previous data, we sought to test whether Wnt-induced destruction complex localization is an initial event of Wnt signal transduction and whether APC-loss affects the dynamics of Wnt-induced β-catenin accumulation.

As noted previously (Chapter 2), our understanding of Wnt signaling dynamics as it relates to intestinal epithelial cells has been limited. Many studies have utilized overexpression of Wnt pathway components or relied on treatment of cells with soluble Wnt in the media which limits the ability to elucidate dynamics of the endogenous destruction complex and its localization in response to Wnt. Here, we examine the initial response of endogenous β-catenin and the destruction complex to Wnt-3a. We demonstrate that APC and β-catenin localize to a Wnt bead within 15 minutes if β-catenin levels are sufficient for detection. Further, our data suggest that either newly synthesized β-catenin or previously existing pools of β-catenin can be recruited to the destruction complex once it is at the cell membrane. We also demonstrate that β-catenin levels accumulate within 1 hour of Wnt treatment in cells with an intact Wnt pathway, but that Wnt-induced β-catenin accumulation and degradation is impaired in APC-knockout cells. Our work provides useful information regarding the kinetics of the Wnt response in CRC cells and provides evidence that newly synthesized or previously existing β-catenin is maintained within the destruction complex following Wnt treatment.

## Results

### ***Wnt pathway activation occurs within 2 hours of Wnt-3a treatment***

Previously, we demonstrated that the β-catenin destruction complex relocalizes toward a Wnt-source within 4-14 hours in RKO colorectal cancer cells which harbor an intact Wnt signaling pathway [21]. It is possible that destruction complex localization toward Wnt is not a direct consequence of the initial Wnt response, but rather a downstream consequence of Wnt target gene expression. Wnt target genes are reported to be induced within 30 minutes to 1 hour

in other cell types [24]. Thus, we used the TOPflash β-catenin activity luciferase-reporter assay [25] to determine the timing of the transcriptional response to a Wnt ligand in our setting. RKO cells were co-transfected for ~24 hours with either the TOPflash or the negative control FOPflash reporter plasmids and with the Renilla-luciferase control plasmid. Treatment with recombinant Wnt-3a resulted in a 50% increase in TOPflash activity by 2h (Fig. 4.1A). Since the TOPflash reporter assay relies on transcription and translation of the luciferase gene, it is likely that transcriptional activation of Wnt-responsive genes occurs prior to 2h of Wnt treatment. By 4h, luciferase activity reached 4.4-fold higher than control. We find that the half-life of TOPflash firefly luciferase in RKO cells is ~167 minutes (Fig. 4.1B), thus it is likely that the enhanced luciferase signal seen after 4h Wnt3a is in part due to accumulation of the luciferase protein (Fig. 4.1A). Renilla and FOPflash firefly luciferases had similar half-lives (Fig. 4.1B). To determine if Wnt pathway activation affects the stability of luciferase protein, we utilized RKO-APC$^{KO}$ cells which have high endogenous Wnt pathway activity and found half-lives of the various luciferases to be ~180 minutes (Fig. 4.1C). Taken together, these data demonstrate that RKO cells are transcriptionally responsive to Wnt within 2 hours.

***Destruction complex recruitment to Wnt is an early event in Wnt signal transduction***

APC and β-catenin localize to Wnt-beads in ~70-80% of RKO cells treated for 12-14 hours [21]. Given that transcriptional activation of the Wnt pathway occurs in RKO cells in less than 2 hours, it is possible that Wnt-induced destruction complex localization is a secondary effect of the Wnt response rather than an initial event. We found APC localization toward Wnt-beads in 68% of cells treated for 15 minutes, the shortest interval we were able to analyze (Fig. 4.2A, C). At earlier time points, most beads do not adhere to the cells and are lost during

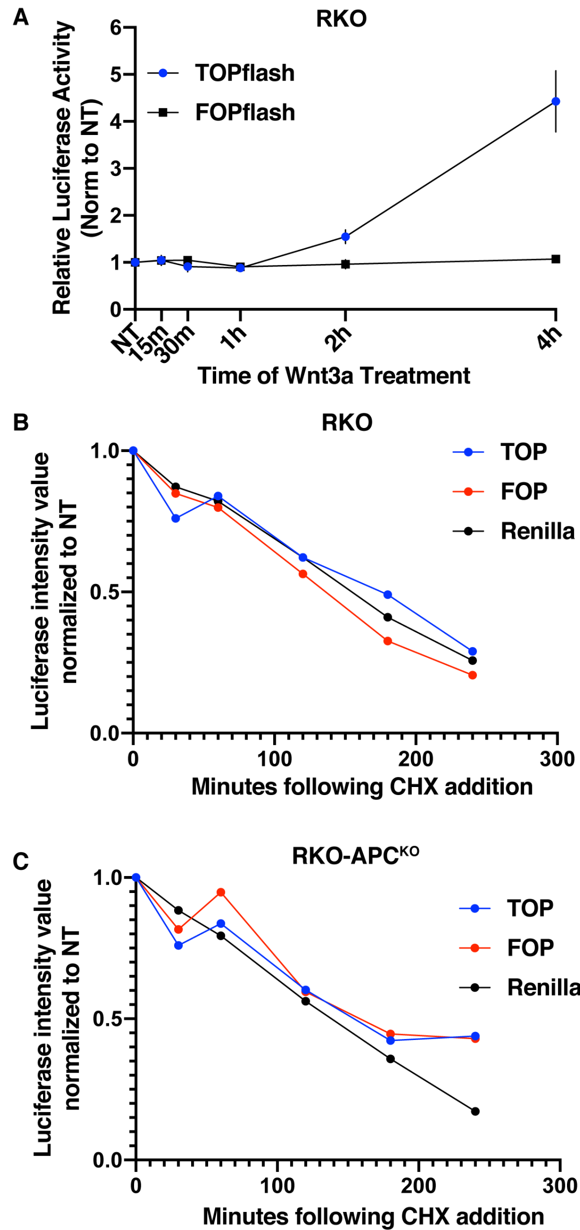


**Figure 4.1** *Wnt pathway activation in RKO cells.* (A) Luciferase activity in RKO cells treated with 71ng recombinant Wnt3a. Cells were transfected with the TOPflash reporter plasmid or the negative control FOPflash and normalized to Renilla-luciferase expression. N=2 experiments. (B) Degradation of Firefly luciferase (TOP and FOPflash) and Renilla-luciferase in RKO cells following 100µg/ml cycloheximide treatment. (C) Degradation of Firefly luciferase and Renilla-luciferase in RKO-APC$^{KO}$ cells following cycloheximide treatment.

immunofluorescent staining. By 30 minutes of Wnt treatment, 77% of cells displayed APC near the Wnt-bead, a pattern that persisted throughout the duration of treatment (Fig. 4.2C) These data demonstrate that APC, a key component of the β-catenin destruction complex, localizes to the Wnt receptors soon after Wnt stimulation and maintains association with Wnt-beads. Unexpectedly, β-catenin localization only occurred in 32% of Wnt-bead treated cells by 15 minutes and steadily increased in Wnt-bead localization until 4h (Fig. 4.2B, C). These data are consistent with the destruction complex localizing toward a Wnt-cue early on, but β-catenin joining the complex at later times. Alternatively, RKO cells possess a functional destruction complex and the low β-catenin levels may limit the detection of β-catenin re-localized toward the Wnt-bead.

***Wnt-beads induce immediate localization of previously synthesized β-catΔS45***

HCT116βm cells contain a single allele of mutant β-catenin harboring a Ser45 deletion (referred to as β-catΔS45) which results in its stabilization [26]. β-catΔS45 and APC localize to Wnt-beads in HCT116βm cells [21] and β-catΔS45 continues to be Wnt-regulated, albeit less efficiently than in RKO cells (Chapter 3). Thus, HCT116βm cells have levels of β-catΔS45 higher than RKO and may be useful to determine localization kinetics at earlier times post Wnt addition. Moreover, HCT116βm cells would allow us to distinguish localization of β-catenin present at the time of Wnt addition from newly synthesized. HCT116βm cells were treated with translation inhibitor cycloheximide and Wnt-beads at the same time. Interestingly, we find that both APC and β-catenin localize to a Wnt-bead in the presence or absence of cycloheximide within 15 minutes (Fig. 4.3A, B). These results suggest that β-catenin can quickly localize to a Wnt source and Wnt bead localization is not limited to newly synthesized β-catenin. Together

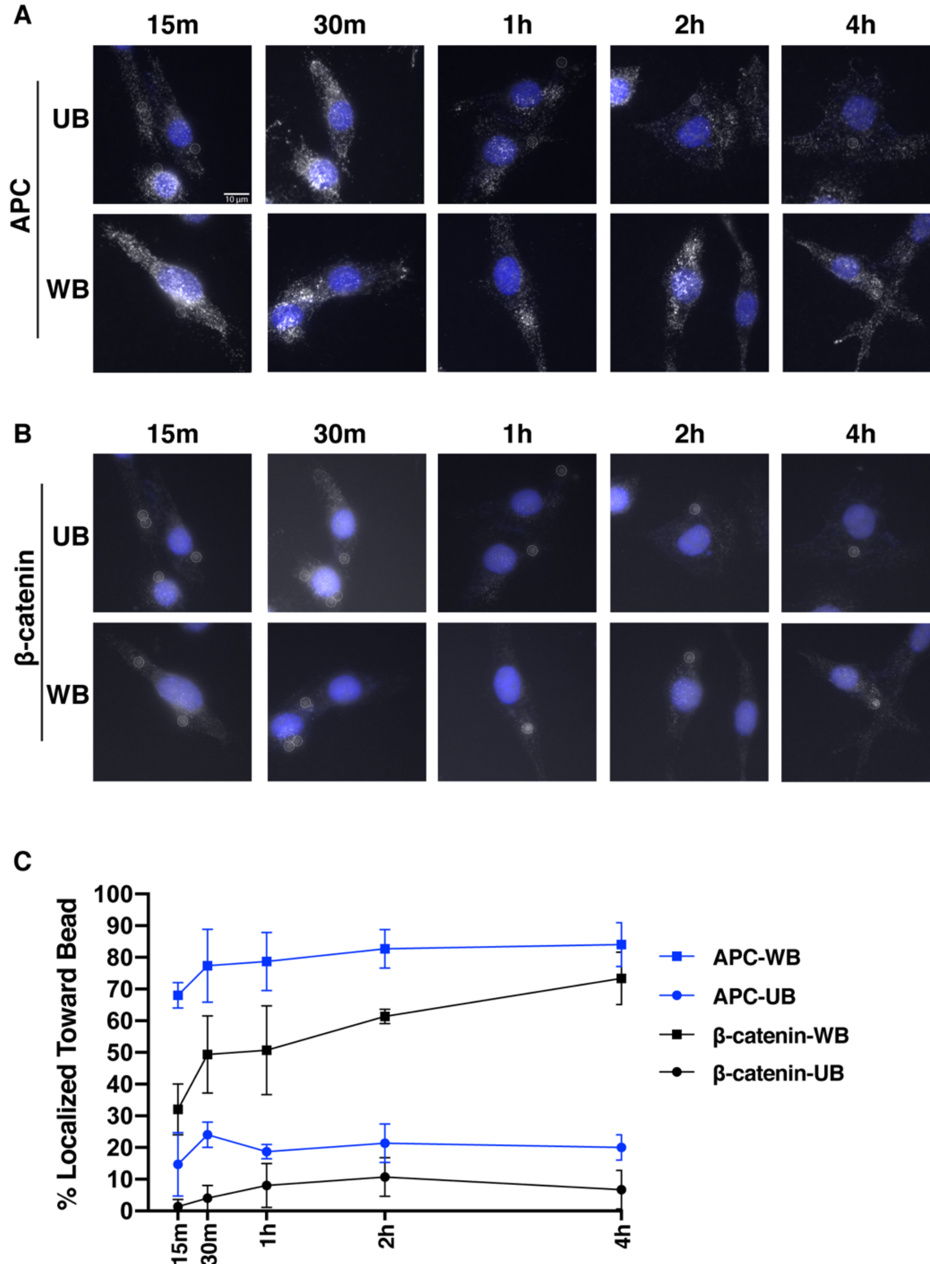


**Figure 4.2** *Wnt-induced APC localization is followed by β-catenin localization in RKO cells.* Representative images of RKO cells treated with UB or WB for the indicated times. Representative images shown for (A) APC, and (B) β-catenin. (C) Scoring of APC and β-catenin localization. Scoring was performed as in Chapter 2 and the percent of cells localizing toward the bead is shown. Data averaged from three independent experiments (n=25 cells per condition). Error bars, SEM.

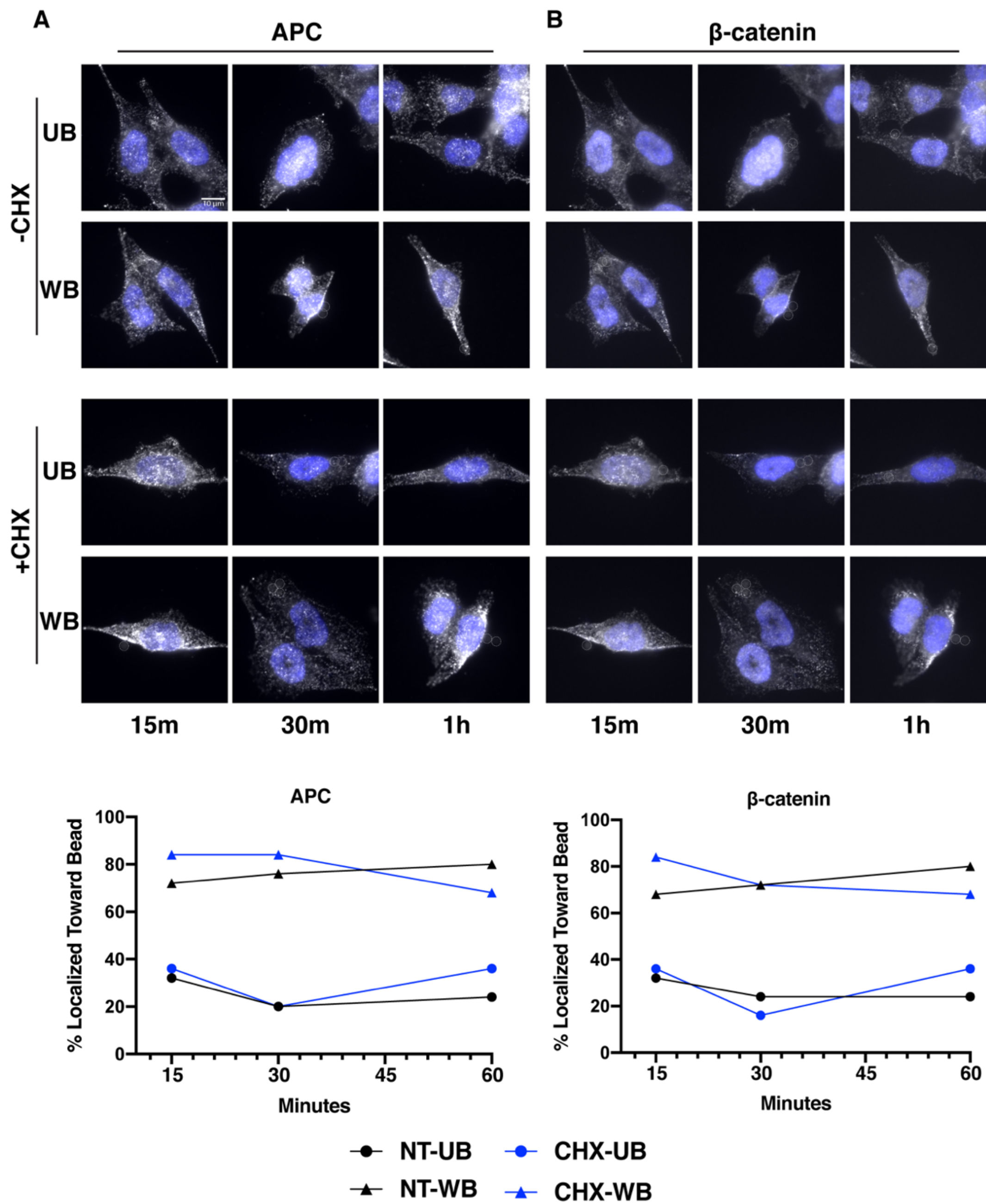


**Figure 4.3** *Wnt-induced APC and β-catenin localization are independent of synthesis of new proteins in HCT116βm cells.* Representative images of HCT116βm cells treated with UB or WB in the presence or absence of cycloheximide for the indicated times. Representative images and scoring shown for (A) APC, and (B) β-catenin. (C) Scoring of APC and β-catenin localization. Scoring was performed as in Chapter 2 and the percent of cells localizing toward the bead is shown. Data from one experiment (n=25 cells per condition).

with the data in RKO cells, these findings suggest that previously synthesized β-catenin localizes with destruction complex component APC toward a Wnt-bead but also that newly synthesized β-catenin can be bound by the destruction complex after it has localized to the membrane.

***β-catenin accumulation and degradation is dependent on APC***

So far, our data demonstrated that Wnt-ligand presentation can induce the Wnt transcriptional program within 2 hours and that destruction complex localization to a Wnt-ligand is an early event in Wnt signal transduction. Further, we previously showed that APC controls the Wnt-induced complex localization to the membrane [21]. Given these results, we sought to examine the APC influence on the β-catenin accumulation and degradation kinetics.

To examine the rate of β-catenin accumulation in response to Wnt, we treated RKO and RKO-$APC^{KO}$ cells with Wnt for 0-4 hours and examined β-catenin levels. By 1 hour of Wnt treatment, RKO cells exhibit a 1.47-fold increase in β-catenin compared to control with a further increase up to 3.7-fold by 2h (Fig. 4.4A, B). APC-loss renders RKO cells transcriptionally unresponsive to Wnt (see TOPflash assay in Fig. 3.4B). In addition, RKO-$APC^{KO}$ cells do not exhibit any β-catenin accumulation when exposed to Wnt (Fig. 4.4A, B). While RKO-$APC^{KO}$ cells have high basal levels of β-catenin, APC loss may limit further accumulation by interfering with the response to Wnt.

The β-catenin destruction complex functions to promote β-catenin degradation. Inhibiting protein translation in RKO cells by treatment with cycloheximide results in a 60% decrease in β-catenin within 15 minutes (Fig. 4.4C, D). This data suggests that the destruction complex is highly active in RKO cells. In contrast, RKO-$APC^{KO}$ cells only exhibit a 6% decrease in β-

catenin by 15m and a 21% decrease by 1h (Fig. 4.4C, D). These data demonstrate that APC loss greatly reduces the degradation of β-catenin.

The primary regulatory mechanism of the β-catenin destruction complex is to phosphorylate β-catenin at four conserved sites – Ser45 by CK1-α followed by Ser33/Ser37/Thr41 by GSK-3β [7]. In chapter three, we identified phosphorylated β-catenin in RKO cells and showed that proteasome inhibition resulted in increased total and GSK-3β-phosphorylated β-catenin (see Fig. 3.5D). To test if β-catenin phosphorylation is impaired upon APC loss or mutation, we treated RKO-APC$^{KO}$ cells or DLD1 cells (truncated APC) with MG132 in the presence or absence of Wnt-3a (Fig. 4.4E). Treatment of RKO-APC$^{KO}$ cells with MG132 resulted in 1.9-fold increase in β-catenin level and a 5.7-fold increase in phospho-S33/S37/T41-βcatenin compared to untreated cells (Fig. 4.4F). DLD1 cells exhibit a similar increase in β-catenin level, but phosphorylated β-catenin does not accumulate to the level seen in RKO-APC$^{KO}$ (Fig. 4.4F). The ratio of phosphorylated β-catenin to total β-catenin shows that phosphorylated β-catenin accumulates more than total β-catenin in RKO-APC$^{KO}$ but not in DLD1 (Fig. 4.4G).

**Discussion**

In the current study, we sought to examine the dynamics of the initial Wnt response in colorectal cancer cells and test roles for APC within the β-catenin destruction complex. Previously, we demonstrated that the β-catenin destruction complex localizes to a Wnt-bead within 12-14 hours in human intestinal epithelial cells in an APC-dependent manner [21]. This work demonstrated that APC controls the localization of the β-catenin destruction complex

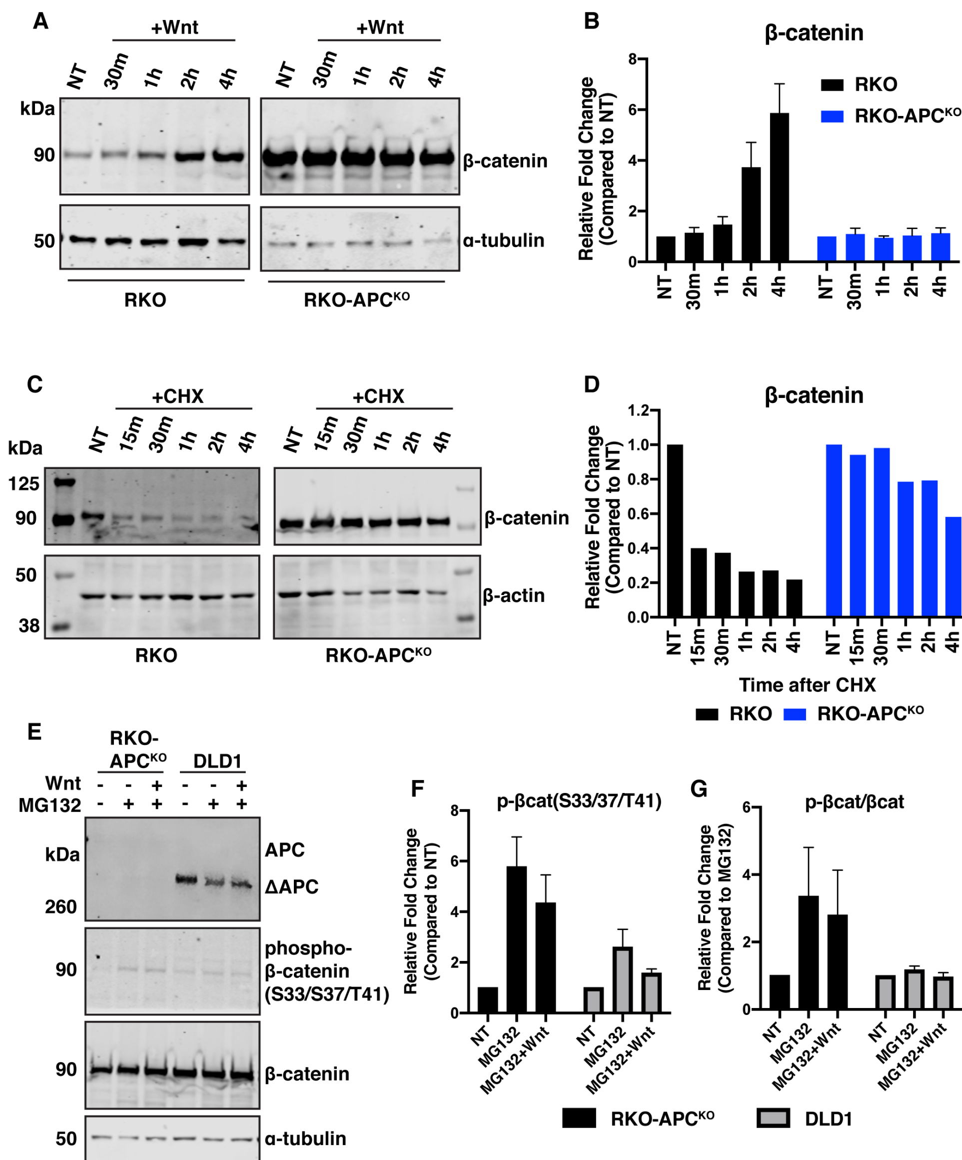


**Figure 4.4** *APC loss impairs Wnt-induced β-catenin accumulation and β-catenin degradation.* (A) β-catenin accumulation in RKO and RKO-APC$^{KO}$ cells treated with 25ng/ml Wnt-3a for the indicated times. (B) Quantification of β-catenin accumulation averaged from three independent experiments. Relative fold-change calculated by dividing each lane by α-tubulin and normalizing to NT. (C) β-catenin degradation in RKO and RKO-APC$^{KO}$ following 100µg/ml cycloheximide treatment. (D) Quantification of β-catenin degradation in one experiment. (E) Treatment of RKO-APC$^{KO}$ and DLD1 cells with 10µM MG-132 and 25ng/ml Wnt-3a as indicated. (F) Quantification of phospho-S33/S37/T41-β-catenin levels representative of two independent experiments. (G) Quantification of p-β-catenin over total β-catenin levels representative of two independent experiments. The same experiment was performed on RKO and HCT116βm cells in Chapter 3. Error bars, SEM.

following Wnt stimulation and that the destruction complex can maintain association with β-catenin following Wnt stimulation. However, the dynamics of destruction complex localization to Wnt are not known and may occur much faster. Further, our previous data suggests that APC may play a positive role in the transduction of a Wnt signal. Given that APC is involved in movement of the destruction complex to the Wnt-stimulated receptors, it is possible that APC-loss may be responsible for the inability of APC-mutant cells to respond to Wnt.

Here, we utilized RKO cells with an intact Wnt signaling pathway to examine the kinetics of the Wnt response. We found that Wnt-induced APC membrane localization occurs rapidly within 15 minutes. We, and others, postulate that β-catenin maintains association with the destruction complex following Wnt stimulation [21,24]. However, we find that Wnt-induced β-catenin localization is infrequent until four hours of Wnt-bead treatment. These data prompted us to ask whether newly synthesized β-catenin is brought into membrane-bound destruction complexes or if previously existing pools of β-catenin could localize to Wnt-beads. To ask this, we utilized Wnt-responsive HCT116βm cells which express β-catΔS45. β-catΔS45 is present at higher concentrations than wild-type β-catenin in RKO cells, continues to be bound by the β-catenin destruction complex, and relocalizes upon extended Wnt stimulation [21]. When treated with Wnt-beads, β-catΔS45 in HCT116βm cells relocalizes to the Wnt-bead within 15 minutes, as seen with APC. Further, inhibition of translation does not interfere with this Wnt-induced response, indicating that pre-existing β-catΔS45 is trafficked to the Wnt-bead as part of the initial Wnt-response. The incorporation of β-catenin by four hours in RKO cells indicates that newly synthesized β-catenin can be sequestered by membrane-bound destruction complexes, whereas the data in HCT116βm cells indicates that pre-existing β-catenin can be trafficked as well. The ability of the membrane-bound destruction complex to continue binding newly

synthesized β-catenin may act as a buffer to ensure that the saturation of available destruction complexes must occur before β-catenin accumulation can take place.

Further, we analyzed the kinetics of Wnt-induced β-catenin accumulation in RKO and RKO-APC$^{KO}$ cells. While RKO cells accumulate β-catenin within 1-2 hours, RKO-APC$^{KO}$ cells are defective in Wnt-induced β-catenin accumulation. RKO-APC$^{KO}$ cells are unable to degrade β-catenin despite the ability to phosphorylate β-catenin at its GSK-3β phosphorylation sites. These data suggest APC-loss renders cells unresponsive to further Wnt stimulation, possibly through the inability of APC to traffic destruction complex components to the membrane. Additionally, these data agree with previous studies that APC may play a key role in promoting β-catenin ubiquitination, given the findings that RKO-APC$^{KO}$ cells maintain high basal levels of β-catenin, are defective in its degradation, and are able to phosphorylate β-catenin [19].

In the current study, we have clarified that β-catenin destruction complex localization is an immediate response to Wnt signaling rather than a downstream event due to transcriptional feedback from Wnt pathway activation. Future studies are needed to determine the mechanism by which APC traffics the destruction complex to the membrane given that this response is lost in APC-mutant cells and may be responsible for the inability to respond to Wnt stimulation. Previous studies have primarily suggested that Axin is responsible for destruction complex membrane association given that Axin interacts with phosphorylated LRP5/6 receptors and Dvl [23,27,28]. Our data does not support this model, but rather supports a model in which APC traffics the complex to the membrane. It is possible that Axin-binding to LRP5/6 and Dvl docks the complex to the membrane following APC trafficking, however we previously demonstrated that Axin1-knockdown had no effect on destruction complex localization in colon epithelial cells [21]. Taken together, our data indicate that the C-terminus of APC may contribute to destruction

complex trafficking, possibly through its interactions with cytoskeletal proteins [29–36]. Overall, these findings demonstrate the importance of understanding the kinetics of the Wnt response and the multiple roles of APC in β-catenin degradation as well as Wnt signal transduction.

## Materials and Methods

### *Cell Culture and Treatments*

HCT116βm, RKO, DLD1, and RKO-$APC^{KO}$ cells were cultured in DMEM (with L-Glutamine and 4.5g/L Glucose; without Sodium Pyruvate) supplemented with 10% FBS and were maintained at 37°C and 5% $CO_2$. Wnt treatment was performed by adding Recombinant Wnt-3a (Peprotech #315-20) at the indicated concentration/time prior to cell lysis or immunofluorescence analysis. MG-132 treatment was performed by addition of 10mM MG-132 to a final concentration of 10μM in cell media for four hours. Cycloheximide treatment was performed by addition of 100μg/ml cycloheximide to the cell media for the indicated times.

### *Immunoblotting*

Cells were washed 1x in PBS prior to harvesting in pre-heated high-salt sample lysis buffer (20% glycerol, 2% SDS, 30% 10X PBS, 2.5% β-mercaptoethanol). Scraped cells were transferred to Eppendorf tubes, heated at 95°C for 1min, pulled through an insulin syringe three times, and heated again. Samples were separated on 7.5% SDS-PAGE (Bio-Rad, TGX FastCast Acrylamide Kit) using Tris-Glycine running buffer and transferred to a nitrocellulose membrane (GE) with a 0.45-μm pore size. Antibodies were diluted in Odyssey Blocking Buffer TBS (LI-COR) as follows: anti-APC-M2 Chicken pAb (1:2,000), anti-β-catenin mouse mAb (1:1,000),

anti-phospho-Ser33/Ser37/Thr41-β-catenin rabbit pAb (Cell Signaling, 1:500), anti-α-tubulin DM1A mouse mAb (Santa Cruz, 1:1,000), anti-β-actin mouse mAb (Sigma, 1:1,000), and IRDye 680LT and 800CW anti-rabbit, anti-mouse, or anti-chicken secondary antibodies (1:15,0000). Immunoblots were imaged on a LI-COR Odyssey CLx imaging system.

***Luciferase Reporter Assay***

Cells were seeded into 12-well plates 24 hours prior to transfection. On day of transfection, cell culture media was changed one hour prior to siRNA and plasmid transfection. Both reporter plasmids and siRNAs were transfected concurrently using Lipofectamine 3000 according to manufacturer's instructions. For luciferase reporters, cells were co-transfected with TOPflash (450ng) and Renilla (50ng) expression plasmids. As a control, identical wells were transfected with FOPflash (450ng) and Renilla (50ng) expression plasmids to validate that a scrambled TCF-binding sequence does not result in increased reporter activity upon treatment. Cells were treated with Wnt3a or cycloheximide for the indicated times and lysed using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase signal was normalized to Renilla luciferase signal, and data were normalized to control (set to 1).

***Immunofluorescence and Analysis***

Cells were briefly rinsed in PBS prior to fixation. Cells were fixed in 4% PFA in Brinkley's Buffer 1980 (80mM PIPES pH 6.8, 1mM $MgCl_2$, 1mM EGTA) for 20min at RT, washed two times in PBS prior to permeabilization in TBS/0.2% Triton X-100 for 5min. Cells were washed in TBS two times prior to incubation for 1hr at RT in blocking buffer containing TBS/0.2% Triton X-100, 1% BSA, and 3% Normal Goat Serum. Primary and secondary

antibodies were incubated for 1hr at RT. Cells were washed three times in TBS following primary and secondary antibody incubations. Mounting of coverslips was performed using Prolong Diamond Antifade Mountant with DAPI (Invitrogen). Antibody was diluted in blocking buffer as follows: anti-β-catenin mouse mAb (1:250, BD Transduction Laboratories, #610154), anti-APC rabbit pAb (1:4,000, generated previously in Neufeld lab). Stained cells were examined using an Axioplan microscope (Zeiss) with a X100 objective. Images of stained cells were captured using an Orca $R^2$ digital camera (Hamamatsu).

### *Quantification and Statistical Analysis*

Fluorescent-detection and quantification of immunoblots was performed using the LI-COR Odyssey CLx imaging system and Image Studio™ Lite software (LI-COR). Graphs were generated using Graphad Prism (GraphPad Software, Inc.). Scoring of APC and β-catenin localization was performed as done in Chapter 2 [21].

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# Chapter 5

# Discussion

## Chapter 5. Discussion

Mutation of the tumor suppressor *APC* occurs in the majority of human colorectal cancers and is considered to be an initiating event in colorectal tumorigenesis [1–3]. Since the discovery of APC in the early 1990s [4,5], many studies have attempted to uncover its functional roles within and outside of Wnt/β-catenin signaling (reviewed in [6]). The primary known role of APC is as a negative regulator of the Wnt/β-catenin signaling pathway in which APC participates as part of the β-catenin destruction complex to maintain low cellular levels of the transcriptional co-factor β-catenin. In many human CRCs, *APC* mutation frequently results in the expression of a truncated protein product absent of important functional domains involved in β-catenin and Axin binding, nuclear localization, and cytoskeletal interaction. APC truncations result in elevated β-catenin levels and consequently lead to aberrant activation of the canonical Wnt signaling pathway. It is intriguing that *APC* mutations appear to be selected in CRC, given that alteration to other Wnt pathway components can also lead to elevated β-catenin levels and transcriptional activity. For these reasons, it has been proposed that *APC* mutations predominate in CRC based on their ability to maintain a specific level of β-catenin regulation so that a "just-right" amount of Wnt/β-catenin signaling takes place for optimal tumor growth [7]. Given that APC has no known enzymatic activity and its primary function is thought to be involvement as a scaffolding protein, the roles APC performs in normal Wnt/β-catenin regulation and in Wnt signal transduction remain elusive. Perhaps the loss of other C-terminal domains of APC such as those mediating cytoskeletal interactions or nuclear localization are important for intestinal tumorigenesis. Dissecting less characterized functions of APC in β-catenin regulation and Wnt signal transduction is a major focus of this work.

The mechanism by which the β-catenin destruction complex is inhibited following Wnt stimulation is not well understood and remains one of the largest questions in the Wnt signaling field. While numerous models of destruction complex inhibition following Wnt receptor activation have been proposed, the specific molecular events that occur continue to be debated for myriad reasons. The use of overexpression of Wnt pathway components, global addition of Wnt to cell culture media, or the wide range of cell and tissue types used may affect stoichiometry of the destruction complex, improperly represent a polarized Wnt cue, or lead to results that are specific to a single cell type. As such, models of destruction complex inactivation have included kinase inactivation or partial disassembly [8–12], targeting to the Wnt receptors [13–16], or endocytosis of the complex[17,18]. While it is possible that some or all of these models are accurate depending on the cell or tissue type, the ongoing debate and lack of mechanistic understanding highlight the necessity for further studies in cells that represent the cancer tissue of origin to resolve the inactivation of the β-catenin destruction complex following Wnt signaling.

Through the study of colon epithelial cells of both normal and malignant origin, I have uncovered a novel role for APC in destruction complex trafficking following Wnt stimulation (Chapter 2). Further, I have found that a so called "stabilizing" mutation in β-catenin does not completely evade regulation by the destruction complex (Chapter 3). Cells containing stabilizing β-catenin mutations continue to be Wnt-responsive, despite having an activating mutation downstream in the pathway. My studies also have provided additional support for nuclear export roles of APC and led us to propose a model whereby β-catenin mutations can also act in a "just-right" signaling manner, similar to that previously suggested for APC [7]. Finally, I performed analyses of the kinetics of destruction complex trafficking to a localized Wnt source and found

that the β-catenin destruction complex appears to localize rapidly toward a Wnt cue. Further, newly synthesized or previously existing β-catenin is able to be brought into these membrane-tethered complexes (Chapter 4). The work in this dissertation provides important findings regarding the nature and roles of *APC* and β-catenin human colorectal cancer and roles for APC in the transduction of Wnt signals in colon epithelial cells of healthy and malignant origin.

**A role for APC in Wnt-induced β-catenin destruction complex localization**

What molecular events occur to inhibit the β-catenin destruction complex following Wnt stimulation? A primary limitation to addressing this problem is the inability to assess where the β-catenin destruction complex localizes in response to Wnt since previous studies have relied on addition of soluble Wnt in the media. To ask whether Wnt signals can act as polarity cues to induce asymmetric division of mouse embryonic stem cells, the lab of Roel Nusse developed an assay in which recombinant Wnt ligands were conjugated to Dynabeads [16]. Since the Wnt ligand is chemically conjugated to the bead, this technique allows for the analysis of destruction complex localization to a defined placement of Wnt rather than engaging entire cell surfaces with soluble Wnt.

My initial analysis of APC localization in three CRC cell lines – one with an intact Wnt pathway (RKO), one with stabilized β-catenin (HCT116βm), and one with truncated APC (DLD1) – revealed that wild-type APC localizes to Wnt-beads in nearly all cells whereas this localization is impaired for truncated APC. Using immunofluorescence microscopy, I found the localization of destruction complex components (Axin1, CK1-α, GSK-3β), β-catenin, Fzd7, and β-TrCP toward Wnt-beads in RKO cells, indicating that the destruction complex maintains association rather than disassociating upon Wnt stimulation and that β-catenin remains in the

destruction complex. Using the Wnt-beads to pull-down bead-associated proteins, a physical interaction was found between APC and β-catenin with the Wnt-beads, confirming the immunofluorescence microscopy results. These data were confirmed in HCT116βm cells except β-TrCP localization was impaired. Given that the Ser45 deletion in HCT116βm interferes with downstream phosphorylation of the β-TrCP recognition site, this result demonstrates that β-TrCP is not an inherent member of the complex but is recruited upon β-catenin phosphorylation. Destruction complex localization in DLD1 cells was highly impaired, indicating that full-length APC may be important for this process. Indeed, either CRISPR/Cas9 knock-out of APC or siRNA-mediated APC depletion resulted in loss of destruction complex localization. Depletion of Axin1, a key scaffolding protein in the complex, did not interfere with APC or β-catenin localization to Wnt-beads, indicating that this is an APC-specific role in Wnt signal transduction. These results demonstrated that the endogenous β-catenin destruction complex is recruited to Wnt in an APC-dependent manner in colonic epithelial cells of both normal and malignant origin.

**Stabilized β-catenin is susceptible to regulation by APC and Wnt**

Surprisingly, Wnt-bead treatment of HCT116βm cells resulted in destruction complex localization that included β-catΔS45 and appeared to result in elevated β-catΔS45 protein levels (Chapter 2). For these reasons, I hypothesized that mutations suggested to result in a “stabilized” β-catenin may not be completely resistant to destruction complex regulation or Wnt stimulation. While *APC* mutations in CRC are prevalent, mutations altering the β-catenin protein are relatively rare. It has been previously proposed that *APC* mutations occur in a “just-right” fashion, but this model has not been proposed for other members of the Wnt signaling pathway.

In Chapter 3, I sought to test the “just-right” model of β-catenin mutation to determine if stabilizing β-catenin mutations continue to allow some degree of Wnt-responsiveness and destruction complex regulation.

HCT116βm cells harbor a single copy of mutant β-catenin with a Ser45 deletion, resulting in the absence of its CK1-α phosphorylation site [19–21]. When treated with Wnt3a, HCT116βm cells accumulate β-catΔS45 and further activate the TOPflash Wnt reporter. Additionally, APC-depletion results in β-catΔS45 accumulation and nuclear translocation but does not increase β-catΔS45 transcriptional activity unless combined with Wnt treatment. These experiments confirm previous findings that β-catΔS45 can continue to be phosphorylated by GSK-3β [22]. Treatment with proteasome inhibitor results in β-catΔS45 accumulation, indicative that β-catΔS45 is otherwise targeted to the proteasome. These results demonstrate that a Wnt signal leads to secondary effects which further activate nuclear β-catenin. I propose that APC inhibits this activity through nuclear export mechanisms and/or by sequestering β-catenin or its transcriptional co-factors TCF/LEF in the cytoplasm. This work provides insight to the mechanism of β-catenin regulation in both the Wnt-ON and Wnt-OFF states, but also provides an explanation for the prevalence of *APC* mutations compared to β-catenin mutations. Cells expressing mutant β-catenin continue to be Wnt-responsive, whereas cells expressing mutant APC are unable to be further stimulated by Wnt. Taken together findings from Chapters 2 and 3 clearly highlight key differences between mutation to *APC* or other members of the Wnt pathway, such as β-catenin. Our findings also demonstrate that the Wnt/β-catenin signaling pathway does not act in a strictly linear fashion in which mutation to a downstream component causes aberrant signaling and lack of Wnt-responsiveness. Instead, we find that mutation to the

key downstream effector, β-catenin, still allows regulation by upstream components such as APC or Wnt.

**The kinetics and dynamics of the Wnt response**

In chapter 2, I identify that the β-catenin destruction complex re-orients toward a Wnt cue in an APC-dependent manner. This work was performed with 4-14 hours of Wnt3a-bead treatment (4h for WB pull-down, 12-14h for IF), however earlier studies in a variety of cell types have suggested that a Wnt response may occur on the order of minutes [13,14,17,23]. Because of these findings, I sought to determine if the destruction complex localization observed in chapter 2 was due to an active trafficking of the complex to the Wnt-receptors, or if downstream Wnt transcriptional activation resulted in a feedback response that resulted in a delayed localization of the destruction complex.

Initially, I performed a time-course of Wnt treatment in RKO cells transfected with the TOPflash reporter plasmid and found that Wnt pathway activation is not detectable until two hours of treatment. However, time-course analysis of destruction complex localization revealed that Wnt-beads induce APC localization within 15 minutes of their addition to RKO and HCT116βm cells. In RKO cells, however, β-catenin localization lagged behind APC localization, and did not occur until 2-4 hours of treatment. RKO cells have low levels of β-catenin due to an actively functioning destruction complex. For this reason, I hypothesized that downstream β-catenin accumulation must occur before it can be detected near the Wnt-beads in RKO cells. Indeed, using HCT116βm cells which express stabilized β-catenin at higher basal levels than RKO cells, β-catenin localization occurs within 15 minutes of Wnt-bead treatment in the presence or absence of cycloheximide. These data demonstrate that β-catenin can be trafficked

with the destruction complex to the membrane or incorporated into membrane-bound destruction complexes after synthesis. Further, this data is in agreement with a previously proposed model of destruction complex inhibition in which β-catenin is unable to be released to the proteasome following Wnt stimulation and eventually leads to saturation of available destruction complexes and β-catenin accumulation. In support of this, β-catenin protein accumulation is not detectable until 30 minutes to 1 hour after Wnt stimulation whereas the movement of the complex to the membrane occurs within 15 minutes. Finally, I tested whether APC-loss impacts the ability of cells to further accumulate or degrade β-catenin in response to Wnt stimulation. I find that RKO-$APC^{KO}$ cells are unable to further accumulate β-catenin after Wnt treatment and that degradation of β-catenin is impaired compared to RKO cells.

**Future Directions**

The work presented in this dissertation reveals novel roles for APC in destruction complex localization and Wnt signal transduction. However, several knowledge gaps need to be addressed moving forward. How does APC traffic the β-catenin destruction complex to a Wnt cue? What are the functional consequences of APC truncation and how does this contribute to "just-right" Wnt/β-catenin signaling? Do APC truncations act in a dominant-negative fashion, and can this explain why APC loss in HCT116βm results in elevated β-catenin protein but not elevated activity? What are the kinetic responses of the destruction complex movement to Wnt, β-catenin accumulation/activation, and β-catenin phosphorylation? Finally, is β-catenin activation dependent on saturation of the available destruction complexes?

Understanding how APC regulates the trafficking of the destruction complex to a Wnt cue will provide novel insight into Wnt signal transduction and may assist in future therapeutic

discovery. The C-terminal domains of APC are commonly lost in CRC and contain domains which mediate interactions with cytoskeletal components like microtubules and actin but also with cytoskeletal interacting proteins like EB-1. APC is involved in mechanisms of cargo transport and microtubule assembly, making it possible that APC utilizes actin or microtubule polymerization to traffic toward Wnt-bound Fzd/LRP receptors [24–29]. Further, actin-dependent membrane association of APC has been demonstrated [30]. I have carried out preliminary studies to test the hypothesis that if APC utilizes microtubule or actin-based transport to the membrane upon Wnt stimulation. However, use of polymerization inhibitors nocodazole (microtubules) or cytochalasin D (actin) has resulted in cytotoxicity and interfered with interpretation of results. Moving forward, studies may need to be performed in which actin or microtubule interacting proteins are identified that interact with APC or with Fzd and LRP5/6. Using an approach in which specific interactors are depleted would provide an advantageous system compared to whole-cell cytoskeletal disruption.

Another possible mechanism of APC-mediated trafficking to the membrane could be through APC interaction with clathrin-coated pits. Endocytosis of the Wnt receptors was previously reported as a key event in Wnt signal transduction and is thought to be essential for formation of the Wnt signalosome [18,31]. Further, APC inhibition of clathrin-mediated endocytosis has been proposed and it is well established that the actin cytoskeleton is heavily involved in clathrin-mediated endocytosis [32,33]. Of note, immunoprecipitated APC shows association with both Clathrin and its adaptor protein, AP-2 [32], and Dvl interaction with AP-2 and involvement with cytoplasmic vesicles has been implicated [34,35]. My preliminary evidence suggests that Wnt-beads may pull-down the Clathrin heavy-chain. Determining whether this interaction is an APC-dependent process and which region of APC is involved may resolve unanswered questions

regarding destruction complex membrane-targeting as well as mechanisms by which the destruction complex is inhibited.

A region of APC referred to as "M3" (amino acids 1,211-2,075) contains all of the 20 amino acid repeats involved in β-catenin binding, the SAMP motifs, as well as the nuclear localization signals [36,37]. While this region is primarily considered to be involved in β-catenin binding and regulation, recent results from Ethan Lee's research group at Vanderbilt suggest that M3 may be involved in destruction complex trafficking. Saito-Diaz et al. utilized a Myc-tagged fragment of APC that is nearly identical to M3-APC (amino acids 1,265-2,060) [32]. Expression of this region in RKO-$APC^{KO}$ cells rescues β-catenin degradation. Targeted degradation of the APC fragment through an auxin-inducible degradation system resulted in β-catenin accumulation but also phosphorylation of LRP6, indicating that this region of APC is able to localize to LRP6 and prevent its phosphorylation which is an indication of activated Wnt receptors [32,38]. While this data does not directly demonstrate that M3-APC is responsible for targeting of the destruction complex, it does indicate that an interaction between the Wnt receptors and M3-APC occurs. Further studies treating APC-depleted cells expressing M3-APC with Wnt or Wnt-beads would be necessary to answer if this region of APC is in part responsible for destruction complex trafficking and inhibition.

Lastly, the many roles of APC within and outside of Wnt/β-catenin signaling cannot be overstated [6,39]. To fully understand the effects of APC loss or truncated APC expression, transcriptomic and proteomic data need to be acquired in isogenic colon epithelial cells. I have performed preliminary single-cell sequencing studies in a series of HCEC cells (cells originally derived from human normal colon tissue, described in Chapter 2). Four groups of 200-250 HCEC 1CT cells were subjected to single-cell RNA-sequencing. Groups KN1 and KN2

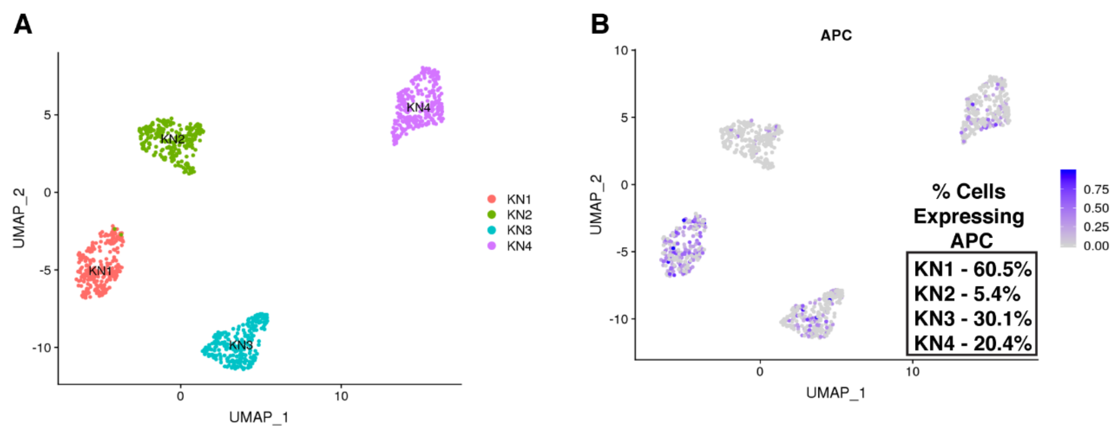


**Fig. 5.1** *Single-cell RNA sequencing reveals distinct clustering of HCEC 1CT populations with varying APC status and identifies population heterogeneity of APC expression.* (A) UMAP clustering using Seurat of HCEC 1CT cells with APC knockdown or additional alterations including KRAS mutation and p53 depletion. Coloring is by group, revealing that each group clusters together. Each data point represents a single cell. (B) The same UMAP clustering as in A but displaying *APC* expression with dark blue as high expression and light blue as low expression. Percentages of cells expressing *APC* within each population listed in chart.

represent HCEC 1CT cells transfected with control siRNA (KN1) or APC siRNA (KN2). Groups KN3 and KN4 represent HCEC 1CTRPA (KN3) or HCEC 1CTRPA-A1309 (KN4) where "RPA" designates stable expression of $KRAS^{V12}$, shTP53 (small hairpin targeting tumor suppressor p53), and shAPC, and "A1309" designates expression of truncated APC (amino acids 1-1,309) [40]. Surprisingly, only 60% of the parental HCEC 1CT line (KN1) express detectable *APC* by RNA sequencing (Fig. 5.1). Nonetheless, initial gene ontology analysis of differential gene expression between the KN1 and KN2 groups reveals many genes involved in cell-cell adhesion, cell proliferation, cytoskeletal organization and the actin cytoskeleton, protein trafficking, membrane rafts, focal adhesions, and endosomal membranes. While these data need to be experimentally repeated and validated, it is intriguing that APC loss results in such widespread differences in gene expression. APC nuclear import and export have previously been characterized by our lab and others, and the possible roles of APC in modulation of gene expression may provide important insight into APC function.

Our work highlights clear roles for APC in Wnt signal transduction and β-catenin regulation and reveals important mechanistic insight into the initial steps involved in Wnt signaling. We have added valuable information to the Wnt signaling field that provides rationale for the prevalence of *APC* mutation in colorectal cancer, and this mechanistic work may aid in the therapeutic targeting of the Wnt pathway. Further studies are needed to clarify the means in which APC exerts its destruction complex trafficking role and to better understand the mechanisms by which a Wnt signal causes activation of β-catenin as a transcriptional co-factor, rather than just its accumulation.

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