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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Materials and Methods
Figs. S1 to S7
Tables S1 to S4
References

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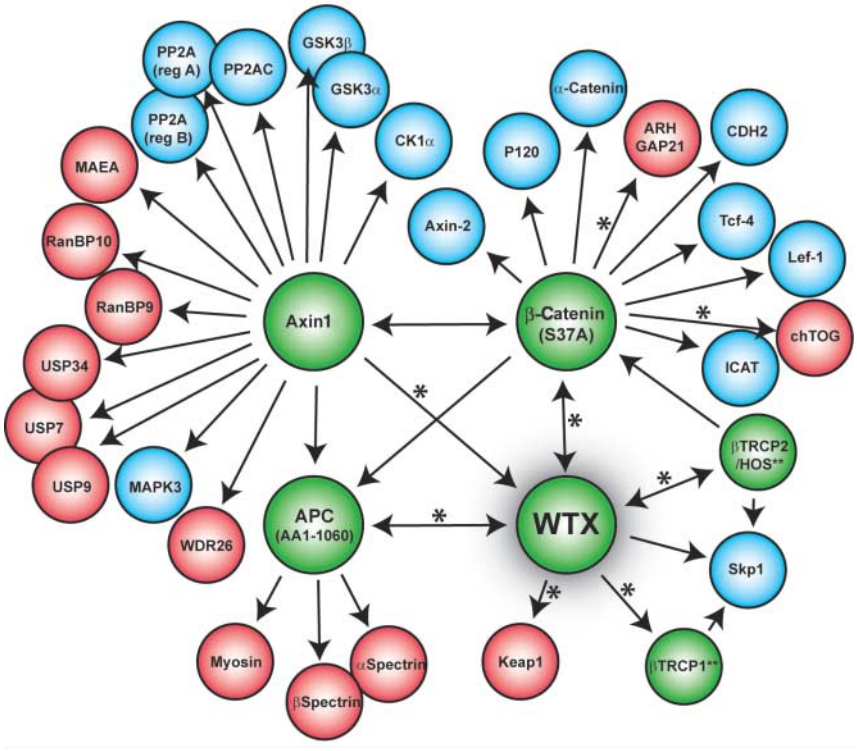
Fig. 1. The β-catenin protein interaction network. Green circles represent proteins used as bait in the tandem affinity purification, blue circles represent known interactors, and red circles represent novel interactors. The arrows indicate directionality for the bait-interactor discovery, and the single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



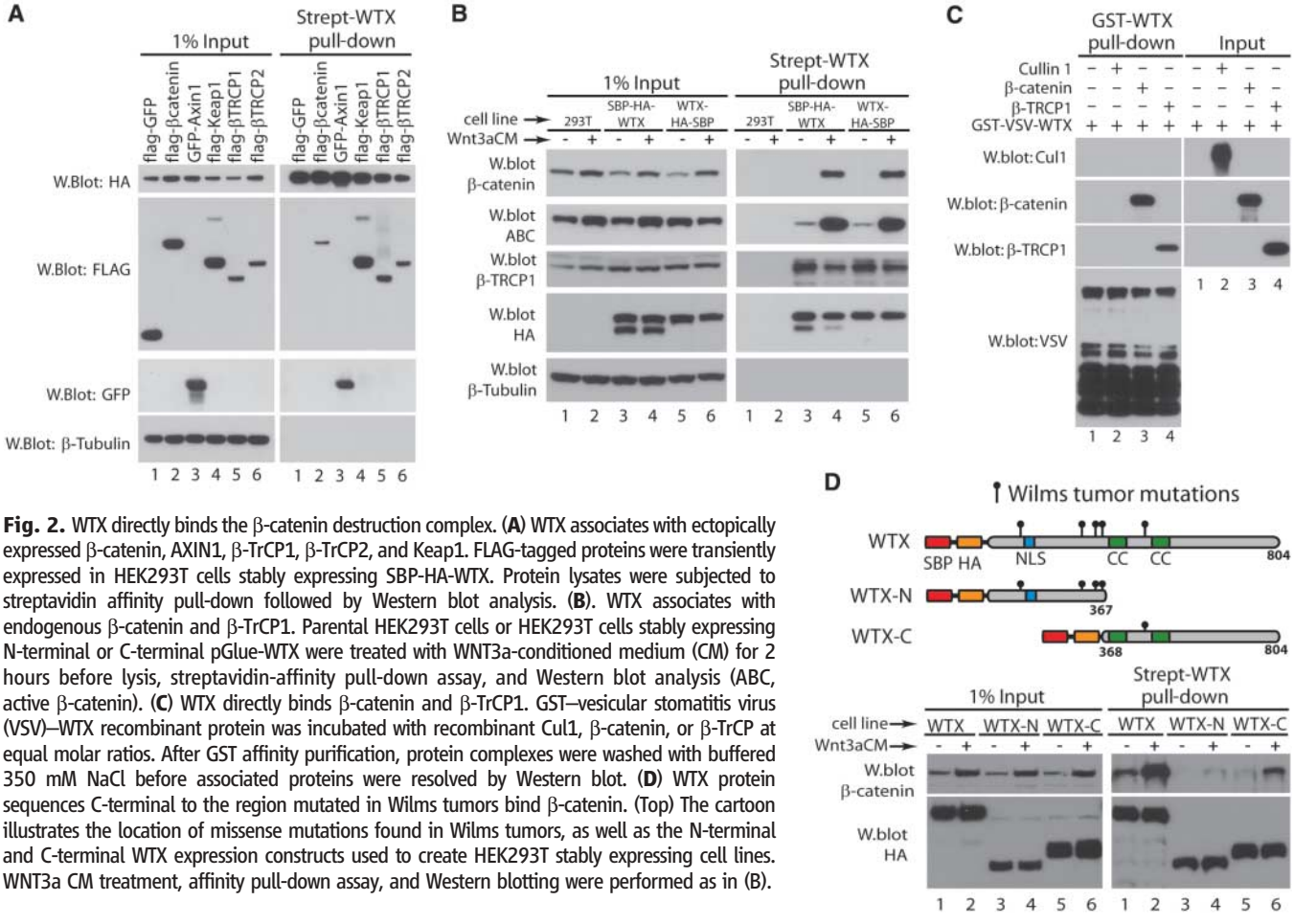


Fig. 2. WTX directly binds the β-catenin destruction complex. (**A**) WTX associates with ectopically expressed β-catenin, AXIN1, β-TrCP1, β-TrCP2, and Keap1. FLAG-tagged proteins were transiently expressed in HEK293T cells stably expressing SBP-HA-WTX. Protein lysates were subjected to streptavidin 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

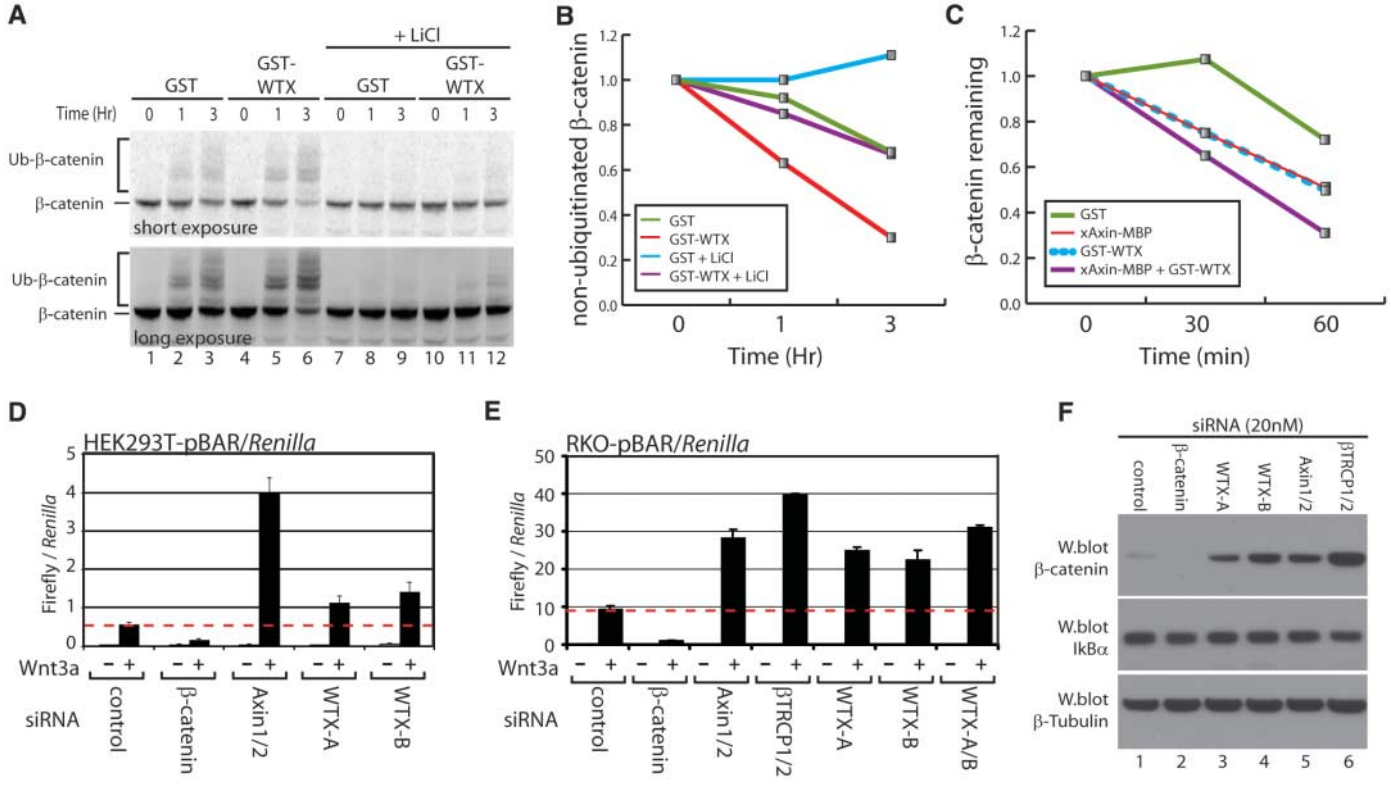


Fig. 3. WTX promotes β-catenin ubiquitination and degradation. (**A**) A cell-free system of *Xenopus* egg extracts was used to monitor β-catenin ubiquitination as a function of time. In vitro transcribed and translated ^{35}S-labeled β-catenin was added to *Xenopus* egg extracts in the presence of methylated 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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Materials and Methods
SOM Text
Figs. S1 to S6
Tables S1 and S2
References

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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*),

Supporting Online Material for

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Materials and Methods

Plasmids

All expression constructs, including human WTX (AK097146), were created with standard PCR-based cloning strategies. β-catenin$^{(SA)}$ contains alanine substitutions at residues 33, 37, 41 and 45. Detailed descriptions, maps and sequences of the expression plasmids can be found on the Moon Lab website (http://faculty.washington.edu/rtmoon/). The β-catenin activated reporter (pBAR) contains 12 TCF binding sites (5'-AGATCAAAGG-3') separated by distinct 5 base linkers. These elements are directly upstream of a minimal thymidine kinase promoter which then drives the expression of firefly luciferase. The reporter contains a separate PGK promoter that constitutively drives the expression of a puromycin resistance gene. The reporter is in a lentiviral platform.

Tissue culture, transfections and small interfering RNAs

HEK293T cells and RKO cells were grown in DMEM supplemented with 10% fetal bovine serum in a 37°C humidified incubator with 5% CO_2. Selection and passage of stable cell lines was performed with 2 μg/ml puromycin for 10-14 days, or until cell death was no long apparent. Transient transfection of siRNA was performed with RNAiMAX, as directed by the manufacturer (Invitrogen, Carlsbad, CA). All siRNA sequences used are listed in Table S2. Expression constructs were transiently transfected in HEK293T cells with LF2000 as directed by the manufacturer (Invitrogen).

Tandem-affinity purification and mass spectrometry

Minor modifications were made to the purification scheme previously reported to maximize elution during the first and second rounds of affinity purification (*1*). Protein complexes were eluted from the straptavidin beads with 50 mM biotin in the absence of TEV protease. 0.1% RapiGest (Waters Corp, MA) was included in the final elution from the calmodulin beads. Prior to mass spectrometry, RapiGest was acid cleaved at 37°C for 30 minutes.

Affinity pull-downs and Western blotting

For streptavidin affinity purification, $2x10^6$ HEK293T cells were lysed in radio-immunoprecipitation buffer (RIPA; 25 mM Tris-HCl at pH 8.0, 150 mM NaCl, 10% glycerol, 1% TritonX-100, 0.25% deoxycholic acid, 2 mM EDTA and protease inhibitor cocktail (Roche, Switzerland), and phosphatase inhibitor cocktail (Calbiochem, San Diego, CA). Cell lysates were cleared by centrifugation and incubated with streptavidin-resin (Amersham, Piscataway, NJ) before washing and eluting in 2XLDS NuPAGE (Invitrogen) loading buffer. Detection of proteins by Western blot was performed using the following antibodies: anti-FLAG M2 monoclonal (Sigma, St Louis, MO), polyclonal anti-VSV (V4888; Sigma), anti-HA polyclonal (1867423; Roche), anti-GFP polyclonal (ab290, abcam), anti-β–catenin polyclonal (9562; Cell Signaling Technology), anti-Active-β-catenin (anti-ABC,clone 8E7; Upstate), anti-βTrCP monoclonal (37-3400; Invitrogen), and anti β-TUBULIN monoclonal (T7816; Sigma).

RNA isolation, reverse transcription and quantitative real-time PCR.
Total RNA from tissue culture cells or zebrafish embryos were harvested in Trizol (Invitrogen) reagent according to manufacturer's instructions. RNA quality and quantities were monitored by denaturing gel electrophoresis and UV spectrophotometry, respectively. Singled stranded cDNA was synthesized from 1 µg total RNA using superscript III (Invitrogen) and a 1:1 mixture of random hexamers and oligo-dT. Real-time PCR was performed using the Roche Light Cycler II instrument and software (Roche Diagnostics). PCR was performed in duplicate with the LightCycler FastStart DNA SyBr Green kit (Roche). The PCR conditions are as follows: 35 cycles of amplification with 1 second denaturation at 95°C, and 5 second annealing at 58 °C. A template-free negative control was included in each experiment. Quantitative light cycler PCR primers used were as follows: human *WTX* (GAC CCA AAA GGA TGA AGC T; and reverse CCC CTC CAA AGA AAC TAG GC), β-catenin gene (TGG ATA CCT CCC AAG TCC TG; and reverse CAG GGA ACA TAG CAG CTC GT), *GAPDH* (TGA AGG TCG GAG TCA ACG GA; and reverse CCA TTG ATG ACA AGC TTC CCG), zebrafish wtx (TGT GAC GGA CAA GAT GGA AA; and reverse TTT TCA CAG AAG GGG GTG AC), *dGFP* (TAT ATC ATG GCC GAC AAG CA; and reverse GAA CTC AGC AGG ACC ATG T), zebrafish 18S (CGC TAT TGG AGC TGG AAT TAC C; and reverse GAA ACG GCT ACC ACA TCC AA).

Xenopus *and zebrafish experiments*
Xenopus egg extracts were prepared as previously described (*2*). *In vitro* transcription and translation of ^{35}S-labeled β-catenin was performed using the coupled transcription-translation T7 system (Promega). For sense RNA injections in *Xenopus* and zebrafish, mRNA was synthesized using the mMessage machine kit (Ambion). For zebrafish loss-of-function studies, 2.0 ng of morpholino was co-injected with mRNA at the one-cell stage (GeneTools, Inc., Philomath, OR). The MO sequences are WTX-MO1 (5'-ACA GGT GAC TGT GGC CTA ATG GAG CA) and WTX-MO2 (5'-CAT GTT CTA CCT GTA AAA GAA ATA G). *In situ* hybridizations using digoxigenin-labelled *zWTX* probes were performed using standard methods. For *in situ* probe synthesis, the 5' end of the zebrafish *wtx* mRNA coding sequence was cloned from zebrafish cDNA using the following primers: forward, 5'-AAA ATA CGG AAG ACA GCT TTC AAA T; reverse, 5'-GAT ATG TGA CAA AAC ATG AGC TAC G.

In vitro binding experiments
Human GST-VSV-WTX was purified from *Escherichia coli* and mixed with previously purified CULLIN 1, βTrCP1, or β-catenin in buffered 150mM NaCl. Following 30 minute 4°C incubation, complexes were washed with 5 bed-volumes of 350mM buffered NaCl before elution and Western blot analysis.

Supporting Text

Generation of WTX stable HEK293T cells
It is presumably due to the low level of protein expression in our stable lines that we were successful in generating the WTX line, as we and others observe rapid apoptosis following transient over-expression of WTX in several cell lines(*3*).

Enhanced association between WTX and β-catenin following addition of WNT3A-CM
WNT3a stimulation increased the relative amount of β-catenin purifying with WTX (Fig. 2C). We believe the increased β-catenin signal reflects increased levels of WNT3a-induced cytosolic β-catenin as treatment of cells with the proteosome inhibitor MG132 similarly increased the relative amount of β-catenin bound to WTX (fig. S1B).

β-Catenin binds the C terminus of WTX
The absence of association between WTX-N and β-catenin was not due to altered subcellular localization, as pGlue-WTX and pGlue-WTX-N both localize to the plasma membrane and less strongly to the cytoplasm (fig S2). Interestingly, WTX-C localized to nuclear punctuate structures.

WTX enhances β-catenin ubiquitination in Xenopus *egg extracts*
The addition of methylated ubiquitin (MeUb) to the extracts, which terminates poly-ubiquitin chain growth and consequently inhibits proteosomal degradation, allowed us to quantitatively assess the effect of WTX on β-catenin ubiquitination (Fig. 3A,B).

siRNA-mediated silencing of WTX sensitizes human cells to WNT3a stimulation
Additional controls showed that, as expected, siRNA-mediated silencing of the negative regulators AXIN1/2 and βTrCP1/2 increased WNT3a-mediated β-catenin transcriptional activity (Fig. 3D,E). We validated the extent of siRNA-mediated silencing of WTX by measuring the levels of wtx mRNA and HA-WTX protein in cells following siRNA transfection (fig. S4).

WTX inhibits Wnt8-induced axis duplication in Xenopus *embryos*
The experiments in *Xenopus* cell free extracts and in mammalian cells establish that WTX is an important negative regulator of WNT/β-catenin signal transduction. To extend these experiments to organisms, we first performed gain-of-function experiments in *Xenopus* embryos, where WNT/β-catenin activity is critical for establishing the embryonic axes(*4*). Ectopic activation of WNT/β-catenin signaling by injection of *Xenopus Wnt8* mRNA in ventral blastomeres induces duplication of the embryonic axis, yielding two-headed tadpoles (fig. S5). When negative regulators of WNT signaling are co-injected with *Wnt8* mRNA this blocks the WNT-mediated axis duplication. In the present study, injection of *WTX* mRNA effectively blocked *Xenopus Wnt8*-induced axis duplication, thereby supporting its activity as an antagonist of β-catenin (fig. S5).

WTX silencing activates Wnt/β-catenin signaling in zebrafish

To investigate the function(s) of endogenous zebrafish wtx as a modulator of WNT signaling, we first cloned the zebrafish *wtx* homologue and performed *in situ* hybridizations that demonstrated a broad pattern of expression (fig. S6). We next designed two morpholino antisense oligonucleotides (MO), one targeted to the translational start codon of *wtx* (MO-1) and a second targeted against an internal splice site (MO-2). In zebrafish embryos, it is well-established that ectopic injection of *wnt8* mRNA leads to anterior truncations(*5*). Phenocopying this effect of *wnt8* mRNA, microinjection of either WTX MO significantly reduced anterior cell fates, as indicated by the presence of small eyes (fig. S5). Remarkably, co-injection of either of the two MOs along with a sub-phenotypic dose of *wnt8* mRNA resulted in severe anterior truncations in the majority of embryos. These results in developing embryos are consistent with the activation of β-catenin signaling attributable to loss of function of endogenous wtx.

If this phenotype in zebrafish embryos were indeed attributable to activation of WNT/β-catenin-mediated transcription, then β-catenin responsive reporters should be activated. To test this prediction, we employed a transgenic zebrafish line that expresses a WNT-responsive GFP reporter [*Tg(TOP:GFP)*^{w25}] (*6*). We asked if reducing expression of wtx would sensitize the embryos to ectopic expression of WNT and found that the co-injection of a low dose of *wnt8* mRNA along with the WTX MOs quantitatively increased *dgfp* transcript expression (fig. S5). Importantly, using cDNAs generated from the same embryos, we validated the reduction of wild-type *wtx* transcript by WTX MO-2, which was designed to disrupt pre-mRNA splicing (fig. S5). Together, data from both *Xenopus* and zebrafish embryos suggest that endogenous wtx inhibits WNT/β-catenin signaling *in vivo*.

Supporting References

1. S. Angers *et al.*, *Nat Cell Biol* 8, 348 (2006).
2. A. Salic, E. Lee, L. Mayer, M. W. Kirschner, *Mol Cell* 5, 523 (2000).
3. M. N. Rivera *et al.*, *Science* 315, 642 (2007).
4. A. P. McMahon, R. T. Moon, *Cell* 58, 1075 (1989).
5. C. E. Erter, T. P. Wilm, N. Basler, C. V. Wright, L. Solnica-Krezel, *Development* 128, 3571 (2001).
6. R. I. Dorsky, L. C. Sheldahl, R. T. Moon, *Dev Biol* 241, 229 (2002).

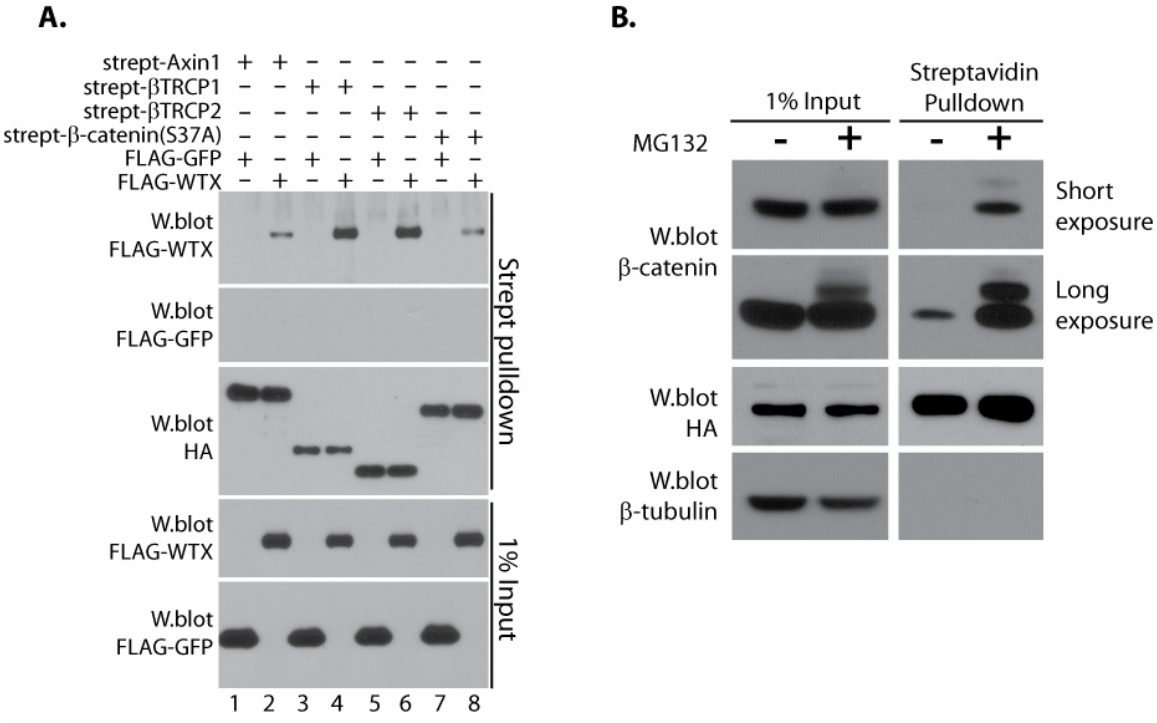

Fig. S1. Validation of the WTX protein interaction network. (A) WTX associates with the β-catenin destruction complex. HEK293T cells that stably express pGlue-Axin1, pGlue-β-catenin$^{(SA)}$, pGlue-βTRCP1 or pGlue-βTRCP2 were transiently transfected with FLAG-GFP or FLAG-WTX. Protein lysates were subjected to streptavidin affinity pulldown followed by Western blot analysis. (B) Proteosome inhibition increases the amount of endogenous β-catenin within the WTX complex. pGlue-WTX HEK293T cells were treated with 10μM MG132 for 1 hour prior to streptavidin affinity purification. WTX associated proteins were visualized by Western blotting.

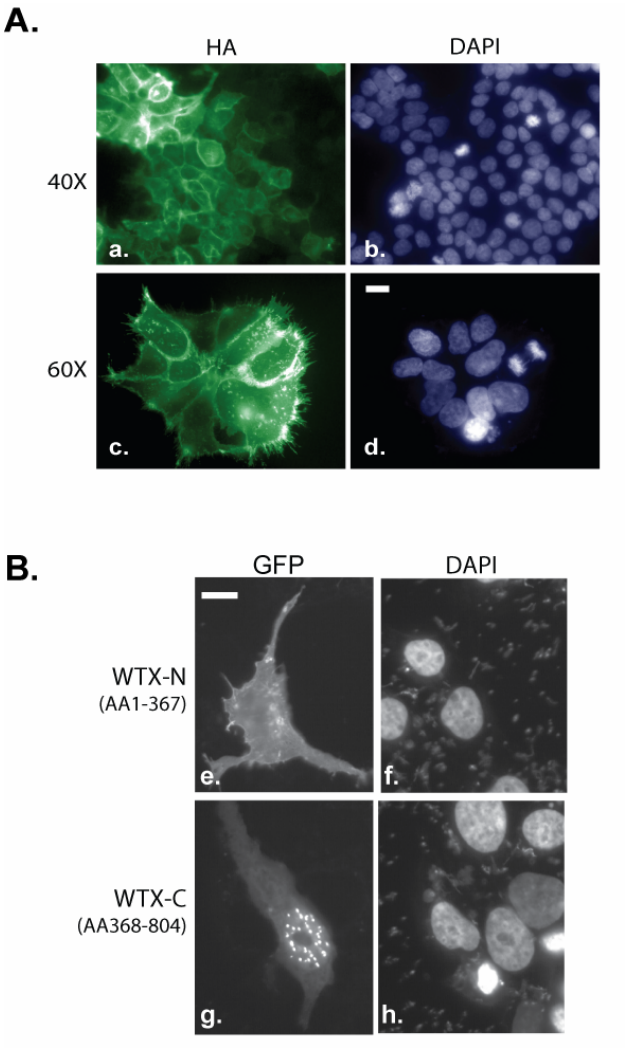

Fig. S2. WTX localizes to the plasma membrane and cytoplasm. **(A)** pGlue-WTX HEK293T stable cells were stained by immunofluorescence with an HA antibody and DAPI to mark nuclei. The scale bar represents 10μM. **(B)** WTX-N and WTX-C localize to the plasma membrane and nucleus, respectively. Twelve hours after HEK293T cells were transiently transfected with GFP-tagged WTX-N or WTX-C, the cells were fixed, stained with DAPI and imaged. The scale bar represents 10μM. The short time between transfection and fixation was necessary because in longer incubations we observed pronounced cell death with full length WTX and WTX-N, but not with WTX-C.

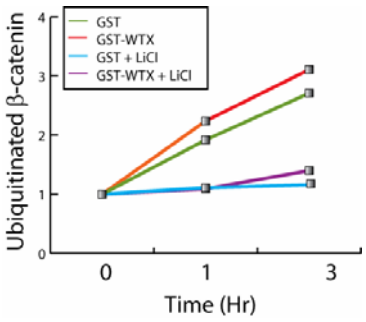

Fig. S3. WTX promotes ubiquitination of β-catenin in *Xenopus* cell free extracts. A plot showing the quantitation of ubiquitinated S^{35}-β-catenin levels from panel A, Figure 3.

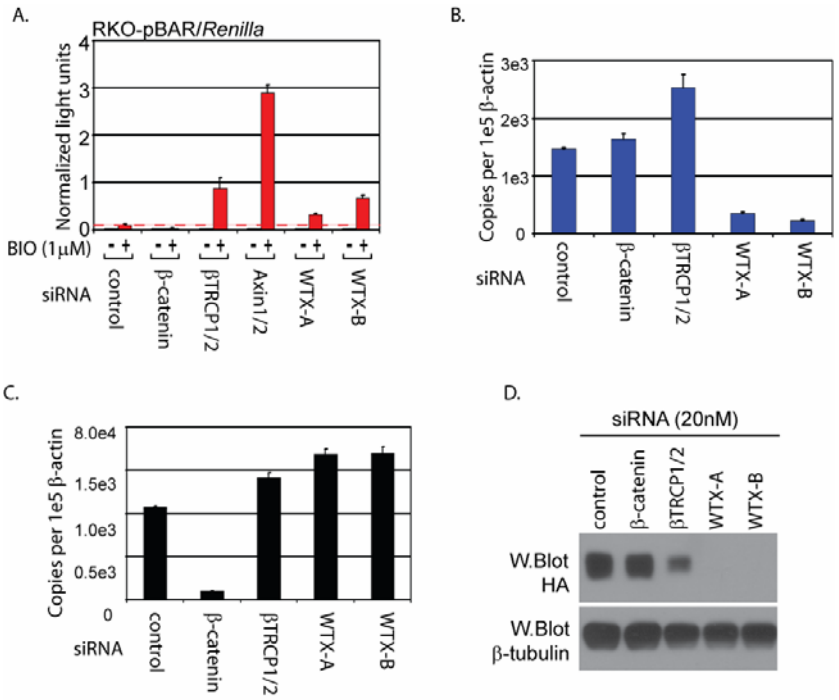

Fig. S4. Silencing WTX expression activates a β-catenin dependent transcriptional reporter. **(A)** RKO-BAR/*Renilla* stable cells were transfected with siRNAs (20nM) for 36 hours before the addition of a GSK3 pharmacological inhibitor (BIO; 1μM). At 48 hours post transfection, firefly luciferase and *Renilla* luciferase were measured and plotted. Error bars show standard deviation for triplicate samples. **(B and C)** siRNAs targeting WTX silence endogenous WTX mRNA expression. RKO cells were transfected with the indicated siRNAs. Forty-eight hours later, total RNA was harvested for a reverse transcription reaction and quantitative real time PCR. The mRNA copy number of WTX (B) and β-catenin (C) were normalized to β-actin and plotted. Error bars represent standard deviation within the PCR reaction. Data reflect two biological replicates. **(D)** siRNAs targeting WTX silence WTX protein expression. SBP-HA-WTX HEK293T stable cells were transfected with the indicated siRNAs. Two days following transfection, cells were lysed and subjected to Western blot analysis. siRNA-mediated silencing of βTRCP1/2 resulted in loss of WTX expression, suggesting that the β-TRCP-WTX association regulates WTX protein stability, as WTX mRNA expression was unchanged by β-TRCP1/2 siRNA transfection (panel B).

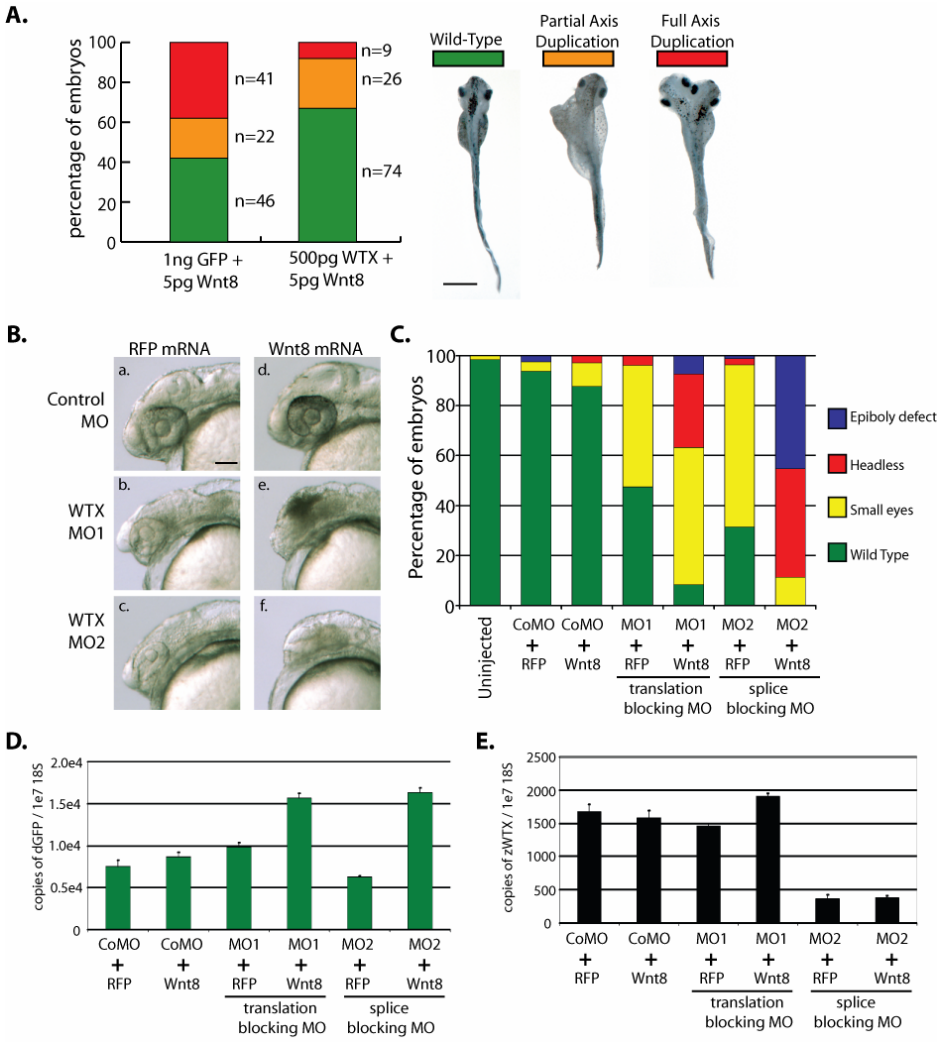

Fig. S5. WTX negatively regulates Wnt signaling in *Xenopus* and zebrafish. (**A**) WTX inhibits Wnt8-induced axis duplication in *Xenopus* embryos. *GFP* or *WTX* mRNA was microinjected into ventral blastomeres at the 4-cell stage, allowed to develop to the 16 cell stage, and then a single ventral cell was injected with *Wnt8* mRNA to induce axis duplication. Embryos were scored for partial and complete axis duplication at stage 33/34. The presence of a second cement gland differentiated full axis duplication from partial duplication. Representative *Xenopus* embryos are shown right of the chart. The scale bar represents 1.0mM **(B)** Morpholino-mediated silencing of zebrafish WTX activates Wnt/β-catenin signaling. Zebrafish embryos at 48 hours post fertilization. (**a-c**) WTX-MO1 (3ng, blocks translation) and WTX-MO2 (3ng, blocks splicing), but not the control MO (coMO), results in partial loss of anterior structures, as evidenced by small eyes. (**d-f**) Co-injection of a sub-phenotypic dose of *Wnt8* mRNA (10pg) and either WTX-MO1 or WTX-MO2 results in complete loss of anterior structures. *RFP* mRNA(10pg) was used as a negative control. The scale bar represents 100μM. (**C**) Percentages of embryos displaying specific phenotypes following MO and mRNA injections. (**D**) Silencing WTX activates the Wnt/β-catenin reporter in TOPdGFP transgenic fish. Normalized real time PCR quantitation of *dGFP* mRNA in injected embryos, harvested at 30% epiboly for RNA. (**E**) WTX-MO2 inhibits WTX splicing. Normalized real time PCR quantitation of *zWTX* mRNA following MO and mRNA injections.

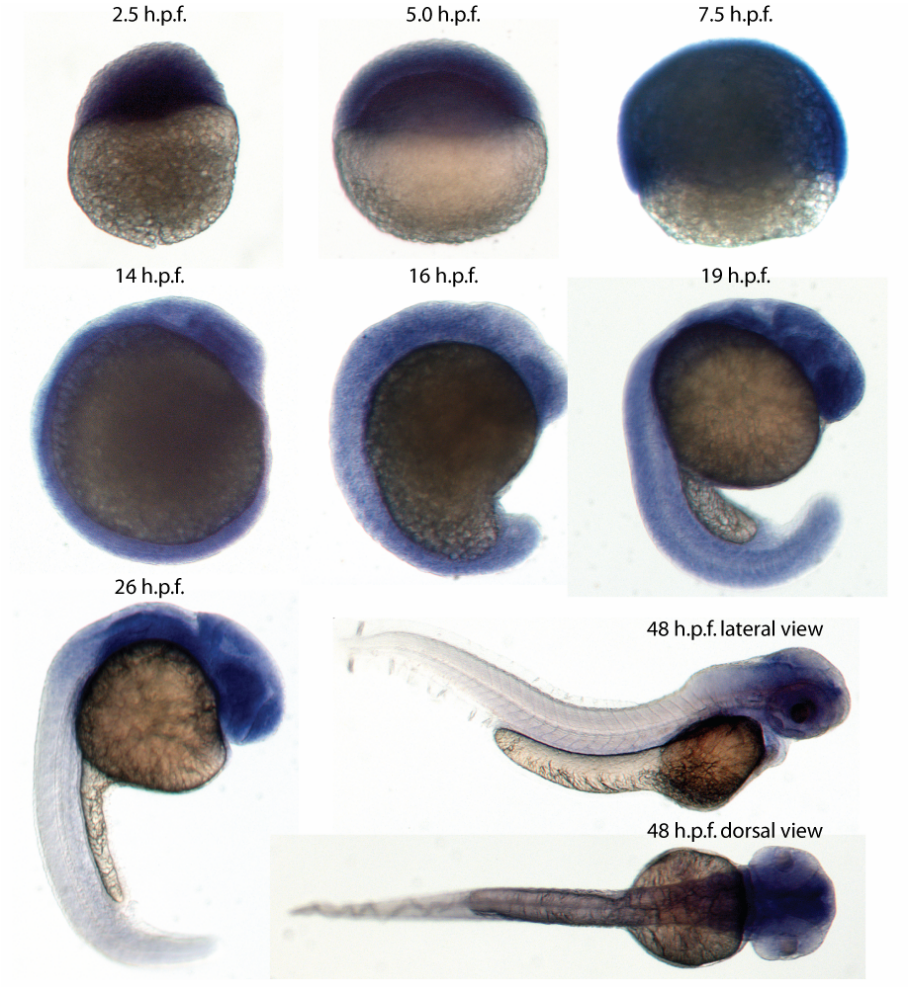

Fig. S6. Spatial and temporal expression of zWTX in developing zebrafish embryos as determined by *in situ* hybridization. zWTX is maternally expressed. Early zygotic expression is spatially unrestricted; however, as development proceeds, zWTX expression becomes anteriorly localized. The developmental stage of the embryos is shown above each image (hours post fertilization; h.p.f).

Supplemental Table 1. Representative mass spectrometry results. The total number of peptides identified are shown.

	β-catenin(S37A)	Axin1	APC(1-1060)	βTRCP2	WTX	WTX(368-804)
α-catenin	206					
APC	306	8	140		15*	
ARHGAP21	9					
Axin1	68	88				
Axin2	43					
β-catenin	864	7		5	3	2
βTRCP2				291	8	4
C20ORF11		7				
chTOG	2					
CK1α	4	10				
ERK1		2				
GSK3α	3	14				
GSK3β	11	14				
ICAT	45					
Keap1					16	
LEF1	5					
MAEA		4				
myosin			16			
N-cadherin	106					
p120	7					
plakoglobin	42					
PPP2CA		3				
PPP2R1A		8				
PPP2R2A		3				
PPP2R5C		1				
RanBP10		4				
RanBP9		4				
SART1					2*	
Skp1				179	5	1
spectrin			53			
TCF-7	2					
UBC		18		27	9	
USP34		46				
USP7		4				
USP9		4				
WDR26		7				
WTX	7	2	11	1	40	46

* peptides identified in 1%TritonX-100, as opposed to 0.1%NP40.

Supplemental Table S2: Sequences of siRNA sense strands

siRNA mRNA Target	Sequence (5'->3')
Axin1	GGUGUUGGCAUUAAAGGUGdTdT
Axin2	GGGAGAAAUGCGUGGAUACdTdT
βcatenin	GGUGGUGGUUAAUAAGGCUdTdT
βTRCP1/2	GUGGAAUUUGUGGAACAUCdTdT
WTX-A	GGAGCCAAGAACAAGGCAGdTdT
WTX-B	GGCCUAAAAGGCUUUUUUAdTdT