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Wnt Signaling through Inhibition of β-Catenin Degradation in an Intact Axin1 Complex

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SUMMARY

Degradation of cytosolic β-catenin by the APC/Axin1 destruction complex represents the key regulated step of the Wnt pathway. It is incompletely understood how the Axin1 complex exerts its Wnt-regulated function. Here, we examine the mechanism of Wnt signaling under endogenous levels of the Axin1 complex. Our results demonstrate that β-catenin is not only phosphorylated inside the Axin1 complex, but also ubiquinated and degraded via the proteasome, all within an intact Axin1 complex. In disagreement with current views, we find neither a disassembly of the complex nor an inhibition of phosphorylation of Axin1-bound β-catenin upon Wnt signaling. Similar observations are made in primary intestinal epithelium and in colorectal cancer cell lines carrying activating Wnt pathway mutations. Wnt signaling suppresses β-catenin ubiquitination normally occurring within the complex, leading to complex saturation by accumulated phospho-β-catenin. Subsequently, newly synthesized β-catenin can accumulate in a free cytosolic form and engage nuclear TCF transcription factors.

INTRODUCTION

The canonical Wnt (Wnt/β-catenin) signaling pathway controls many biological processes, including cell fate determination, cell proliferation, and stem cell maintenance (Clevers, 2006). Deregulation of this pathway occurs in cancer and underlies multiple hereditary syndromes (Clevers, 2006; MacDonald et al., 2009). The key regulatory step involves the phosphorylation, ubiquitination, and subsequent degradation of its downstream effector protein, β-catenin, by a dedicated cytoplasmic destruction complex. This complex consists of the central scaffold protein Axin and three other core components, adenomatous polyposis coli (APC) and the kinases glycogen synthase kinase-3 alpha/beta (GSK-3) and casein kinase-1 (CKI). Mutations in components of the β-catenin destruction complex (APC, AXIN, or β-catenin) result in cancer (Kinzler and Vogelstein, 1996; Korinek et al., 1997; Liu et al., 2000; Morin et al., 1997; Rubinfeld et al., 1996), most notably of the colon.

In resting cells, despite the gene being continuously transcribed, vanishingly low levels of free β-catenin protein are present in the cytosol. This pool of β-catenin is efficiently captured by the destruction complex and phosphorylated by CKI at Ser45, which in turn primes GSK3 phosphorylation of β-catenin on the more N-terminal Thr41, Ser37, and Ser33 residues (Liu et al., 2002). Phosphorylated β-catenin is ubiquitinated by the F-box-containing protein β-TrCP ubiquitin E3 ligase to be degraded by the proteasome (Aberle et al., 1997; Kitagawa et al., 1999).

Axin1 is the rate-limiting factor of the destruction complex (Lee et al., 2003). Axin1 directly interacts with all other core components of the destruction complex (β-catenin, APC, CKα, and GSK3), thus being the central scaffold of the complex (Ikeda et al., 1998; Kishida et al., 1998; Liu et al., 2002; Sakanaka et al., 1998). As the least abundant component, Axin1 can regulate its rapid assembly and disassembly. For this reason, it has been proposed that degradation of Axin1 in Wnt-activated cells may be the immediate cause of β-catenin stabilization (Mao et al., 2001; Tolwinski et al., 2003). Although multiple roles have been proposed for the genetically essential APC protein, there is no consensus as to its key activity.

Wnt ligands bind to the frizzled (FZD) and low-density-lipoprotein-related protein 5/6 (LRP5/6) coreceptor complex to activate the canonical Wnt signaling pathway. Through an incompletely

resolved mechanism that involves Dishevelled, the activated receptor complex disrupts or functionally inactivates the destruction complex, leading to the accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin engages TCF/LEF transcription factors to activate the Wnt transcriptional program (Molenaar et al., 1996; Behrens et al., 1996).

Several models describe the events following Wnt receptor activation that lead to stabilization of β-catenin.

Membrane Sequestration of Axin1-GSK3

Sequestration of Axin1 by binding LRP5/6 reduces the availability of cytoplasmic destruction complexes, thereby causing β-catenin accumulation. Membrane translocation of Axin1 may also mediate its Wnt-induced dephosphorylation and destabilization (Mao et al., 2001; Zeng et al., 2005).

Axin1 Degradation

Mediated by activated Wnt receptors or Dishevelled (Lee et al., 2003; Mao et al., 2001; Tolwinski et al., 2003). Also, the poly-ADP-ribosylating enzyme tankyrase can mediate Axin1 degradation (Huang et al., 2009). By contrast, SUMOylation of Axin1 was reported to protect it from polyubiquitination and increase its stability (Kim et al., 2008). An endocytic adaptor protein Dab2 prevents Axin1 membrane translocation, thereby leading to Axin1 stabilization (Jiang et al., 2009).

Dissociation of Axin1 and/or APC from GSK3 or β-Catenin

Dishevelled, which binds Axin1 directly, may disrupt the destruction complex upon Wnt activation (Liu et al., 2005; Logan and Nusse, 2004; Malbon and Wang, 2006). Alternatively, Frat/GBP family members may compete with Axin1 for GSK3 binding, thus disrupting the destruction complex (van Amerongen and Berns, 2005; van Amerongen et al., 2005). CKI and GSK3 can phosphorylate Axin1 and APC, enhancing their binding affinity for GSK3 and β-catenin. Wnt stimulation results in dephosphorylation of both Axin1 and APC. The catalytic subunits of the phosphatases PP1 and PP2A directly bind and dephosphorylate Axin1, promoting the disassembly of the destruction complex (Luo et al., 2007; Strovel et al., 2000).

Inhibition of GSK3 Kinase Activity

In vitro phosphorylation of β-catenin by GSK3 is inhibited by PPPSPxS motif peptides or by phosphorylated LRP6 cytoplasmic domain (Cselenyi et al., 2008; Piao et al., 2008; Wu et al., 2009). In addition, suppression of GSK3 kinase activity by protein kinase B (PKB) directly or through Dishevelled may mediate Wnt signal transduction (Desbois-Mouthon et al., 2001; Fukumoto et al., 2001).

Dephosphorylation of Phosphorylated β-Catenin

CKIα/GSK3-phosphorylated β-catenin can be dephosphorylated by phosphatase PP2A upon Wnt induction (Su et al., 2008).

GSK3 Sequestration

Taelman et al. propose that sequestration of GSK3 from the cytosol into multivesicular bodies inhibits GSK3 activity during Wnt signal transduction (Taelman et al., 2010).

Complex Disassembly

Roberts et al. suggest that disassembly of the destruction complex by APC through transferring phosphorylated β-catenin to the E3 ligase recycles the destruction complex for renewed β-catenin phosphorylation and degradation (Roberts et al., 2011).

Thus, all previously proposed models (MacDonald et al., 2009) either assume a physical dissociation of the β-catenin destruction complex or propose an interference with β-catenin phosphorylation, leading to stabilization of β-catenin. All studies cited above, however, utilize overexpression strategies. Studies on the endogenous complex are hampered by the fact that Axin1 is expressed at vanishingly low levels, whereas APC, CK1, and GSK3, as well as β-catenin, occur abundantly outside of the destruction complex. Here, we study the endogenous destruction complex during Wnt signaling.

RESULTS

β-Catenin Stabilization and Wnt Target Gene Transcription Precede Axin Degradation

HEK293T cells carry an intact Wnt signaling cascade. We previously generated high-affinity immunoprecipitating (IP)/western blotting monoclonal antibodies against Axin1 (Ng et al., 2009). Pooled HEK293T cells from two 15 cm culture dishes allow visualization of endogenous Axin1 in a single lane by western blotting after immunoprecipitation (Ng et al., 2009). This strategy allowed us to probe the composition of the destruction complex in Wnt-active versus nonactive HEK293T cells. Axin1 protein is degraded upon Wnt stimulation (Jiang et al., 2009; Kim et al., 2008; Mao et al., 2001). We stimulated HEK293T cells with Wnt3A-conditioned medium or control medium and monitored Axin1 protein and cytosolic β-catenin by western blot analysis (Figure S1A available online and Figure 1A). Of note, the overwhelming amount of β-catenin resides in the membrane-bound E-cadherin complex, a highly stable pool that is irrelevant to Wnt signaling (van de Wetering et al., 2001). To increase detection sensitivity, we performed Axin1 immunoprecipitation on whole-cell lysates prior to western blot analysis (Figures 1B and 1C). Though cytosolic accumulation of β-catenin was detectable as early as 30 min following Wnt treatment (Figure 1A), we first observed a significant decrease in endogenous Axin1 protein at 4 hr post-Wnt stimulation (Figures 1B, 1C, and S1A), implying that degradation of Axin1 protein is not causal to the initial activation of the Wnt pathway.

To extend these findings, we examined the endogenous Wnt target genes *AXIN2* (Lustig et al., 2002), *CCND1* (Tetsu and McCormick, 1999), *EPHB3* (van de Wetering, M et al., 2002), *TCF7* (Roose et al., 1999), and *ZCCHC12* (Mahmoudi et al., 2009) by quantitative RT-PCR in a time course experiment (Figure 1D). Significant mRNA increases were detected from 0.5–2 hr post-Wnt stimulation (Figure 1D), when Axin1 protein levels were still unchanged.

Phosphorylated β-Catenin Accumulates in the Axin1 Complex in Response to Wnt

We then asked how the interaction between Axin1 and other components of the Wnt cascade is influenced by Wnt

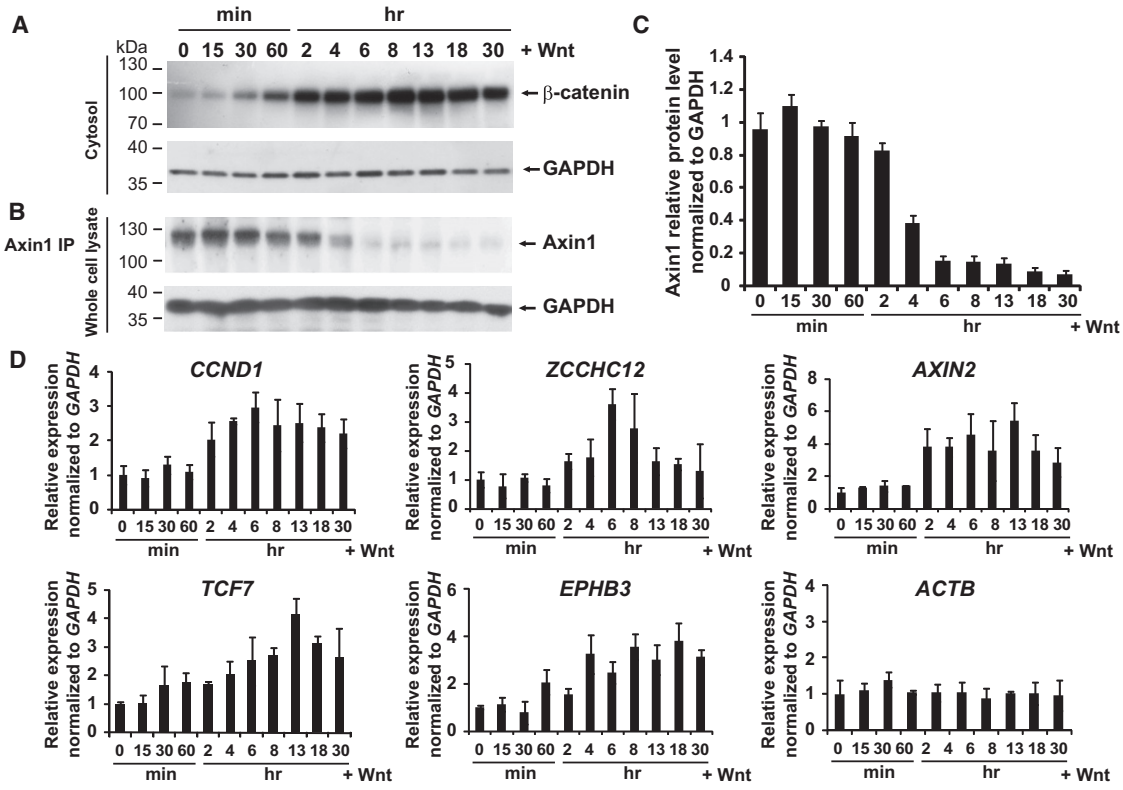

Figure 1. Wnt-Induced Stabilization of β-Catenin Occurs prior to and Independently of Axin1 Degradation

HEK293T cells were stimulated with Wnt according to the indicated time points.

(A) Cytosolic β-catenin protein levels begin to accumulate 30 min after Wnt stimulation, peaking at ~2 hr. GAPDH protein levels were used as loading control. Note that there is vanishingly detectable free β-catenin in cytosol in the absence of Wnt.

(B) Axin1 immunoprecipitation was performed from whole-cell lysates for detection of endogenous Axin1 level upon Wnt induction. GAPDH input was used as input loading control.

(C) Quantitation of Axin1 protein level relative to GAPDH. Axin1 degradation becomes significant at 4 hr post-Wnt induction. Error bars represent ±SD.

(D) Wnt-induced activation of target genes *CCND1*, *TCF7*, *ZCCHC12*, *EPHB3*, and *AXIN2* and, as control, *ACTB* was examined in HEK293T cells by quantitative RT-PCR at the indicated time points. Time course expression data are presented as fold induction normalized to *GAPDH* control in triplicate and are representative of at least two independent experiments. Error bars represent ±SD.

See also Figure S1.

stimulation. We immunoprecipitated the Axin1 complex from HEK293T cells before and during Wnt treatment, followed by western blotting (Figure 2A). In the absence of Wnt, Axin1 interacted with GSK3 (last panel) and APC (second panel), but not LRP6 (fourth panel). Phosphorylated LRP6 was only detected in the Axin1 complex after Wnt stimulation (third panel) (Mao et al., 2001; Tamai et al., 2004; Tolwinski et al., 2003). Dishevelled 3 coimmunoprecipitated with Axin1 in the absence and presence of Wnt (fifth panel). In contrast to previous reports (Liu et al., 2005; Logan and Nusse, 2004), we did not find a significant decrease in binding of either APC (second panel) or GSK3β (last panel) to Axin1 in response to Wnt stimulation. This observation was inconsistent with models in which dissociation of the destruction complex or modulation of Axin1 binding to GSK3β or APC mediate functional inactivation of the destruction complex.

β-catenin coimmunoprecipitating with Axin1 was hardly detectable in the absence of Wnt stimulation, highlighting the dynamic nature of the β-catenin/Axin1 interaction (Figure 2, eighth panel). Surprisingly, we found a significant Wnt-induced increase in β-catenin immunoprecipitating with Axin1. We further analyzed this β-catenin pool using antibodies that specifically recognize P-Ser45 β-catenin or P-Ser33/Ser37/Thr41 β-catenin (Figure 2, sixth and seventh panel). Counter to prediction, we found an increase in phosphorylated β-catenin in the Wnt-stimulated Axin1 complex (Figure S2A). This finding was consistent with a previous report noting phosphorylated β-catenin in high-molecular-weight complexes, whereas non-phospho-β-catenin accumulated in a monomeric form upon Wnt signaling (Maher et al., 2010). Thus, the critical kinases CK1 and GSK3β remain present and active within the destruction complex upon Wnt signaling.

We also combined Axin1 immunoprecipitation with mass spectrometry (MS) to obtain a global picture of the Axin1 complex in HEK293T. Consistent with our IP results, we readily detected the core components of the destruction complex (APC, GSK3β, CK1, and β-catenin) in both Wnt-inactive and -activated cells (Table S1). Quantitative MS using a label-free approach with extracted ion chromatograms further confirmed a significant

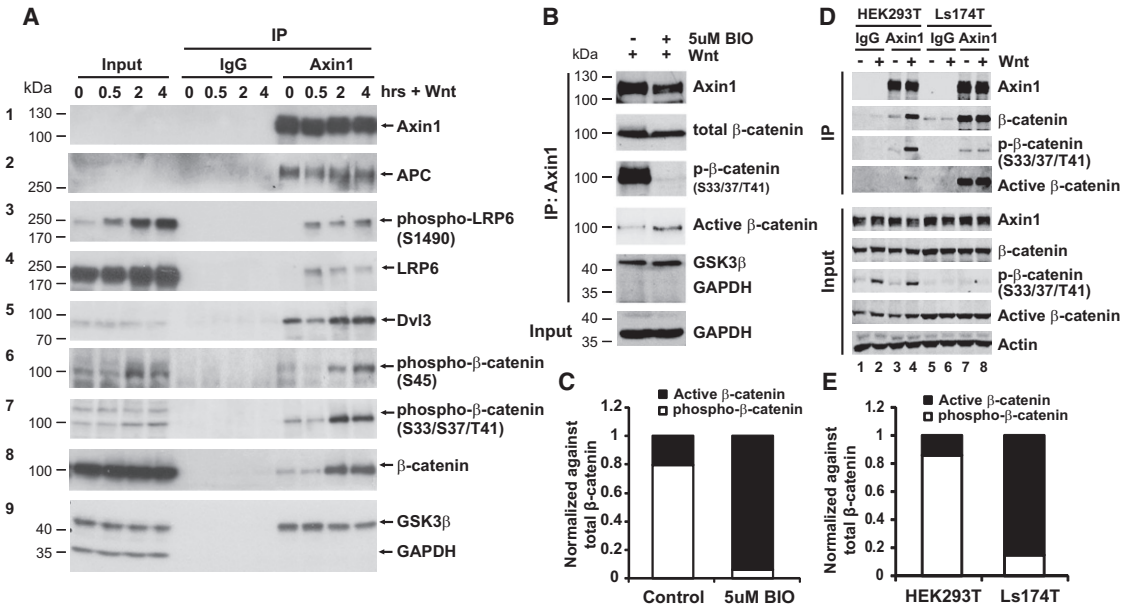

Figure 2. Accumulation of Phosphorylated β-Catenin in Axin1-β-Catenin Destruction Complex upon Wnt Activation

(A) HEK293T cells were exposed to Wnt3A-conditioned medium or control-conditioned medium at different time points as indicated. Stimulated lysates were subjected to immunoprecipitation using an Axin1-specific antibody and IgG as control and were subjected to SDS-PAGE followed by western blot analysis using the indicated antibodies.

(B) Wnt-stimulated HEK293T cells with or without GSK3 inhibitor BIO treatment were subjected to Axin1 immunoprecipitation followed by western blot analysis using the indicated antibodies.

(C) Quantitation of the amount of β-catenin phosphorylation in the Axin complex upon Wnt stimulation by comparing the ratio between phospho-β-catenin (ser33/37/thr41) and active non-phospho-β-catenin with total β-catenin. Error bars represent ±SD.

(D) HEK293T and Ls174T cells treated with control- or Wnt-conditioned medium were subjected to Axin1 immunoprecipitation followed by western blot analysis using the indicated antibodies.

(E) Quantitation of β-catenin phosphorylation in the Axin complex upon Wnt stimulation in HEK293T and Ls174T cells was done as described in (C).

See also Figure S2 and Tables S1 and S2.

increase of β-catenin detected within the Axin complex after Wnt stimulation. Other components (e.g., APC, GSK3β, and CK1) remained unchanged (Figures S2C, S2D, and Table S2). Similar observations were reported recently (Hilger and Mann, 2012).

To quantify the relative amount of phosphorylated β-catenin bound to the Axin-β-catenin destruction complex upon Wnt stimulation, we treated HEK293T with 6-bromoindirubin-3′-oxime (BIO), a GSK3 inhibitor. HEK293T cells, either untreated or treated with BIO overnight, were induced to Wnt3A followed by Axin1 immunoprecipitation. Axin-bound β-catenin was then analyzed by Western blotting (Figure 2B). Confirming our earlier observations, phospho-β-catenin was strongly present in the Axin complex after Wnt induction. Importantly, BIO treatment completely abolished β-catenin phosphorylation in the Axin complex (Figure 2B), implying that GSK3 remained active under Wnt stimulation. An antibody, recognizing non-phospho-β-catenin (van Noort et al., 2002), confirmed the accumulation of non-phospho-β-catenin in the Axin-complex after BIO treatment. Quantitation of the ratio between phospho- and non-phospho β-catenin relative to total β-catenin demonstrated that ~80% of β-catenin bound to the Axin complex upon Wnt induction was phosphorylated (Figure 2C).

To further quantify β-catenin phosphorylation within the Axin destruction complex, we compared the ratio of phospho- to non-phospho β-catenin in the presence or absence of Wnt in HEK293T and in Ls174T colorectal cancer (CRC) cells (Figure 2D). In Ls174T, β-catenin is homozygously mutated at Ser45 and cannot be phosphorylated by CKIα and consequently by GSK3. β-catenin is thus not recognized by the phospho-β-catenin antibody in these cells. As expected, phospho-β-catenin was detected in HEK293T after Wnt induction, but not in Ls174T cells, whereas non-phospho β-catenin was present in Ls174T cells only (Figure 2D, lanes 4 and 8). Confirming our results using BIO treatment of HEK293T cells (Figure 2C), quantitation of the ratio between phospho- and non-phospho β-catenin in HEK293T versus Ls174T cells revealed that ~80% of β-catenin within the destruction complex was phosphorylated in HEK293T cells (Figure 2E). Taken together, these results imply that Wnt stimulation does not affect the destruction complex kinases GSK3 or CK1 but, rather, causes accumulation of phospho-β-catenin in the Axin-complex.

Wnt Stimulation Abrogates Ubiquitination of Phosphorylated β-Catenin

To determine whether the β-catenin pool bound to Axin1 is subject to ubiquitination and degradation within the complex, we treated HEK293T cells with a combination of Wnt and the proteasome inhibitor MG132. This compound blocks

proteasomal degradation of ubiquitinated proteins. First, we examined whether treatment with proteasome inhibitor allowed detection of ubiquitinated Axin1-bound β-catenin in non-Wnt stimulated HEK293T cells. We immunoprecipitated Axin1 from lysates of MG132-untreated or -treated cells and probed for the association of S33/S37/T41 phosphorylated β-catenin with Axin1 (Figure S3A). If ubiquitination and degradation of β-catenin occurs within the destruction complex, we should detect the ubiquitinated forms of phosphorylated β-catenin upon Axin pull-down. Indeed, we readily observed ubiquitinated, phosphorylated β-catenin in immunoprecipitated Axin1 complexes after MG132 treatment (Figure S3A). This implied that β-catenin is not only phosphorylated, but also ubiquitinated and degraded within the destruction complex, suggesting a proteasome-dependent mechanism for complex regeneration. In support of this notion, we detected a number of proteasome complex subunits in our Axin1 immunoprecipitation/MS experiment in HEK293T cells (Figure S1B and Table S1). Independently, tandem-affinity purification of SBP-HA-CBP-tagged Axin coupled to mass spectrometry has identified the ubiquitin protease USP34 as an Axin1-interacting protein (Lui et al., 2011). These data imply that the phosphorylated and ubiquitinated form of β-catenin is removed from the Axin1 complex by proteasomal degradation, thus recycling the destruction complex.

As additional evidence, we performed a time course experiment for MG132 treatment, followed by Axin IP and western blotting for ubiquitinated phospho-β-catenin (Figure 3A). We reasoned that, if our hypothesis was correct, direct blockage of proteasomal degradation within the Axin1-complex by MG132 treatment should cause an immediate phospho-β-catenin accumulation within the Axin complex, with faster kinetics than the accumulation induced by Wnt (Figure 2A). Indeed, we found that, even after only 0.5 hr of MG132 treatment, phospho-β-catenin occurred in the Axin complex (Figure 3A). Ubiquitination of β-catenin was confirmed using an anti-ubiquitin antibody (Figure S3B). Cells remained healthy, as 7 hr of MG132 in the absence of Wnt readily activated Wnt/TCF-driven transcription as determined by qRT-PCR for Wnt target gene expression and by TOPFlash luciferase assay (Figures 3B, S3E, S3F).

To test the effect of Wnt on ubiquitination of β-catenin within the Axin complex, we immunoprecipitated Axin1 from lysates treated with either MG132 alone or combined with Wnt. There was a much stronger presence of phosphorylated β-catenin that coimmunoprecipitated with Axin1 in MG132-treated cells without Wnt than in cells treated with MG132 with Wnt (Figure 3C). Of note, the polyubiquitination of phospho-β-catenin is shown by multiple band shifts of ~8 kDa (Figure 3D, arrows). This result suggested that Wnt stimulation interferes with ubiquitination of phosphorylated β-catenin within the destruction complex.

To document the ubiquitination state of phospho-β-catenin within the destruction complex, we overexpressed His-tagged ubiquitin in HEK293T cells 12 hr prior to treatment with MG132 and Wnt. Antibodies specific for Axin1, β-catenin, or control isotype-matched IgG were used to immunoprecipitate the specific complexes (Figure 3E). The immunoprecipitated complexes were washed and eluted followed by a His pull-down assay. The His pull-down samples were analyzed by western blotting using an antibody specific for β-catenin (Figure 3F). We found significantly less ubiquitinated β-catenin in Wnt-treated cells than in untreated samples (Figure 3F, lanes 7 and 8). Ubiquitinated β-catenin occurred only in Axin1 complexes when cells were treated with only MG132 and not with MG132 and Wnt (Figure 3F, lanes 5 and 6). These data demonstrated that Wnt stimulation interferes with ubiquitination of phosphorylated β-catenin within the destruction complex.

We then examined whether Wnt stimulation has an effect on β-TrCP, the E3 ligase that ubiquitinates β-catenin (Aberle et al., 1997; Kitagawa et al., 1999). As shown in Figures 3G and 3H, whereas β-TrCP coimmunoprecipitated with both β-catenin (Figure 3G, lane 5) and Axin1 (Figure 3H, lane 5) in the presence of MG132 in non-Wnt-treated cells, Wnt treatment abrogated the β-TrCP interaction with β-catenin (Figure 3G, lane 6) and Axin1 (Figure 3H, lane 6). The dissociation of β-TrCP upon Wnt stimulation was seen with two β-TrCP antibodies (Figures 3H and S3C). As expected, β-TrCP also dissociated from exogenously expressed Flag-tagged β-catenin upon Wnt stimulation (Figure S3D). Wnt-driven suppression of β-catenin ubiquitination and β-TrCP dissociation from the complex was confirmed using an Axin-specific monoclonal antibody (A6) recognizing a distinct epitope (Figure S4A) as well as with Flag-tagged human Axin1 (Figure S4B).

Wnt-Induced Accumulation of Phosphorylated β-Catenin within the Axin1 Complex in Primary Tissue

The small intestinal epithelium is arguably the best described model for mammalian Wnt signaling. Signaling is active in all crypt cells and inactive in all villus cells (Reya and Clevers, 2005) and can be visualized by β-catenin nuclear localization or by Wnt target genes such as *Axin2* (Figure 4A). We performed endogenous Axin1 IP on freshly isolated crypts and villi. Consistent with the HEK293T data, Axin1 complex interacted with phospho-Lrp6 in crypt cells, but not villus cells (Figure 4B). The increased presence of phospho-β-catenin within the Axin1 complex was detected in crypt, but not villus cells. We confirmed this in cultured intestinal organoids (Sato et al., 2009). This culture requires R-spondin (Figure S5A) to enhance Wnt signals through its receptors Lgr4 and Lgr5 (de Lau et al., 2011). We treated the organoids with or without R-spondin and Wnt3A medium for 4 hr followed by Axin1 immunoprecipitation. This confirmed the phospho-Lrp6/axin1 interaction as well as the accumulation of β-catenin in the Axin1 complex in the presence of R-spondin and Wnt3A medium (Figure S5B). The new Wnt regulatory model for the destruction complex is shown in Figure 4C.

A recent report proposes that sequestration of GSK3 into multivesicular bodies controls its activity during Wnt signaling (Taelman et al., 2010). We have previously demonstrated different GSK3 pools; only 3%–5% of endogenous cellular GSK3 resides in the destruction complex (Ng et al., 2009). Our current findings do not support the GSK3 sequestration model during initial Wnt activation. We examined the effect of Wnt on β-TrCP interaction with the Axin1 complex 1 hr poststimulation in HEK293T cells. We detected significant dissociation of β-TrCP from Axin1 at this early time point (Figures 5A and 5B). Interaction of Axin1

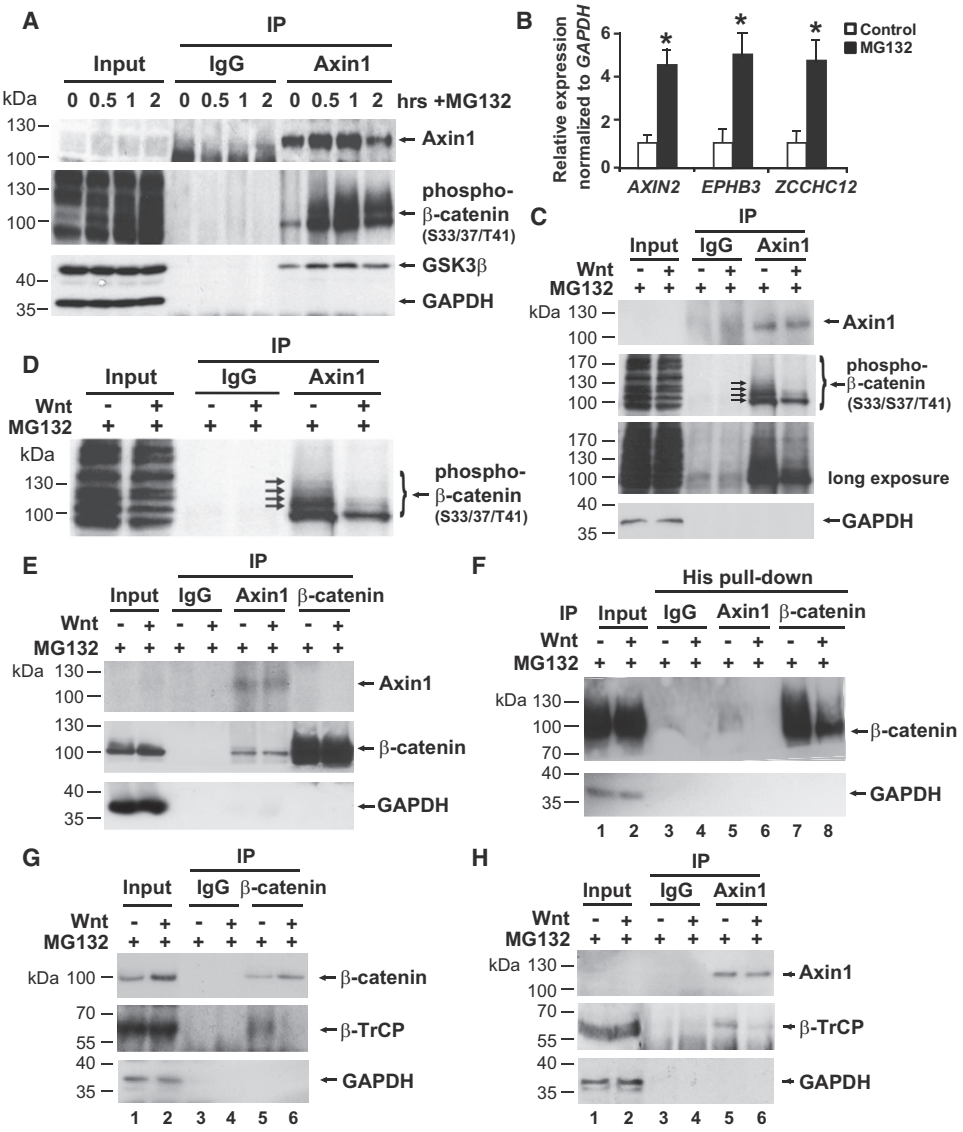

Figure 3. Wnt Stimulation Abrogates Ubiquitination of Phosphorylated β-Catenin and Disrupting the Interaction of β-TrCP with the Axin1-β-Catenin Destruction Complex

(A) HEK293T cells were treated with or without proteasome inhibitor MG132 at indicated time points. Samples were immunoprecipitated with Axin1 antibody followed by western blot analysis using the indicated antibodies.

(B) qRT-PCR results of indicated Wnt target genes with control or MG132 treatment after 7 hr. Error bars represent SD from three independent experiments. *$p < 0.001$.

(C) HEK293T cells were treated with proteasome inhibitor MG132 together with Wnt3A- or control-conditioned medium for 4 hr. Treated cells were collected, lysed, and used for Axin1 immunoprecipitation, followed by western blot analysis using the indicated antibodies. Arrows indicate detection of polyubiquitinated phospho-β-catenin with an ~8 kDa shift.

(D) Enlargement of anti-phospho-β-catenin (Ser33/Ser37/T41) blot from (C) for better resolution.

(E and F) HEK293T cells were transfected with His-tagged ubiquitin. After 12 hr, cells were treated with proteasome inhibitor MG132 together with Wnt3A- or control-conditioned medium for 4 hr. Cell lysates were then used for immunoprecipitation with antibodies specific for Axin1, β-catenin, or control IgG as indicated (E). Axin1 and β-catenin complexes were eluted and used in His pull-down assays. Samples were separated by SDS-PAGE and analyzed by western blotting with anti β-catenin antibody (F).

(G and H) HEK293T cells treated with MG132 together with Wnt3A or control-conditioned medium for 4 hr were lysed and immunoprecipitated with an antibody against β-catenin (G) or Axin1 (H). Immunoprecipitated complexes were analyzed by western blotting using antibodies directed against β-TrCP, β-catenin, Axin1, and GAPDH as control as indicated.

See also Figures S3 and S4.

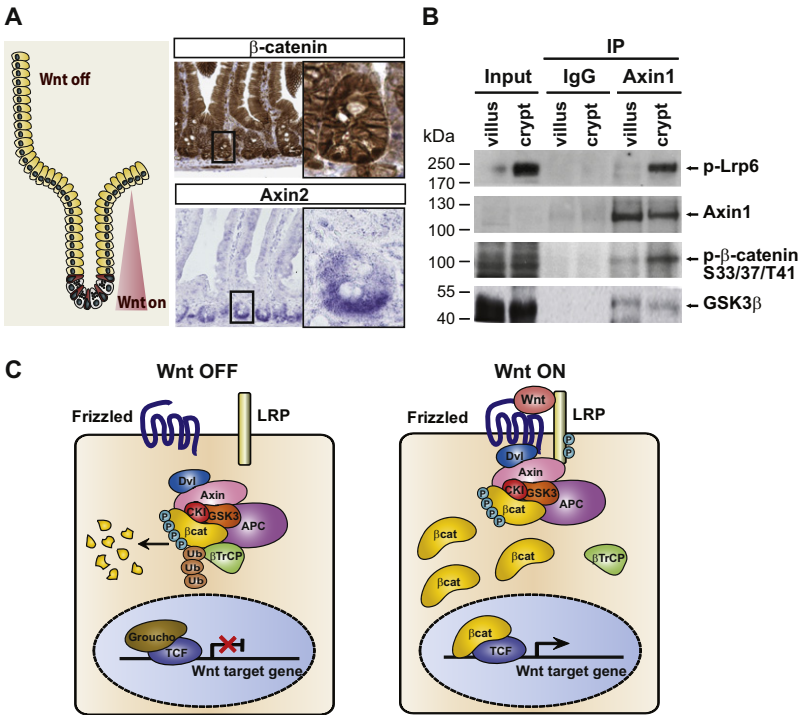

Figure 4. Wnt-Induced β-Catenin Phosphorylation within the Destruction Complex Is Confirmed in Primary Tissues

(A) Schematic representation of small intestine showing Wnt signal is "on" in crypt and "off" in villus compartment. Immunohistochemical staining showing β-catenin is nuclei-localized in the crypts. The Wnt target gene *AXIN2* mRNA is also expressed at the bottom crypts, as shown by in situ hybridization.

(B) Axin1 complexes were immunoprecipitated from isolated crypts and villi fractions followed by western blot analysis using the indicated antibodies.

(C) Schematic diagram showing a novel regulatory model of Wnt/β-catenin signaling. Refer to the text for details.

See also Figure S5.

with phospho-Lrp6 was also detected (Figure 5A). We then analyzed the sequestration of the Axin1 destruction complex into MVB at early Wnt induction time points (1 and 4 hr post-Wnt stimulation) by studying colocalization with LysoTracker, a dye marking MVB and lysosomes. We did not detect colocalization of endogenous β-catenin (Figures 5C–5E) or GSK3β (Figures 5F–5H) with LysoTracker after 1 or 4 hr. We further analyzed the localization of Axin1 by overexpressing Axin1-YFP in the cells. Axin1-YFP puncta were detected in the cytosol (Figures 5I–5K). However, no colocalization of Axin1 with LysoTracker was observed even after 4 hr. We therefore concluded that β-TrCP dissociation from the destruction complex is the immediate molecular response following Wnt stimulation. Our data do not exclude that sequestration of the destruction complex in MVB may occur as a later event.

Axin1 Complex Remains Compositionally Intact in APC and β-Catenin Mutant Colon Cancer Cells

Activating mutations of Wnt pathway components constitute the gatekeeper event in human colorectal cancer (Fodde and Brabletz, 2007). The data in Figure 2D suggested that the Axin complex remains intact upon mutational Wnt activation in CRC. We examined the Axin1 complex in various CRC cell lines in which the Wnt pathway is locked in the "on" state. We immunoprecipitated Axin1 from Ls174T (homozygous activating β-*catenin* mutation; WT *APC*), HCT116 (Ser45 deletion in one β-*catenin* allele and one WT allele; WT *APC*) and from DLD1 cells (truncated *APC*; WT β-*catenin*). We then probed the Axin1-immunoprecipitated complexes for components of the destruction complex (Figure 6A). In all CRCs, the destruction complex contained the core components GSK3β and β-catenin despite the constitutive activity of the Wnt pathway. More interestingly, the destruction complex of DLD1 cells contained the mutationally truncated APC, lacking the Axin-binding SAMP motifs and most β-catenin 20 aa repeats (Miyoshi et al., 1992; Nagase and Nakamura, 1993). We excluded the possibility that Axin1 interacts with APC and β-catenin in distinct subcomplexes (Figures S6A and S6B). Does GSK3 still phosphorylate β-catenin within the Axin1 complex in these APC-mutated CRCs? In HCT116, there remains one WT allele of β-catenin. Indeed, phospho-β-catenin was readily detectable in the Axin1 complex. In DLD1, a P-Ser33/Ser37/Thr41 β-catenin coimmunoprecipitated with Axin1 (Figures 6A and S6C). Thus, disruption of the interaction between APC and β-catenin is not the mechanism of tumorigenesis caused by APC mutations. Rather, these observations suggested that the Axin1 complex may be saturated by phospho-β-catenin in CRCs due to improper function of APC.

Unlike the situation in exogenous Wnt-activated HEK293T cells, Lrp6 did not coimmunoprecipitate with Axin1 in CRCs (Figure 6A, top). We hypothesized that the upstream pathway in the Wnt cascade may be functionally intact and can further be induced by Wnt treatment. Consistent with this hypothesis, all three CRCs showed Wnt receptor activation upon Wnt stimulation, as read out by Lrp6 phosphorylation (Figure 6B). HCT116 could be further activated by exogenous Wnt based on TOP/Flash luciferase assays (Figure 6C). This can be explained by the fact that HCT116 cells retain one wild-type β-catenin allele.

Failure of Axin-Bound β-Catenin Ubiquitination in APC-Truncated Cell Lines

It is commonly believed that APC truncation in CRCs causes dissociation of the destruction complex because of the loss of the 20 aa repeat regions that bind β-catenin (Munemitsu et al., 1995) and/or the SAMP repeats that bind Axin (Behrens et al., 1998). Contrasting with this notion, we find that the Axin complex remains intact in CRCs. This prompted us to study whether APC truncation would rather affect β-catenin ubiquitination. Re-expression of the central region of APC is sufficient for Wnt pathway regulation (Munemitsu et al., 1995). We therefore

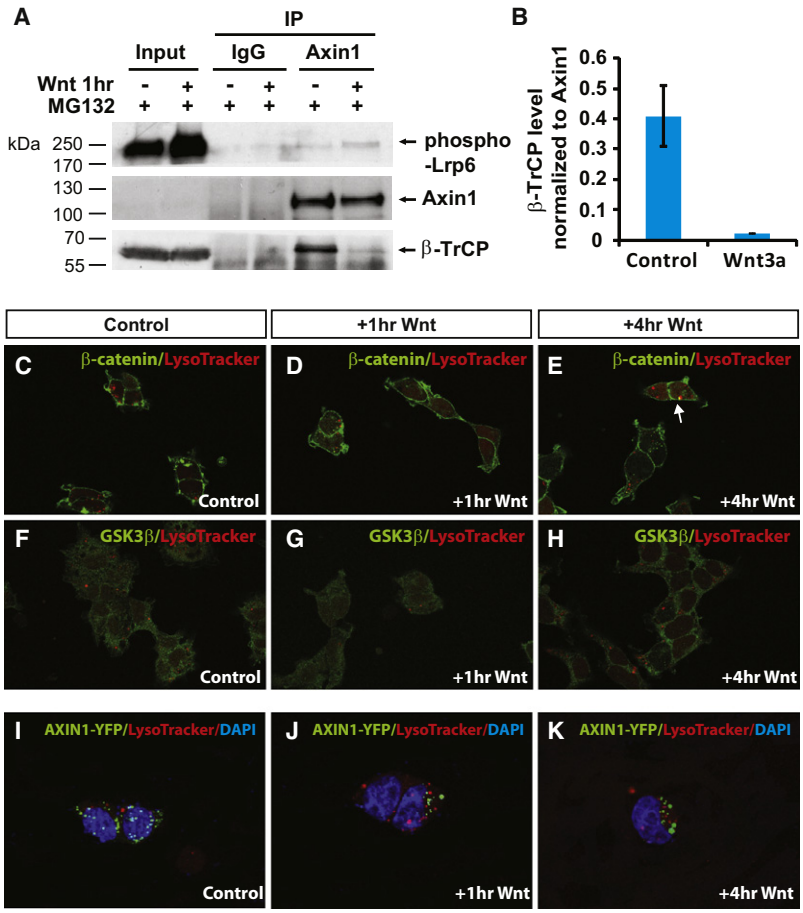

Figure 5. β-TrCP Dissociation from the Complex Is the Immediate Response of Wnt Stimulation, prior to Sequestration of the Complex to Multivesicular Endosomes

(A) HEK293T cells were treated with proteasome inhibitor MG132 together with Wnt3A- or control-conditioned medium for 1 hr. Treated cells were collected, lysed, and used for Axin1 immunoprecipitation, followed by western blot analysis with anti-phospho-Lrp6, Axin, and β-TrCP antibodies.

(B) Quantitation of β-TrCP level normalized to Axin1 after control or Wnt3A medium treatment for 1 hr from (A). Error bars represent SD from three independent experiments. Error bars represent ±SD.

(C–K) HEK293T cells were treated with control- (C, F, and I) or Wnt3A-conditioned medium for 1 (D, G, and J) and 4 hr (E, H, and K). Co-immunostaining of Wnt components (green) β-catenin (C–E), GSK3β (F–H), and Axin1-YFP (I–K) with LysoTracker (red) was performed after Wnt treatment at different time points. Colocalization of β-catenin and LysoTracker is indicated by white arrow.

cloned this central region (1265–2060 amino acids) containing the 20 aa repeats and the SAMP motifs (Figure 7A). TOP/Flash luciferase arrays confirmed that this central region of APC could significantly suppress the downstream β-catenin/TCF transcription in both SW480 (Figure 7B) and DLD1 cells (Figure S7A) to a level comparable to full-length APC.

To examine the β-catenin ubiquitination state in these two APC truncated cell lines, we pretreated the cells with MG132 for 4 hr followed by endogenous Axin1 immunoprecipitation. Western blot analysis revealed that β-catenin was present in the Axin1 complex in both SW480 and DLD1 cells (Figures 7C and S7B, lane3). Strikingly, β-catenin ubiquitination was readily detectable in the Axin1-complex after reintroducing APC 1265–2060 (Figures 7C and S7B, lane 4). Our data demonstrate that APC truncations in CRCs activate Wnt signal by impairing β-catenin ubiquitination within the destruction complex, thereby saturating the complex, as Wnt treatment does to normal cells.

DISCUSSION

In the current study, we have not used overexpression analyses but, for the first time, study the endogenous Axin complex. We make a number of observations that are not compatible with existing models of Wnt signal transduction. Specifically: (1) The composition of the Axin complex does not change upon Wnt signaling. (2) The activities of the kinases (GSK3/CKI) are not inhibited upon Wnt signaling. Rather, a phosphorylated form of β-catenin accumulates in the intact destruction complex upon Wnt signaling. (3) The E3 ligase β-TrCP acts within the intact destruction complex. (4) It has been unknown how β-catenin, once targeted for destruction, is removed from the complex. We now show that removal of β-catenin from the intact complex is executed by the proteasome upon ubiquitination. (5) In contrast to current belief, the Axin complex remains compositionally intact in APC mutant colorectal cancer. Again, it is the failure of β-catenin ubiquitination that is the principle biochemical activating event.

Our current data thus support an alternate Wnt-induced regulatory model for the destruction complex, as shown in Figure 4C. In the absence of Wnt stimulation, β-catenin is sequentially phosphorylated within the destruction complex by the serine/threonine kinases CK1 and GSK3α/β. Phosphorylated β-catenin is then recognized by β-TrCP, a component of the E3 ubiquitin ligase complex, and ubiquitinated (Ub) within the destruction complex. Subsequently, ubiquitinated β-catenin is rapidly degraded by the proteasome. The removal of β-catenin from the destruction complex is accomplished simply by direct degradation by the proteasome. This step recycles the destruction complex for another round of β-catenin degradation. We show that Wnt receptor-ligand interaction leaves the destruction complex compositionally unchanged and does not affect the activity of its kinases. The only change that we observe is the association of Axin1 with phosphorylated Lrp6 and the dissociation of β-TrCP. Indeed, phosphorylated β-catenin—still bound to the Axin1 complex—is no longer ubiquitinated and degraded. It saturates and thus effectively inactivates the Axin1 complex. We have previously reported that only β-catenin that is newly synthesized after initiation of the Wnt signal is signaling competent

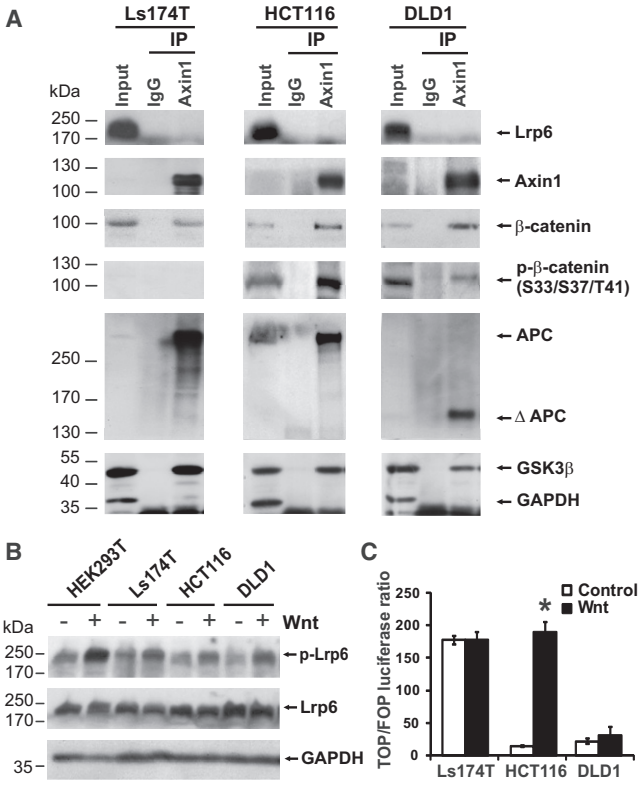

Figure 6. The Axin1-β-Catenin Destruction Complex Remains Compositionally Intact despite Constitutive Wnt Signaling in Various Colorectal Cancer Cell Lines

(A) Cell lysates from colorectal cancer cell lines Ls174T, HCT116, and DLD-1, as indicated, were immunoprecipitated with a specific antibody directed against endogenous Axin1 and IgG as a negative control. The immunoprecipitated complex was analyzed by western blotting using antibodies directed against Lrp6, Axin1, β-catenin, phosphorylated β-catenin, β-TrCP, APC, and GSK3β and, as a negative control, GAPDH. ΔAPC indicates the truncated form of APC expressed in DLD-1 (140 KD) cells.

(B) HEK293T cells together with three CRCs, Ls174T, HCT116, and DLD1 cells, were treated with control or Wnt3A medium for 4 hr followed by western blot analysis to probe for Lrp6 phosphorylation.

(C) Relative TOP/FOP activities were shown after control (white bars) or Wnt3A (black bars) treatment to the three CRCs. Error bars represent SD from three independent experiments.*$p < 0.001$.

See also Figure S6.

(Staal et al., 2002). This notion is in agreement with our current model, which predicts that newly synthesized, nonphosphorylated β-catenin will be stable in a free cytosolic form once the destruction complex is saturated. It can then translocate to the nucleus to associate with TCF and activate the Wnt transcriptional program.

Comparing these molecular events to those resulting from mutations in β-catenin and APC in CRC reveals mechanistic differences in these two "Wnt on" systems. In HEK293T, Wnt stimulation leads to a temporary inactivation-by-saturation of the destruction complex, followed by degradation of the Axin1 scaffold itself. By contrast, the destruction complex is compositionally intact and stable in all CRC lines examined. Contrary to common belief, truncated APC remains present within the Axin1 complex in APC mutant CRC cells. Our data support a model in which mutations in Wnt pathway components in CRC cause the Axin1 complex to be locked in a compositionally intact but functionally inactive form, leading to aberrant cytoplasmic accumulation and nuclear localization of β-catenin.

What is the essential role played by APC during the β-catenin degradation/elimination process? Our observations indicate that APC protein function is most likely required at a step after β-catenin phosphorylation. APC was reported to act as a "ratchet" to remove phosphorylated β-catenin from Axin for ubiquitination, making Axin available for further rounds of β-catenin phosphorylation (Kimelman and Xu, 2006; Xing et al., 2003). We do not confirm this observation. Rather, we observe that removal of ubiquitinated β-catenin from the Axin1 complex requires functional proteasomes. Interestingly, it has recently been shown that β-TrCP can only interact with β-catenin in the presence of ectopically expressed wild-type APC in three APC-truncated cell lines. This study lends support to the notion that APC may function by mediating β-catenin's ubiquitination by β-TrCP (Su et al., 2008). Indeed, our current data prove that APC truncation results in failure of β-catenin ubiquitination within the Axin destruction complex.

EXPERIMENTAL PROCEDURES

Antibodies

APC (Calbiochem), Dishevelled3 (Santa Cruz), GSK3b (Cell Signaling), phospho-LRP6 (S1490, Cell Signaling), LRP6 (Cell Signaling), phospho-β-catenin S45 (Cell Signaling), phospho-β-catenin S33/S37/T41 (Cell Signaling), β-catenin (BD Transduction), active-β-catenin (Millipore, 8E7), β-TrCP (ITK Diagnostics BV and Cell Signaling), ubiquitin (Millipore, FK2), and GAPDH (Abcam) were used in immunoprecipitations or Western blot analysis.

Cell Culture

HEK293T, DLD-1, HCT116, and Ls174T cells were maintained in RPMI 1640 (Invitrogen) supplemented with 5% fetal calf serum (FCS). Wnt3A-producing L cells (kind gift from R. Nusse) or control L cells were cultured according to the manufacturer's instructions (ATCC).

Cell Fractionation

HEK293T cells were stimulated with Wnt3A-conditioned medium or control-conditioned medium as indicated. Cells were washed and collected in ice-cold PBS. Cell pellets were then resuspended in hypotonic lysis buffer (10 mM KCl, 10 mM Tris [pH 7.5], and 2 mM EDTA) containing protease inhibitor and phosphatase inhibitor cocktail tablets. Cell suspensions were incubated on ice for 30 min and dounced multiple times. Lysis of cells was controlled by checking under the microscope. Nuclear proteins, including the unlysed cells, were pelleted by spinning at 2,000 rpm for 2 min at 4°C centrifugation. The supernatant that contained both cytoplasm and membrane proteins was then centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was collected and analyzed further by SDS-PAGE followed by western blotting.

Immunoprecipitation

At 80% confluency, HEK293T cells were treated with either Wnt3A or control-conditioned medium in the absence or presence of 10 μM MG132 as indicated. For GSK3 inhibitor BIO assay, 5 μM BIO was added to the cells 16 hr prior to Wnt induction. HEK293T, Ls174T, DLD-1, and SW480 cells were washed and collected with cold PBS, lysed in cold lysis buffer containing 150 mM NaCl, 30 mM Tris (pH 7.5), 1 mM EDTA, 1% Triton X-100, 10% Glycerol, 0.1 mM PMSF, 0.5 mM DTT, protease inhibitor

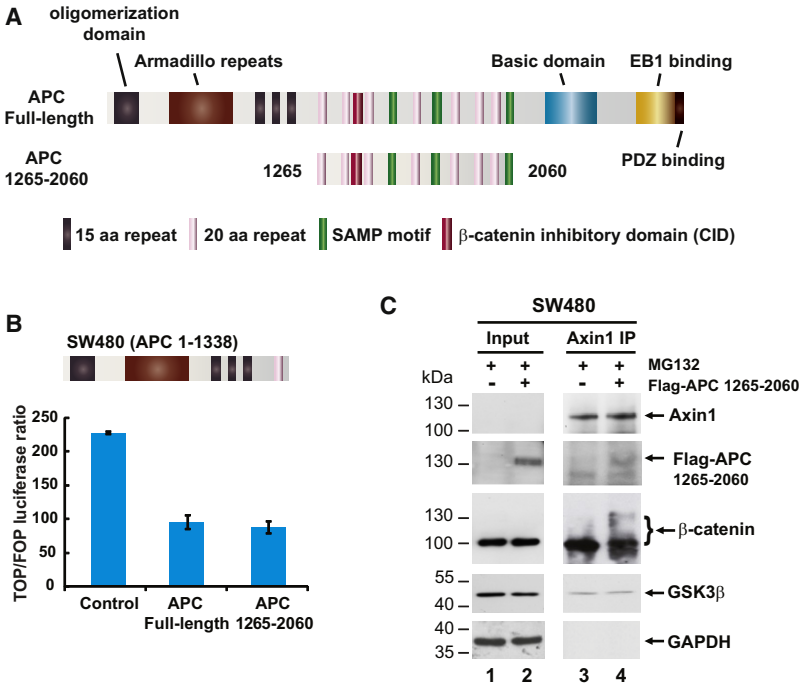

Figure 7. APC Truncation Impairs β-Catenin Ubiquitination within the Axin Complex

(A) Schematic representations of wild-type full-length and the central region (1265–2060 amino acids) of APC.

(B) Relative TOP/FOP activities of the APC truncated cell line SW480 was shown after transfecting empty vector, full-length, or central region of APC plasmids. Error bars represent ± SD.

(C) Axin1 complexes were immunoprecipitated from SW480 cells with or without APC transfection and were analyzed by western blotting using the indicated antibodies.

See also Figure S7.

cocktail tablets (EDTA-free) (Roche), and phosphatase inhibitor cocktail tablets (Roche). After clarification by centrifugation (14,000 rpm for 30 min at 4°C), the cellular lysates were precleared with IgG-agarose beads (Sigma) for at least 6 hr at 4°C. Immunoprecipitation of endogenous complexes was carried out by incubating the cellular lysates with anti-Axin1 antibody, mouse IgG, or β-catenin antibody (BD Transductions) immobilized on Protein G PLUS-Agarose beads (Santa Cruz Biotechnology) at 4°C overnight. Immunocomplexes were washed with cold lysis buffer six times, resuspended in SDS sample buffer, and subjected to SDS-PAGE and western blot analysis.

Immunostaining Assay

HEK293T were grown on 12-well coverslips in 12-well plates precoated with poly-L-lysin (Sigma), fixed with 4% paraformaldehyde (PFA) for 15 min, and permeabilized by 0.2%Triton X-100 in PBS for 10 min. Cells were blocked with 5% goat serum and 0.5% BSA in PBS for 1 hr and then primary antibodies overnight at 4°C. Cells were washed three times PBS followed by secondary antibodies incubation at room temperature for 1 hr. Coverslips were washed another three times with PBS and were then mounted with Vectashield with DAPI (Vector Lab). For LysoTracker staining, live cells were treated with 1:1,000 diluted LysoTracker Red DND-99 (Invitrogen) for 1 hr at 37°C prior to 4%PFA fixation.

In Vivo and Ex Vivo Wnt Induction Using Mouse Small Intestines

Intestinal crypts and villi fractions were isolated as previously described (Mahmoudi et al., 2009) followed by Axin1 immunoprecipitation. Wild-type isolated crypts were cultured into organoids according to the protocols invented by our group (Sato et al., 2009). Organoids were pretreated with or without Rspondin and Wnt-conditioned medium for 4 hr followed by Axin1 immunoprecipitation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.cell.2012.05.002.

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