Wilms Tumor Suppressor WTX Negatively Regulates WNT/ß-Catenin Signaling

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gene amplification of a kinase that is not a direct or downstream target of gefitinib or erlotinib. Moreover, MET has not previously been shown to signal through ERBB3. These findings may have important clinical implications for NSCLC patients who develop acquired resistance to gefitinib or erlotinib. Our findings also suggest that irreversible EGFR inhibitors, which are currently under clinical development as treatments for patients whose tumors have developed acquired resistance to gefitinib and erlotinib, may be ineffective in the subset of tumors with a *MET* amplification even if they contain an *EGFR* T790M mutation. Therefore, combination therapies with MET kinase inhibitors, which are in early-stage clinical trials, and irreversible EGFR inhibitors should be considered for patients whose tumors have become resistant to gefitinib or erlotinib. Notably, a small percentage of NSCLCs from EGFR TKI–naïve patients have been reported to contain both an *EGFR*-activating mutation and *MET* amplification (*20*, *21*). This situation is analogous to the observation that untreated NSCLCs occasionally have an *EGFR* T790M. These concurrent genetic alterations may help explain why some NSCLCs with *EGFR*-activating mutations fail to respond when initially treated with gefitinib (*22*).

It will continue to be important to study NSCLC primary tumors and cell lines with acquired resistance to EGFR inhibitors for insights into additional resistance mechanisms. Our findings illustrate the value of studying genetic alterations that produce persistent PI3K/Akt signaling in the presence of gefitinib rather than focusing solely on mutations in the *EGFR* gene itself. It will also be important to determine whether *MET* amplification contributes to resistance in other *EGFR*-dependent cancers such as glioblastoma multiforme, head and neck cancer, and colorectal cancer after treatment with EGFR-directed therapies. Finally, since ERBB2-amplified breast cancers also activate PI3K/Akt signaling through ERBB3, it will be interesting to explore whether *MET* amplification also occurs in breast cancers that develop resistance to drugs that target ERBB2, such as trastuzumab and lapatinib (*9*, *23*).

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Supporting Online Material

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Wilms Tumor Suppressor WTX Negatively Regulates WNT/β-Catenin Signaling

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Aberrant WNT signal transduction is involved in many diseases. In colorectal cancer and melanoma, mutational disruption of proteins involved in the degradation of β-catenin, the key effector of the WNT signaling pathway, results in stabilization of β-catenin and, in turn, activation of transcription. We have used tandem-affinity protein purification and mass spectrometry to define the protein interaction network of the β-catenin destruction complex. This assay revealed that WTX, a protein encoded by a gene mutated in Wilms tumors, forms a complex with β-catenin, AXIN1, β-TrCP2 (β-transducin repeat–containing protein 2), and APC (adenomatous polyposis coli). Functional analyses in cultured cells, *Xenopus*, and zebrafish demonstrate that WTX promotes β-catenin ubiquitination and degradation, which antagonize WNT/β-catenin signaling. These data provide a possible mechanistic explanation for the tumor suppressor activity of WTX.

In the absence of WNT ligands, cytosolic β-catenin is constitutively degraded through phosphorylation-dependent ubiquitination and subsequent proteosomal clearance. A complex of proteins including adenomatous polyposis coli (APC), AXIN, casein kinase 1α (CK1α), and glycogen synthase kinase 3 (GSK3) phosphorylates N-terminal serine residues in β-catenin, which creates a substrate efficiently ubiquitinated by the Skp1, Cullin1, F-box protein β-TrCP ($SCF^{\beta TrCP}$) ubiquitin ligase (*1*). The engagement of a Frizzled receptor with WNT ligand initiates a signaling cascade, culminating in the inactivation of the β-catenin destruction complex. Consequently, β-catenin levels increase in the nucleus, where it functions as a transcriptional coactivator for members of the TCF-LEF family of transcription factors (*2*, *3*). Although mutations in APC are common in colorectal cancer, many human malignancies harboring active WNT/β-catenin signaling have no identified causative mutation(s) (*4*, *5*).

To identify proteins associated with the β-catenin destruction complex, we performed a tandem-affinity purification (TAP) of $\beta\text{-catenin}^{(SA)}$, AXIN1, APC (amino acids 1 to 1060), β-TrCP1, and β-TrCP2 in mammalian cells (*6*). The $\beta\text{-catenin}^{(SA)}$ mutant has alanine substituted for serine at codon 37. Specifically, cDNA for each of these "bait" proteins was cloned into the pGlue vector encoding a dual-affinity tag containing streptavidin-binding protein (SBP), calmodulin-binding protein (CBP), and the hemagglutinin (HA) epitope (*7*). Lines of human embryonic kidney cells (HEK293T) expressing low levels of each of the tagged-bait fusion proteins were generated, then detergent-solubilized, subjected to two rounds of affinity purification, trypsinized,

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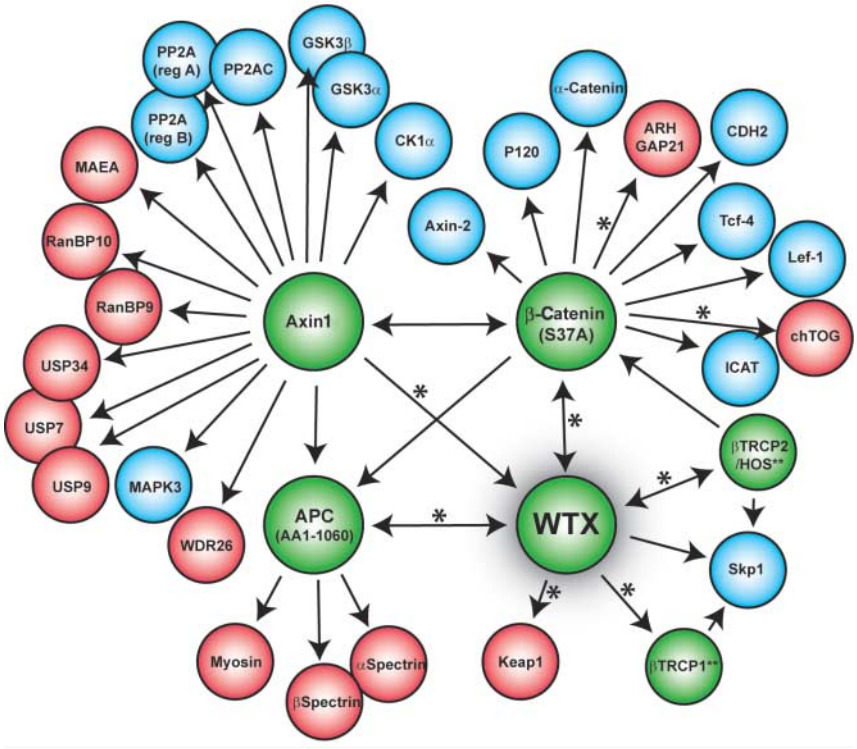
Fig. 1. The β-catenin protein interaction network. Green circles represent proteins used as bait in the tandem affinity purification, blue circles represent known interactors, and red circles represent novel interactors. The arrows indicate directionality for the bait-interactor discovery, and the single asterisks (*) show interactions that were confirmed in secondary assays. **The protein interaction networks for β-TrCP1 and β-TrCP2 are not yet complete.



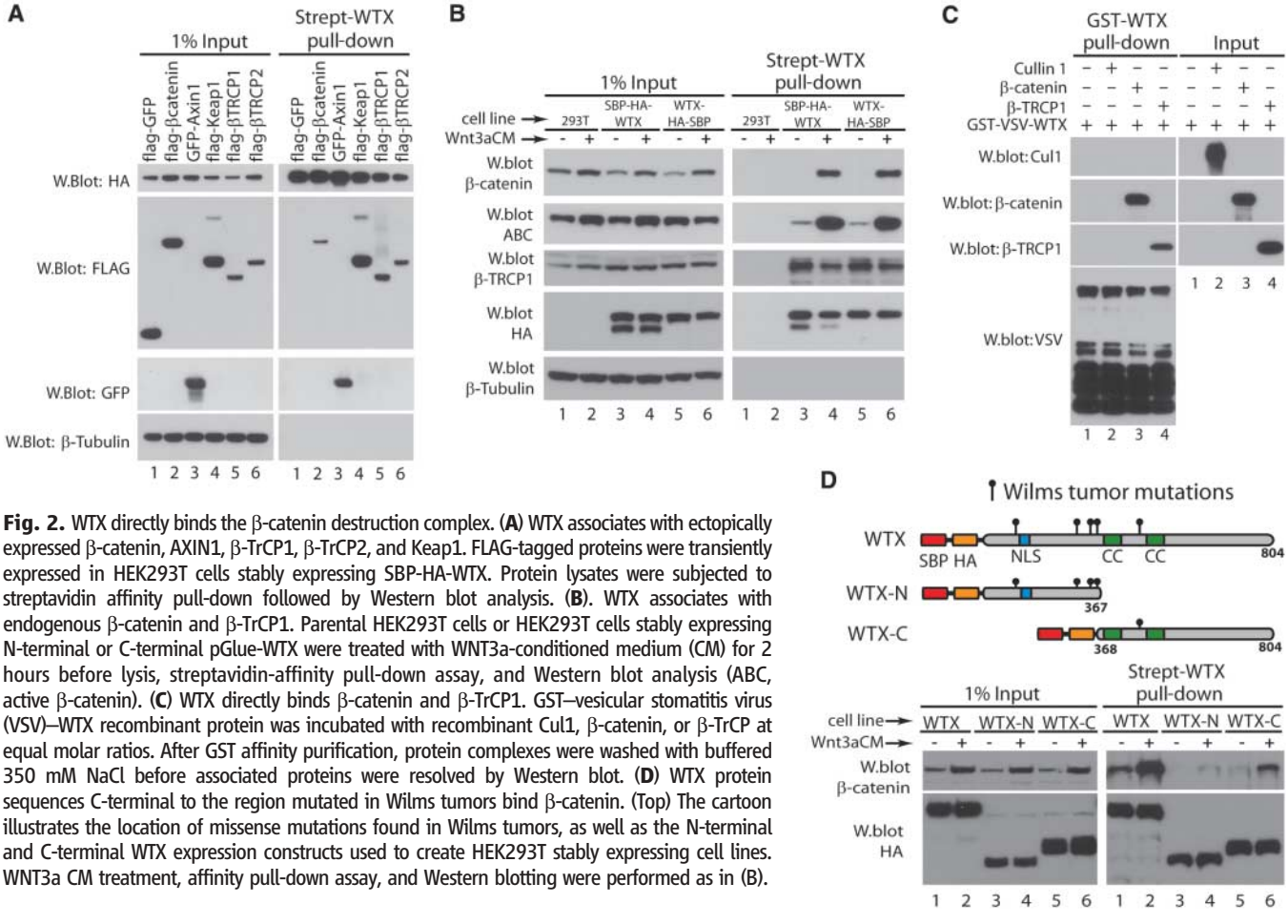


Fig. 2. WTX directly binds the β-catenin destruction complex. (**A**) WTX associates with ectopically expressed β-catenin, AXIN1, β-TrCP1, β-TrCP2, and Keap1. FLAG-tagged proteins were transiently expressed in HEK293T cells stably expressing SBP-HA-WTX. Protein lysates were subjected to streptavidin affinity pull-down followed by Western blot analysis. (**B**). WTX associates with endogenous β-catenin and β-TrCP1. Parental HEK293T cells or HEK293T cells stably expressing N-terminal or C-terminal pGlue-WTX were treated with WNT3a-conditioned medium (CM) for 2 hours before lysis, streptavidin-affinity pull-down assay, and Western blot analysis (ABC, active β-catenin). (**C**) WTX directly binds β-catenin and β-TrCP1. GST–vesicular stomatitis virus (VSV)–WTX recombinant protein was incubated with recombinant Cul1, β-catenin, or β-TrCP at equal molar ratios. After GST affinity purification, protein complexes were washed with buffered 350 mM NaCl before associated proteins were resolved by Western blot. (**D**) WTX protein sequences C-terminal to the region mutated in Wilms tumors bind β-catenin. (Top) The cartoon illustrates the location of missense mutations found in Wilms tumors, as well as the N-terminal and C-terminal WTX expression constructs used to create HEK293T stably expressing cell lines. WNT3a CM treatment, affinity pull-down assay, and Western blotting were performed as in (B).

and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The resulting data for all bait proteins were integrated to yield the protein-protein interaction network of the β-catenin destruction complex (Fig. 1 and table S1). This proteomic analysis confirmed the presence of all the core proteins identified in previous screens (*1*), including β-catenin, APC, AXIN1, AXIN2, protein phosphatase PP2A, GSK3α, GSK3β, and CK1α. In addition, 13 new proteins were found to associate with known components of the destruction complex.

We further explored WTX (FLJ39287/FAM123B) because it copurified with each of the baits examined. The WTX gene was recently discovered to be mutated in ~30% of Wilms tumors, which are pediatric kidney cancers (*8*). Constitutive activation of WNT/β-catenin signaling is common in Wilms tumors; ~10% of tumors harbor activating mutations in β-catenin (*9*), and nuclear β-catenin is observed in ~50% of tumors lacking detectable β-catenin mutations (*10*). Note that WTX and β-catenin mutations were mutually exclusive in the tumor samples examined (*8*).

To test the hypothesis that WTX negatively regulates WNT/β-catenin signaling in normal kidney, we generated HEK293T cells that stably express pGlue-WTX (supporting online text). From these cells, we isolated and identified WTX-associated protein complexes by TAP/LC-MS/MS (Fig. 1 and table S1). β-Catenin and β-TrCP were among the most abundant WTX-interacting proteins, which independently confirms the interactions of β-catenin$^{(SA)}$–WTX and β-TrCP2–WTX. To validate the WTX protein interaction network, we assessed protein binding in HEK293T cells and in vitro. We transiently expressed FLAG-tagged fusion proteins in cells stably expressing pGlue-WTX, isolated WTX by streptavidin affinity chromatography, and detected bound FLAG-tagged fusion proteins by Western blot (Fig. 2A). The reverse pull-down strategy yielded identical results (fig. S1). These data demonstrate that WTX binds both wild type β-catenin and the stabilized β-catenin$^{(SA)}$ mutant (Fig. 2A and fig. S1).

Using cells stably expressing either N-terminal or C-terminal tagged WTX, we next investigated whether endogenous proteins within the destruction complex bound WTX. Streptavidin affinity purification of WTX revealed that it associates with endogenous β-catenin and β-TrCP (Fig. 2B and supporting online text). Additionally, using purified recombinant protein in vitro, we found that WTX directly binds β-catenin and β-TrCP1, but not the Cullin1 scaffold within the E3 ligase complex (Fig. 2C). These results show that post-translational modifications are not required for WTX binding to β-catenin or β-TrCP1.

Although deletion of the *WTX* gene was more commonly found in Wilms tumor samples, five truncating mutations were identified in tumors within the amino-terminal half of the protein (*8*). As such, these mutations are consistent with the existence of a putative tumor suppressor motif within the C terminus of WTX. If WTX regulates

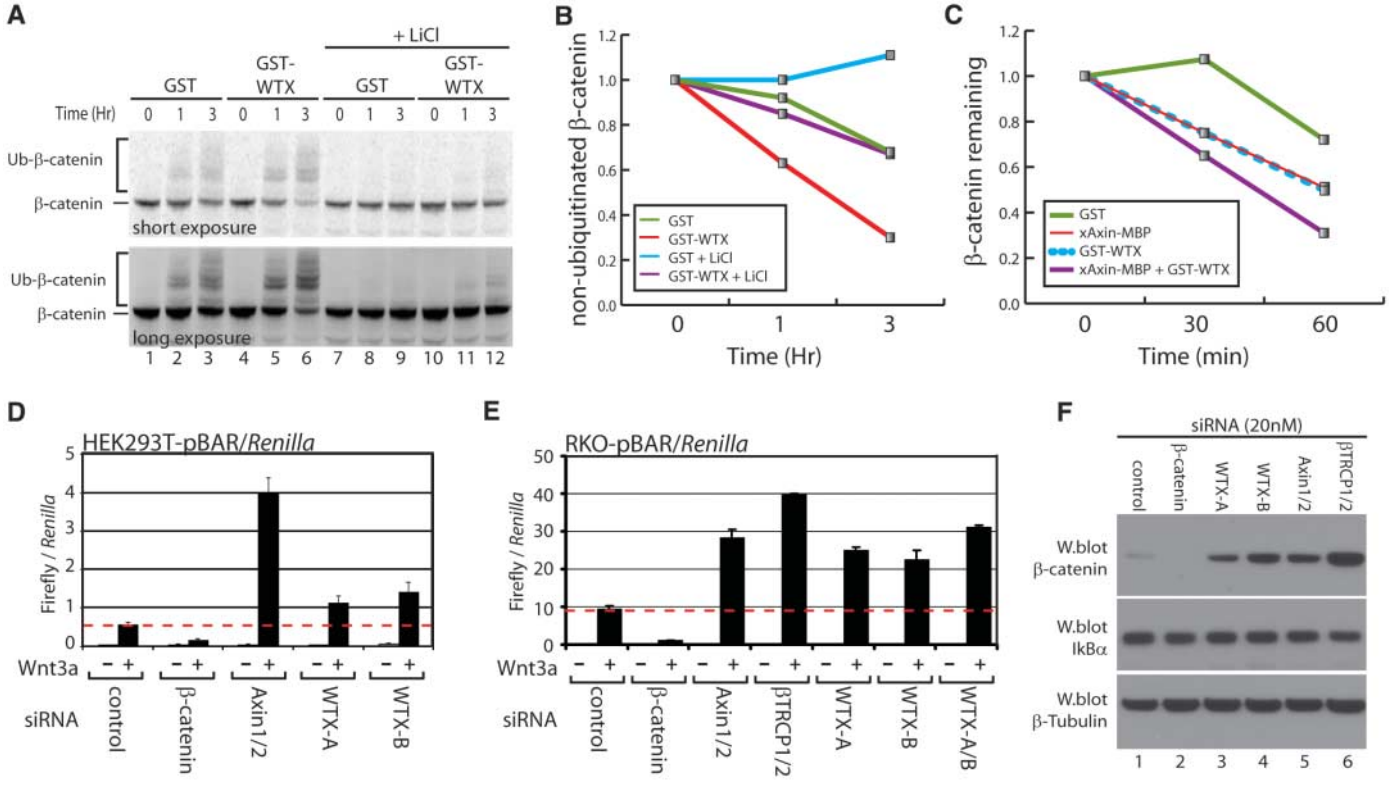


Fig. 3. WTX promotes β-catenin ubiquitination and degradation. (**A**) A cell-free system of *Xenopus* egg extracts was used to monitor β-catenin ubiquitination as a function of time. In vitro transcribed and translated ^{35}S-labeled β-catenin was added to *Xenopus* egg extracts in the presence of methylated ubiquitin (MeUb) and either purified GST or GST-WTX protein. Measuring the extent of ^{35}S-labeled β-catenin ubiquitination was followed by SDS–polyacrymamide gel electrophoresis (SDS-PAGE) and autoradiography. As a measure of specificity, LiCl (10 mM) was added to inhibit β-catenin phosphorylation and subsequent ubiquitination. (**B**) Quantification of nonubiquitinated ^{35}S-labeled β-catenin levels from (A). (**C**) Recombinant GST-WTX and myelin basic protein (MBP)–AXIN1 synergize to degrade ^{35}S-labeled β-catenin in *Xenopus* egg extracts. Graphic representation of ^{35}S-labeled β-catenin degradation as a function of time; note absence of methylated ubiquitin (meUb) in this experiment, as well as difference in time scale. (**D** and **E**) WTX silencing synergizes with WNT3a CM to activate a β-catenin–responsive luciferase reporter (pBAR) in mammalian cells. HEK293T cells (D) or RKO cells (E) stably expressing the pBAR reporter and *Renilla* luciferase were transiently transfected with siRNAs targeting the indicated mRNAs. Two days after transfection, cells were treated with control or WNT3a CM for 14 hours. BAR-luciferase values were normalized to *Renilla* and plotted. Error bars represent standard deviation from the mean. Data are representative of 4 independent experiments for HEK293T cells and 12 independent experiments for RKO cells. (**F**) WTX silencing stabilizes β-catenin. RKO cells were transfected with siRNAs targeting the indicated mRNAs. Two days after transfection, cells lysates were subjected to Western blot analysis for the indicated proteins. IκBα, inhibitor of nuclear factor κB and a β-TrCP substrate induced by tumor necrosis factor–α stimulation, as well as β-tubulin, demonstrate equal protein loading in the blots.

kidney biology through negative regulation of WNT/β-catenin signaling, then we should be able to ascribe a WNT-related function to the C terminus of WTX. Therefore, we mapped the domain of WTX that interacts with β-catenin and found that β-catenin purified with full-length WTX and the C-terminal half of WTX (WTX-C), but interacted poorly with the N-terminal half (WTX-N) (Fig. 2D and fig. S2 and supporting online text). As additional confirmation, we used our TAP–LC-MS/MS analysis on cells expressing pGlue–WTX-C and found both β-TrCP and β-catenin within the protein complex (table S1). Thus, mutational alteration of WTX in Wilms tumor likely reduces its interaction with β-catenin and β-TrCP.

The direct binding of WTX to both β-catenin and to its E3 ubiquitin ligase adaptor, β-TrCP, suggests that WTX regulates β-catenin degradation. We tested this hypothesis using cell-free *Xenopus* egg extracts, an experimental system that allows quantitative monitoring of β-catenin ubiquitination and degradation (*11*). The addition of recombinant glutathione *S*-transferase (GST) in complex with WTX protein increased the rate of β-catenin ubiquitination, but GST control did not (Fig. 3, A and B, and fig. S3). Inhibition of GSK3 by lithium chloride (LiCl) suppressed β-catenin ubiquitination in the presence of GST and GST-WTX. As a scaffold protein, AXIN nucleates the GSK3-CK1-APC phosphorylation complex and thereby dramatically increases β-catenin turnover in *Xenopus* extracts (*11*). When WTX and AXIN1 were added to the extracts individually, each increased the rate of β-catenin degradation (Fig. 3C). When WTX and AXIN1 were added together, the rate of β-catenin degradation was more rapid than observed with either alone. These data suggest that WTX negatively regulates WNT signaling by promoting β-catenin ubiquitination.

If WTX promotes β-catenin degradation, then suppressing WTX expression should activate WNT/β-catenin signaling in mammalian cells. To test this prediction, we measured the activity of a β-catenin–dependent transcriptional reporter after small interfering RNA (siRNA)-mediated silencing of *WTX*. Specifically, HEK293T human embryonic kidney cells and RKO human colon carcinoma cells were transduced with lentiviruses encoding a firefly luciferase–based β-catenin–activated reporter (pBAR), along with *Renilla* luciferase (*Renilla*-Luc) under the control of the constitutively active thymidine kinase promoter for normalization. To validate the dynamic range of this reporter system, stably transduced cell lines were treated with WNT3a-conditioned medium, which activated the reporter by a factor of 100 to 300 (Fig. 3, D and E). As a control, we showed that siRNAs directed against β-catenin abolished this WNT3a-induced reporter activity in both cell lines (fig. S4 and supporting online text). Using this assay system, we found that two different siRNAs targeting WTX produced an increase in WNT3a-induced reporter activity in both cell types. Furthermore, in RKO-pBAR/*Renilla* cells, siRNA-mediated silencing of WTX, AXIN1 and 2, or β-TrCP1 and 2 synergized with a GSK3 inhibitor, (2′*Z*,3′*E*)-6-bromoindirubin-3′-oxime, to activate the pBAR reporter (fig. S4). These data suggest that WTX is a negative regulator of WNT/β-catenin signal transduction in mammalian cells.

We next tested whether silencing of *WTX* with siRNAs increases β-catenin levels in cells. In RKO cells, β-catenin does not localize to the plasma membrane, whereas in other cell types, such as HEK293T cells, it resides with a relatively long half-life at the inner surface of the plasma membrane. Thus, in the absence of membrane-associated β-catenin, total cellular levels of β-catenin in RKO cells are very low, which allows study of cytoplasmic and nuclear β-catenin stability in response to experimental perturbation. We transiently transfected RKO cells with siRNAs targeting WTX, β-catenin, AXIN1 and 2, or β-TrCP1 and 2. Silencing of WTX, AXIN1 and 2, or β-TrCP1 and 2, but not β-catenin, was found to increase β-catenin levels, as determined by immunoblot analysis (Fig. 3F). Thus, WTX is required in these cells as a negative regulator of both β-catenin protein stability and β-catenin–mediated transcription.

To extend these experiments to organisms, we performed gain-of-function experiments in *Xenopus* embryos and loss-of-function experiments in zebrafish (supporting online text). Ectopic activation of WNT/β-catenin signaling by injection of *Xenopus Wnt8* mRNA in *Xenopus* embryo ventral blastomeres induced duplication of the embryonic axis, yielding two-headed tadpoles (fig. S5). Injection of *WTX* mRNA blocked *Xenopus Wnt8*–induced axis duplication. In developing zebrafish embryos, ectopic activation of WNT/β-catenin signaling leads to anterior truncations. When we silenced endogenous zebrafish wtx expression, we observed anterior truncations and the activation of a WNT/β-catenin reporter gene (fig. S5). These results suggest that WTX is a negative regulator of WNT/β-catenin signaling in vivo.

In summary, these data establish that the cancer-associated WTX protein is a required component of the β-catenin destruction complex. Furthermore, our data underscore the power of proteomic approaches for identifying new components of cellular signal transduction pathways that may ultimately provide important mechanistic insights into human disease.

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Supporting Online Material

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Revisiting the Role of the Mother Centriole in Centriole Biogenesis

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Centrioles duplicate once in each cell division cycle through so-called templated or canonical duplication. SAK, also called PLK4 (SAK/PLK4), a kinase implicated in tumor development, is an upstream regulator of canonical biogenesis necessary for centriole formation. We found that overexpression of SAK/PLK4 could induce amplification of centrioles in *Drosophila* embryos and their de novo formation in unfertilized eggs. Both processes required the activity of DSAS-6 and DSAS-4, two molecules required for canonical duplication. Thus, centriole biogenesis is a template-free self-assembly process triggered and regulated by molecules that ordinarily associate with the existing centriole. The mother centriole is not a bona fide template but a platform for a set of regulatory molecules that catalyzes and regulates daughter centriole assembly.

Centrioles are essential for the formation of cilia and flagella and for the organization of the centrosome (*1*). Normally, centrioles duplicate in coordination with the cell cycle. A new centriole, the daughter, arises orthogonally to each old one, the mother (*1*), in S phase. This led to the idea that the mother centriole templates the formation of the daughter (*2*, *3*). However, daughter centrioles do not incorporate a substantial proportion of the mother (*4*),