WTX is a novel regulator of ubiquitination in the Wnt/β-catenin and KEAP1/NRF2 pathways

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Abstract

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Ubiquitination of proteins and subsequent proteosome-mediated degradation is one mechanism cells use to regulate protein steady-state levels. This essential process permits cells to rapidly respond to extracellular cues or cytotoxic insult, through selective modulation of intracellular signaling cascades. For example, the Wnt-β-catenin pathway is controlled by ubuitination. In the absence of a WNT ligand, the transcription factor β-catenin is directed to the SCF^{BTRC} E3 ubiquitin ligase complex where it is poly-ubiqutinated. Poly-ubiquitinated β-catenin is then recognized and rapidly degraded by the proteosome. WNT ligand binding to its cognate receptor prevents β-catenin ubiquitination, allowing it to circumvent proteasome degradation, accumulate in the nucleus and drive transcription of genes required for proliferation and differentiation. My research investigated the functional roles of a novel regulator of the Wnt/β-catenin pathway, Wilms Tumor gene on the X-chromosome (WTX). My study revealed that WTX interacts with two E3 ubiquitin ligase adaptors, BTRC and KEAP1. The WTX/BTRC complex promoted β-catenin ubiquitination, while the WTX/KEAP1 complex inhibited ubiquitination of the stress response transcription factor

NRF2). Mutations in WTX have been proposed to the cause of Wilms tumor, the most common form of pediatric kidney cancer. Therefore, the results of my thesis provide a mechanism explaining how mutations in WTX cause disease, and provide potential solutions to treating this disorder.

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DEDICATION

For my
Parents Bill and Cec Camp,
My Wife Cristi Camp,
My Daughter Calla,
and my unborn Son

CHAPTER 1

Introduction to Wnt/β-catenin signaling

Historical perspective of the Wnt signaling Pathway

Almost three decades have passed since the discovery of the first Wnt protein, Wnt1 (Nusse and Varmus 1982). Wnt1 was initially called "Int1" (for Integrated) in mice after it was found to be overexpressed in mouse mammary tumors caused by proviral integration in a specific locus termed MMTV Int1 (Nusse and Varmus 1982). In parallel with this study, the *Drosophila* Wnt1 homolog, *wingless* (*wg*), was identified in a chemical mutagenesis screen for genes involved in developmental segmentation (Nusslein-Volhard and Wieschaus 1980). The homologic relationship of Int1 and *wg* went undiscovered until 1987 when an attempt to clone the *Drosophila* homolog of Int1 lead Rijsewijk et al. to conclude that *Drosophila* Int1 is identical to *wg* (Rijsewijk, Schuermann et al. 1987). This groundbreaking work has since lead to the identification of many Wnt proteins that are highly conserved from nematodes to humans (Chien, Conrad et al. 2009).

Elucidation of the major components involved in Wnt signaling has depended heavily on the identification of a *Drosophila* orthologue of Wnt1, *wingless* (*wg*) (Wodarz and Nusse 1998). *Drosophila* are relatively easy to manipulate genetically and therefore most of what is known about the pathway was initially observed in fly mutants. During fly development, *wg* is involved in segmentation polarity. Normally, on the ventral side of the fly embryo there is a cuticle pattern of denticle belts separated by naked cuticle. In

loss-of-function *wg* mutants the embryo is covered with denticles, and *wg* overexpression leads to a naked embryo. This developmental read-out has been used in genetic screens to identify other important players in the pathway and their respective hierarchy with epistasis studies. Genes that phenocopy the loss of *wg* include *Dishevelled (Dsh), Armadillo (Arm)/*β-catenin *and Pangolin (Pan*)/TCF.

Later work in *Xenopus* led to the classification of Wnts into two general categories. When certain Wnts are injected into the ventral blastomeres of the embryo, an ectopic body axis is observed (McMahon and Moon 1989). These Wnts are known as canonical Wnts (e.g. Wnt1, 3 & 8) as they can induce an ectopic body axis via the stabilization of β-catenin (Wodarz and Nusse 1998). The second class of Wnts are involved in cell movements but do not induce the formation of an ectopic body axis and are generally known as non-canonical Wnts (e.g. Wnt5 & 11) (Veeman, Axelrod et al. 2003). Less is known about the mechanism of non-canonical Wnts but they are thought to signal via the activation the small GTPase, Rho, and/or the Ca^{2+}/PKC pathway, and not β-catenin.

Wnt/β-catenin signal transduction

Insight into Wnt functional roles in cellular development and disease states has been achieved through genetic and biochemical studies. Genetic screens have identified numerous molecules involved in the transduction of a Wnt signal. For example, *Armadillo (Arm)/*β-catenin and *Wingless* (*Wg*)/Wnt mutant drosophila embryos display similar phenotypes suggesting that both molecules act in the same pathway (Wieschaus

and Riggleman 1987). Subsequent epistasis studies have provided a general order to the genes required for *Wg*/Wnt signal transduction. For example, *Arm*, *Dsh*, *Porc*, and *Zw3* are required for *Wg* signaling (Noordermeer, Klingensmith et al. 1994; Siegfried, Wilder et al. 1994). β-catenin is typically found at adherens junctions associated with members of the cadherin family (McCrea, Turck et al. 1991), but the importance of its role in the Wnt signaling cascade was first suggested by the observation that cytosolic levels of β-catenin accumulate in response to a Wnt ligand (van Leeuwen, Samos et al. 1994). These observations were the first clues that the Wnt signaling pathway is ultimately regulated by controlling the levels of β-catenin.

Absence of a Wnt ligand

In the absence of Wnt, the levels of cytosolic β-catenin protein are maintained at low concentrations through Skp, Cul, F-Box (SCF)-ubiquitin-mediated proteasomal degradation (**Figure 1**). This ubiquitination is dependent on the phosphorylation of β-catenin by casein kinase 1 alpha (CK1α) glycogen synthase kinase 3 beta (GSK3β), which reside in a multi-protein destruction complex that includes Axin and Adenomatous Polyposis Coli (APC) (Rubinfeld, Albert et al. 1996). Axin contains both GSK3β and β-catenin binding sites, allowing for efficient phosphorylation, while APC facilitates the release of phosphorylated β-catenin from the destruction complex (Xing, Clements et al. 2003). Following phosphorylation by CK1α and GSK3β, β-catenin is recruited to the CUL1 E3 ubiquitin ligase by the BTRC family of E3 ubiquitin ligase adaptors. β-catenin

is then ubiquitinated and subsequently degraded by the proteasome (Yost, Torres et al. 1996; Liu, Kato et al. 1999; Winston, Strack et al. 1999).

Wnt signaling at the receptor level

Wnt ligands are secreted glycoproteins, typically released in a paracrine fashion, that bind seven transmembrane domain receptors of the Frizzled (Fz) family as their primary receptor (Bhanot, Brink et al. 1996). Genetic analysis in Drosophila identified a single-pass transmembrane molecule of the LRP family (LRP5 or 6 in vertebrates) that acts as a Wnt coreceptor (Tamai, Semenov et al. 2000; Wehrli, Dougan et al. 2000). While the mechanism that leads to the ubiquitination of β-catenin is apparent and well accepted, the mechanism by which this receptor complex transduces the Wnt signal in the cell is poorly understood. One theory postulates that Frizzled receptors may couple to heterotrimeric G proteins following Wnt binding. Initial studies supporting this hypothesis showed that activation of Fz/β-adrenergic receptor chimeras by β-agonists leads to stabilization of β-catenin in a pertussis toxin-sensitive manner, suggesting the Gαs heterotrimeric G-protein is involved in this pathway (Ahumada, Slusarski et al. 2002). Further evidence from *Drosophila* suggests that Galpha(o) is downstream of Fz/Wnt signaling (Katanaev, Ponzielli et al. 2005).

An alternative theory postulates that the cytosolic phosphoprotein, Dishevelled (Dvl), transduces the signal by interacting directly with Fz receptors. Dvl was initially described as a *Drosophila* segment polarity gene that was required for the transduction of a Wnt signal in a cell-autonomous fashion (Klingensmith, Nusse et al. 1994). Dvl

contains an N-terminal DIX (**DI**shevelled and a**X**in), a basic domain, a central PDZ (**P**ost-Synaptic Density-95, **D**iscs-large, **Z**ona occludens-1), and a C-terminal DEP (**D**ishevelled, **e**gl-10, **p**leckstrin). NMR studies suggest that the PDZ domain of Dvl binds directly to the C-terminal domain tail of Fz (Wong, Bourdelas et al. 2003). Additionally, Wnt stimulates Dvl hyperphosphorylation and relocalization to the plasma membrane (Yanagawa, van Leeuwen et al. 1995). The affinity of this interaction in isolation is relatively weak and it has been suggested that the homo-multimerization of both Dvl and Fz results in an avidity effect.

One other model proposes that LRP recruits Axin to the membrane, thereby leading to dissociation of the β-catenin destructin complex. Wnt stimulation results in phosphorylation of LRP C-terminus, thereby increasing the binding affinity of LRP for Axin (Tamai, Zeng et al. 2004). Thus, this relocalization of Axin could potentially alter the composition of the degradation complex, leading to the stabilization of β-catenin.

Wnt signaling in the cytosol

Although the exact mechanism of transduction of the Wnt signal across the membrane is not clear, it is generally accepted that β-catenin phosphorylation and degradation is inhibited when Wnt signaling is active. The mechanism controlling this pathway remains a topic of active debate, although multiple lines of evidence indicate that Dvl is a necessary component. Frequently Rearranged in Activated T-cells (FRAT) recruitment to the degradation complex by Dvl has been proposed to inhibit GSK3β phosphorylation of β-catenin (Li, Yuan et al. 1999). FRAT binds both GSK3β and Dvl,

and acts as a scaffold between Dvl and the degradation complex. Supporting data demonstrates that casein kinase 1 epsilon (CK1ε), a kinase required for the stabilization of β-catenin, phosphorylates Dvl and enhances FRAT binding (Hino et al., 2003). However, studies performed in FRAT knockout mouse (van Amerongen, Nawijn et al. 2005) and the absence of a FRAT homolog in *Drosophila* diminish enthusiasm for this theory. Alternatively, the destruction complex may be inhibited by Axin destabilization or a lower affinity between Axin and β-catenin. Recent data suggests that the number of Axin molecules in the cell is much lower than other components of the degradation complex and therefore destabilization of Axin would lead to disassembly of the degradation complex and stabilization of β-catenin (Lee, Salic et al. 2003). Destabilization of Axin can be induced upon its recruitment to LRP following Wnt stimulation (Mao, Wang et al. 2001). Although this may contribute to prolonged activation of the pathway, the kinetics of Axin destabilization do not sufficiently mirror the kinetics of β-catenin stabilization.

Wnt signaling in the nucleus

It is thoroughly accepted that Wnt stimulation of Fz increases the protein levels of cytosolic β-catenin, which translocates to the nucleus and interacts with the TCF/LEF DNA binding proteins and promotes the transcriptional activation of select genes (Behrens, von Kries et al. 1996; Molenaar, van de Wetering et al. 1996). β-catenin accumulates due to the inability of GSK-3β to phosphorylate β-catenin. How β-catenin is shuttled to and exported from the nucleus is unclear. β-catenin contains no identifiable nuclear localization signals (NLS) or nuclear export signals (NES). A number of theories

have been proposed explaining how β-catenin moves in and out of the nucleus: 1) β-catenin diffuses freely between the cytoplasm and the nucleus, and its localization is largely determined by the availability and strength of interactions with cytoplasmic and nuclear binding partners (possible when at very high levels in disease states). 2) Legless, a nucleo/cytoplasmic protein, shuttles β-catenin to the nucleus, where they are both retained through an interaction with the nuclear protein Pygopus (Townsley, Cliffe et al. 2004). 3) APC serves as a facilitator of Arm/β-catenin subcellular movement, promoting both nuclear import and export, and the Pygopus/Legless complex prevents APC from exporting Arm.

Termination of the transcriptional activation when the Wnt ligand is no longer present would require β-catenin to be removed from the nucleus and ubiquitinated by a newly formed destruction complex. Compelling evidence suggests that nuclear export of β-catenin is primarily mediated by APC (Henderson 2000), but APC-independent mechanisms have been proposed, although this is most likely restricted to cells with abnormally high levels of stabilized β-catenin.

Wnt/β-catenin signaling in disease

Tight regulation of Wnt/β-catenin signaling is crucial for many aspects of metazoan development, adult homeostasis, and disease (Logan and Nusse 2004; Moon, Kohn et al. 2004; Barker and Clevers 2006). During development Wnt/β-catenin signaling has been implicated in the proliferation, differentiation, and fate designation of

cells in a diversity of developing tissues (Chien and Moon 2007). Homeostatic Wnt/β-catenin signaling plays a crucial role in the maintenance of several stem and progenitor populations as well as the expansion and fate decisions of these populations following injury (Coombs, Covey et al. 2008).

Aberrant Wnt/β-catenin signaling (of both hyper- and hypoactive forms) is associated with a variety of pathological conditions (Moon, Kohn et al. 2004). Myriad cancers have been linked to mutations in core Wnt/β-catenin pathway genes that constitutively activate the pathway in a ligand-independent manner (Reya and Clevers 2005). Inhibiting β-catenin signaling in these instances can reduce proliferation of cancer cells. Thus, there is significant interest to identify small molecule inhibitors of Wnt/β-catenin signaling using high throughput screening assays as potential anti-neoplastic agents (Barker and Clevers 2006). Novel therapeutics that enhance or inhibit Wnt/β-catenin signaling may prove beneficial for other pathologies associated with aberrant Wnt/β-catenin signaling, including low bone mass (Rawadi 2008), familial exudative vitreoretinopathy (Warden, Andreoli et al. 2007), psychiatric disorders (De Ferrari and Moon 2006), neurodegenerative diseases (Boonen, van Tijn et al. 2009), cord blood progenitor expansion (Hofmeister, Zhang et al. 2007), regenerative medicine (Katoh 2008), and malignant melanoma (Chien, Moore et al. 2009).

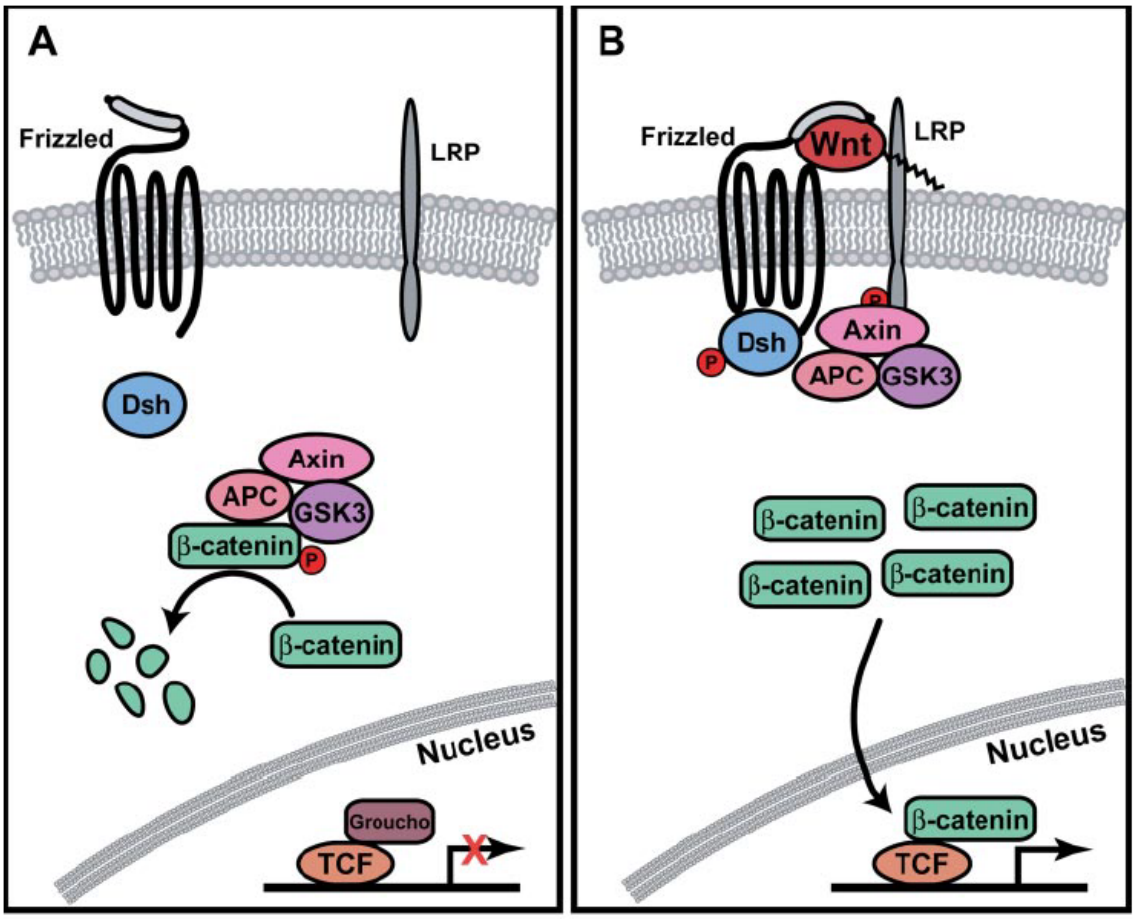

Figure 1. Schematic representation of the WNT/β-catenin signaling pathway. (A) In the absence of a Wnt signal, β-catenin is phosphorylated by the "destruction complex" consisting of Axin, APC and GSK3. The phosphorylated form of β-catenin is subsequently ubiquitinated and degraded. **(B)** In the presence of a Wnt signal, the destruction complex is inhibited and β-catenin accumulates and regulates transcription in the nucleus. Taken from Gordon and Nusse (Gordon and Nusse 2006).

CHAPTER 2

WTX associates with the β-catenin destruction complex and negatively regulates WNT/β-catenin signaling.

Introduction

In the absence of WNT ligands, cytosolic β-catenin is constitutively degraded through phosphorylation-dependent ubiquitination and subsequent proteosomal clearance. A complex of proteins that includes APC, AXIN, CK1α, and GSK3β (collectively known as the "β-catenin destruction complex"), phosphorylates N-terminal residues in β-catenin. Phosphorylated β-catenin is a high-affinity substrate for ubiquitination by the SKP1, CUL1, F-box protein βTrCP (gene symbol BTRC) ubiquitin ligase (SCF^{BTRC}) (Kimelman and Xu 2006). Binding of a Frizzled receptor with WNT ligand initiates a signaling cascade that culminates with inhibition of β-catenin destruction complex. Consequently, β-catenin protein levels increase in the nucleus where it functions as a transcriptional coactivator for members of the LEF/TCF family of transcription factors (Logan and Nusse 2004; Willert and Jones 2006).

Aberrant regulation of β-catenin can result in myriad pathologies ranging from cancer to neurodegeneration. Although mutations in APC that lead to constitutively active β-catenin are common in colorectal cancer, many human malignancies harboring active β-catenin have no identified causative mutation(s) (Moon, Kohn et al. 2004; Clevers 2006). For example, Wilms tumor, the most common pediatric kidney cancer, is characterized by elevated β-catenin levels during kidney development; approximately

50% of all Wilms tumor patient samples contain elevated levels of β-catenin in immunohistochemical analyses (Koesters, Niggli et al. 2003). Several identified mutations in Wilms tumor are gain-of-function mutations in β-catenin or loss of function mutations in the transcription factor WT1 (Wilms Tumor 1) (Haber, Timmers et al. 1992; Koesters, Ridder et al. 1999). However, these mutations account for only 10-15% of all Wilms tumors, and mutations in known regulators of the Wnt/β-catenin pathway have not been identified. Thus, the causative mutations in the majority of patients with elevated β-catenin levels are not known, suggesting that previously unidentified regulators of the Wnt/β-catenin pathway exist and may be mutated in Wilms tumor.

To better understand the molecular mechanisms of β-catenin regulation, we utilized a tandem-affinity purification strategy in conjunction with mass spectrometry to isolate and characterize proteins associated with the β-catenin destruction complex. Specifically, we identified protein complexes associated with β-catenin, APC, AXIN, BTRC, and BTRC2/FBXW11. This strategy identified a novel member of the destruction complex, FAM123B, which was subsequently identified as WTX (Wilms Tumor gene on the X-chromosome).

WTX belongs to a gene family comprised of 3 genes (FAM123A, B, and C) with no significant homology to known functional domains. The full-length transcript (7.5kb) encodes a protein of 1135 amino acids, containing a nuclear localization signal, two coiled-coil domains, an acidic domain that overlaps the first coiled coil domain, and a

proline-rich domain (**Figure 2**). Analysis of the full-length WTX transcript and its encoded protein reveal an N-terminal in-frame alternative splice, internal to the ORF, which leads to an in frame deletion of amino acids 50-326 (Rivera, Kim et al. 2007). Using gain-of-function and loss-of-function approaches *in vitro* and *in vivo*, we show that WTX promotes ubiquitination and degradation of β-catenin.

Experimental Procedures

Plasmids

All expression constructs, including the human WTX, were created with standard PCR-based cloning strategies. β-catenin$^{(SA)}$ contains alanine substitutions at residues 33, 37, 41, and 45. The β-catenin activated reporter (pBAR) contains 12 TCF binding sites (5'-AGATCAAAGG-3') separated by distinct five base linkers. These elements are directly upstream of a minimal thymidine kinase promoter, which then drives expression of firefly luciferase. The reporter contains a separate PGK promoter that constitutively drives expression of a puromycin resistance gene.

Tissue culture and transfections.

All cell lines were grown in DMEM supplemented with 10% fetal bovine serum in a 37°C humidified incubator with 5% CO2. Selection and passage of stable cell lines was performed with 1.5 μg/ml puromycin until cell death was no long apparent. Expression constructs were transiently transfected in HEK293T cells with Lipofectamine 2000 as directed by the manufacturer (Invitrogen, Carlsbad, CA). Transient transfection

of siRNA was performed with Lipofectamine RNAiMAX, as directed by the manufacturer (Invitrogen).

Tandem-affinity purification and mass spectrometry

HEK293T cells (2×10^{8}) expressing the TAP-tagged construct were used for the tandem-affinity purification procedure. The purification scheme was adapted from Gingras *et al* (Gingras, Caballero et al. 2005). Briefly, cell lysates were incubated at 4°C with 100 μL of packed streptavidin resin (Amersham, Piscataway, NJ). Resin beads were washed and protein complexes were then eluted from the streptavidin resin with 50 mM biotin. The second round of affinity purification was performed using 100 μL of calmodulin resin (Amersham). Protein complexes were eluted in buffer containing 25 mM EGTA. Samples were digested overnight at 37°C with RapiGest (Waters Corp, MA). Prior to mass spectrometry, RapiGest was acid cleaved at 37°C for 30 minutes. The resulting peptide mixture was then processed for analysis by LC–MS/MS. The acquired tandem mass spectra were searched against a FASTA file containing the human NCBI sequences (downloaded in April 2004) using a normalized implementation of SEQUEST. The resulting peptide identifications returned by SEQUEST were filtered and assembled into protein identifications using DTASelect.

Affinity pull-downs and Western blotting

For streptavidin affinity purification, cells were lysed in radioimmunoprecipitation buffer (RIPA; 25 mM Tris-HCl at pH 8.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 0.25% deoxycholic acid, 2 mM EDTA) containing 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 NuPAGE loading buffer (Invitrogen). Detection of proteins by Western blot was performed using the following antibodies: anti-FLAG M2 monoclonal (Sigma, St Louis, MO), anti-HA polyclonal (1867423; Roche), anti-CTNNB1 polyclonal (9562; Cell Signaling Technology), anti-GFP polyclonal (ab290, Abcam), anti-VSV polyclonal (V4888, Sigma), anti Active-β-catenin (anti-ABC, clone 8E7, Upstate), anti-BTRC monoclonal (37-3400, Invitrogen) and anti-TUBB1 monoclonal (T7816; Sigma).

RNA isolation, reverse transcription, and semi-quantitative real-time PCR.

Total RNA from Tissue Culture cells or zebrafish embryos was harvested in Trizol (Invitrogen) reagent according to the manufacturer's instructions. RNA was quantified by UV spectrophotometry, and cDNA was created using superscript III (Invitrogen) according to the manufacturer's instructions. PCR was performed in duplicate with the LightCycler FastStart DNA SyBr Green kit (Roche) using the Roche LightCycler 480 instrument (Roche). The PCR conditions are as follows: 35 cycles of amplification with 1 second denaturation at 95 ^{0}C, and 5 second annealing at 58 ^{0}C. A template free negative control was included in each experiment. Quantitative light cycler PCR primers used are as follows: human *WTX* (GAC CCA AAA GGA TGA AGC T; and reverse CCC CTC CAA AGA AAC TAG GC), *β-catenin* (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), and 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 (Salic, Lee et al. 2000). *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 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 vitro binding experiments

Human GST-VSV-WTX was purified from *Escherichia coli* and mixed with purified CUL1, BTRC or β-catenin in buffered 150 mM NaCl. Following incubation for 30 minutes at 4°C, complexes were washed with five bed-volumes of 350 mM buffered NaCl before elution and Western blot analysis.

Results

Elucidation of the β-catenin interaction network

To identify proteins associated with the β-catenin destruction complex, we performed a tandem-affinity purification (TAP) of β-catenin^{SA}, AXIN1, APC$^{(AA1-1060)}$, βTRCP1, and βTRCP2 in mammalian cells. 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 (Angers, Thorpe et al. 2006). Lines of human embryonic kidney cells (HEK293T) expressing low levels of each of the tagged-'bait' fusion protein were generated, detergent solubilized, subjected to two rounds of affinity purification, trypsinized, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) **(Figure 3)**.

The resulting data for all 'bait' proteins were integrated to yield the protein-protein interaction network of the β-catenin destruction complex **(Figure 4 and Table I)**. This proteomic analysis confirmed the presence of all the core proteins identified in previous screens including β-catenin, APC, AXIN1, AXIN2, PP2A, GSK3α, GSK3β and CK1α. In addition, 13 new proteins were found to associate with known components of the destruction complex.

WTX directly binds the β-catenin destruction complex

WTX (FLJ39287/FAM123B) was further explored because it co-purified with each of the baits examined. The WTX gene was recently discovered to be mutated in

approximately 30% of Wilms tumors, a pediatric kidney cancer (Rivera, Kim et al. 2007). Constitutive activation of Wnt/β-catenin signaling is common in Wilms tumors; ~10% of tumors harbor activating mutations in β-catenin and nuclear β-catenin is observed in ~50% of tumors lacking detectable β-catenin mutations. Initial studies found that WTX and β-catenin mutations are mutually exclusive in tumor samples, suggesting that WTX is a "one-hit" tumor suppressor gene (Rivera, Kim et al. 2007). However, subsequent studies have identified patient samples containing both WTX and either β-catenin or WT1 mutations (Ruteshouser, Robinson et al. 2008).

To validate the WTX protein interaction network, we assessed protein binding in HEK293T cells and with recombinant protein. 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 (**Figure 5A**). These data demonstrate that WTX binds both wild type β-catenin and the stabilized β-catenin$^{(SA)}$ mutant. 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 βTRCP (**Figure 5B**). Additionally, using purified recombinant protein *in vitro*, we found that WTX directly binds β-catenin and βTRCP, but not the CUL1 scaffold within the E3 ligase complex (**Figure 5C**). These results show that post-translational modifications are not required for WTX binding to β-catenin or βTRCP.

Although deletion of the entire *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 (Rivera, Kim et al. 2007). As such, these mutations are consistent with the existence of a putative tumor suppressor motif within the carboxy-terminus of WTX. If WTX regulates 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) (**Figure 5D**). Thus, mutational alteration of WTX in Wilms tumor likely reduces its interaction with β-catenin and β-TRCP.

WTX promotes β-catenin ubiquitination and degradation

The direct binding of WTX to both β-catenin and to its E3 ubiquitin ligase, βTRCP, 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 (Salic, Lee et al. 2000). The addition of recombinant GST-WTX protein, but not GST control, increased the rate of β-catenin ubiquitination (**Figure 6A and B**). 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 (Salic, Lee et al. 2000). When WTX and AXIN1 were added to the extracts individually, each increased the rate of β-catenin degradation (**Figure 6C**). 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 following 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 media, which activated the reporter by a factor of 100 to 300 (**Figure 6D and E**). As a control, we showed that siRNAs directed against *β-catenin* abolished this Wnt3a-induced reporter activity in both cell lines. Using this assay system, we found that two different siRNAs targeting *WTX* produced an increase in Wnt3a-induced reporter activity in both cell types (**Figure 6D and E**). 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, while 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, allowing one to study cytoplasmic and nuclear β-catenin stability in response to experimental perturbation. We transiently transfected RKO cells with siRNAs targeting *WTX*, *β-catenin*, *AXIN1/2*, or *βTRCP1/2*. Silencing of *WTX*, *AXIN1/2*, or *βTRCP1/2*, but not *β-catenin*, was found to increase β-catenin levels, as determined by immunoblot analysis (**Figure 6F**). Thus, WTX is required in these cells as a negative regulator of both β-catenin protein stability and β-catenin-mediated transcription.

WTX negatively regulates Wnt signaling in vivo

To extend these experiments to organisms, we performed gain-of-function experiments in *Xenopus* embryos and loss-of-function experiments in zebrafish. Ectopic activation of Wnt/β-catenin signaling by injection of *XWnt8* mRNA in *Xenopus* embryo ventral blastomeres induced duplication of the embryonic axis, yielding two-headed tadpoles (**Figure 7A**). Injection of *WTX* mRNA blocked *XWnt8* induced axis duplication. In developing zebrafish embryos, ectopic activation of Wnt/β-catenin signaling leads to anterior truncations. When we silenced endogenous zWTX expression, we observed

anterior truncations and the activation of a Wnt/β-catenin reporter gene (**Figure 7B**). These results suggest that WTX is a negative regulator of Wnt/β-catenin signaling *in vivo*.

Discussion

Using a tandem-affinity purification (TAP) approach, we identified the cancer-associated WTX protein as a novel interactor of multiple components of the β-catenin destruction complex including β-catenin, APC, AXIN, βTrCP1, and βTrCP2. Furthermore, we determined that WTX directly interacts with β-catenin and βTrCP1, and is a negative regulator of Wnt/β-catenin signaling. Based on these observations, we hypothesize that WTX associates with an intact β-catenin/ βTrCP complex and promotes the ubiquitination of β-catenin, perhaps by enhancing the interaction between β-catenin and BTRC. Although we do not have direct evidence to support this hypothesis, we found that exogenous WTX can enhance the ubiquitination and the degradation of β-catenin in cell-free *Xenopus* extracts.

In parallel with our discovery that WTX associates with the destruction complex and promotes the degradation of β-catenin, Rivera et al. identified *WTX* as a novel gene mutated in Wilms tumor (Rivera, Kim et al. 2007). They found that *WTX* is commonly lost through gene-encompassing deletions, and a small percentage of patients harbor a point mutation within *WTX* that yields a truncated protein. These truncated WTX protein products are not predicted to interact with β-catenin based on our observation that amino acids 368-804 are required to bind β-catenin. Given that elevated levels of β-catenin are

associated with Wilms tumor, we predict that all patients containing some form of a WTX mutation have elevated levels of β-catenin, which may be the driving factor for their Wilms tumor (**Figure 8**). In conclusion, 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.

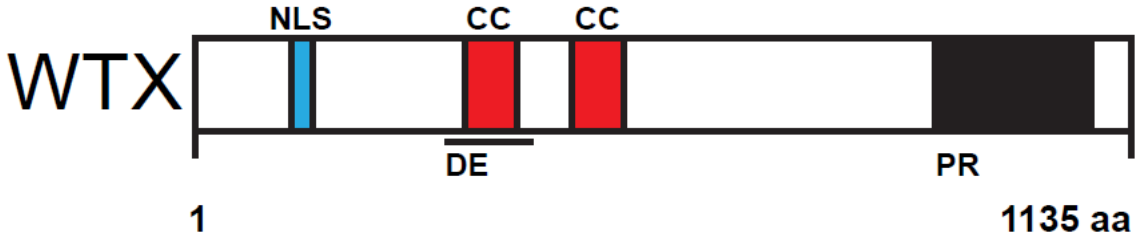

Figure 2. Schematic representation of WTX and its potential functional domains. The full-length WTX protein is 1135 amino acids and contains a nuclear localization signal (NLS), two coiled-coil domains (CC), an acidic domain (DE) that overlaps the first coiled coil domain, and a proline-rich domain (PR).

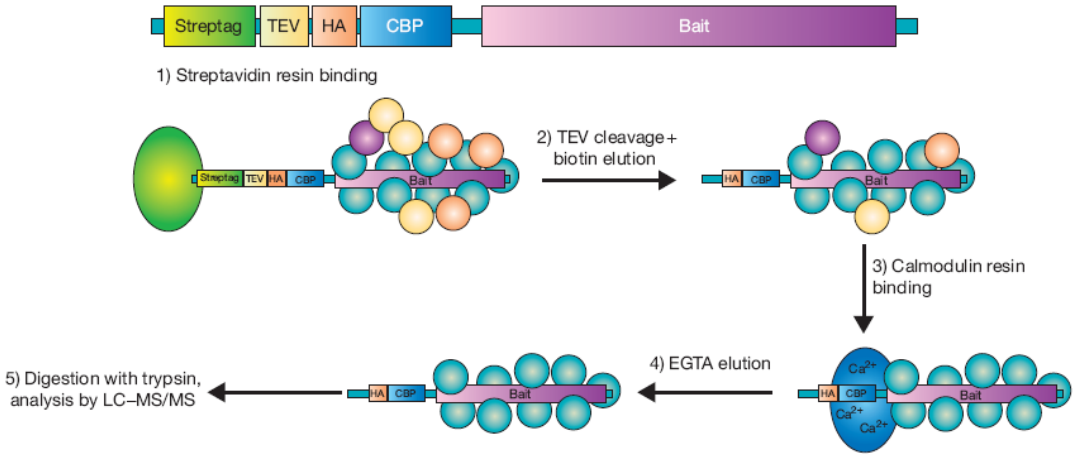

Figure 3. Schematic representation of the tandem-affinity purification strategy. The pGLUE expression vector was designed to have the streptavidin-(streptag) and calmodulin-(CBP) binding affinity tags placed in tandem at the N-terminus of the bait protein. An HA epitope is also present to facilitate protein detection. The first round of purification is performed using streptavidin resin, followed by specific elution with biotin. The second round is done using the calcium-dependent binding to calmodulin resin and specific elution by chelation using ETGA. The eluted complex is directly digested with trypsin and analysed by LC-MS/MS.

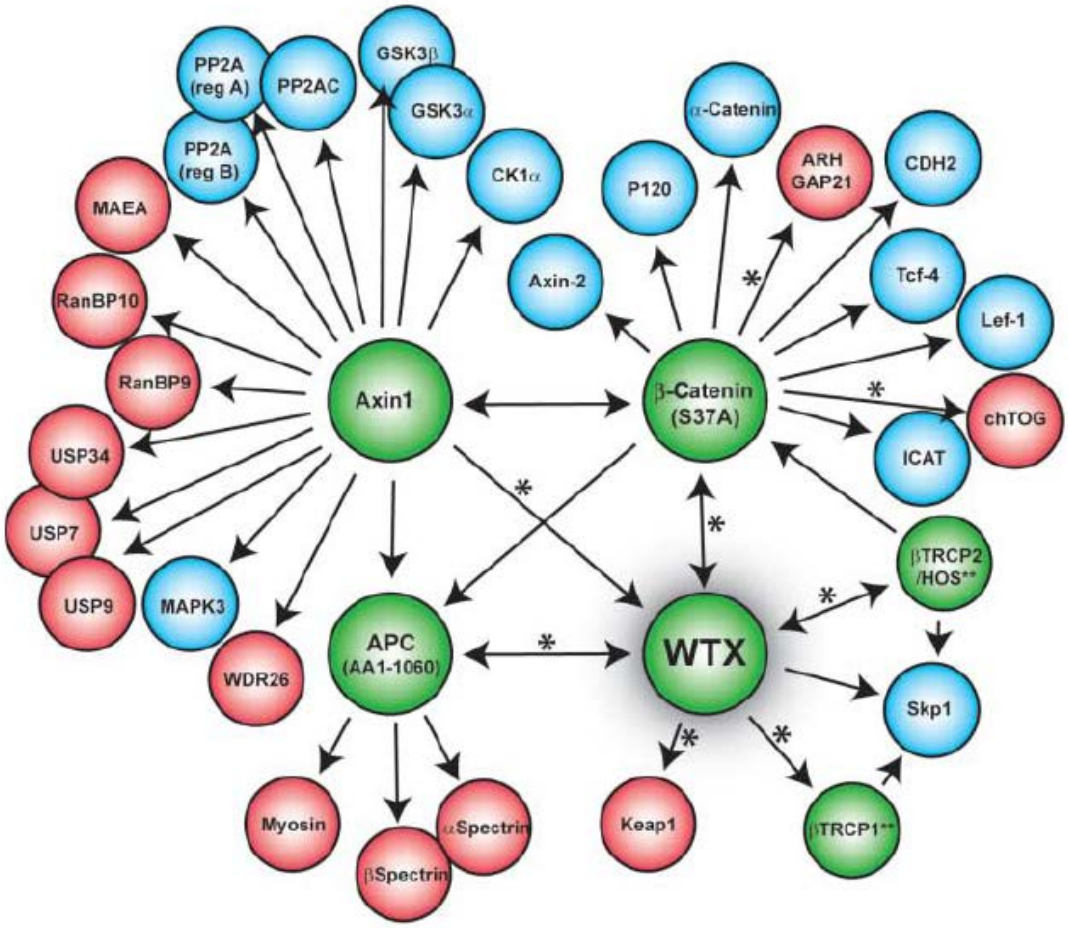

Figure 4. The β-catenin protein interaction network. Green circles represent proteins used as 'bait' in the tandem affinity purification, blue circles represent known interactors and red circles represent novel interactors. The arrows indicate directionality for the 'bait–interactor' discovery and the asterisks show interactions that were confirmed in secondary assays.

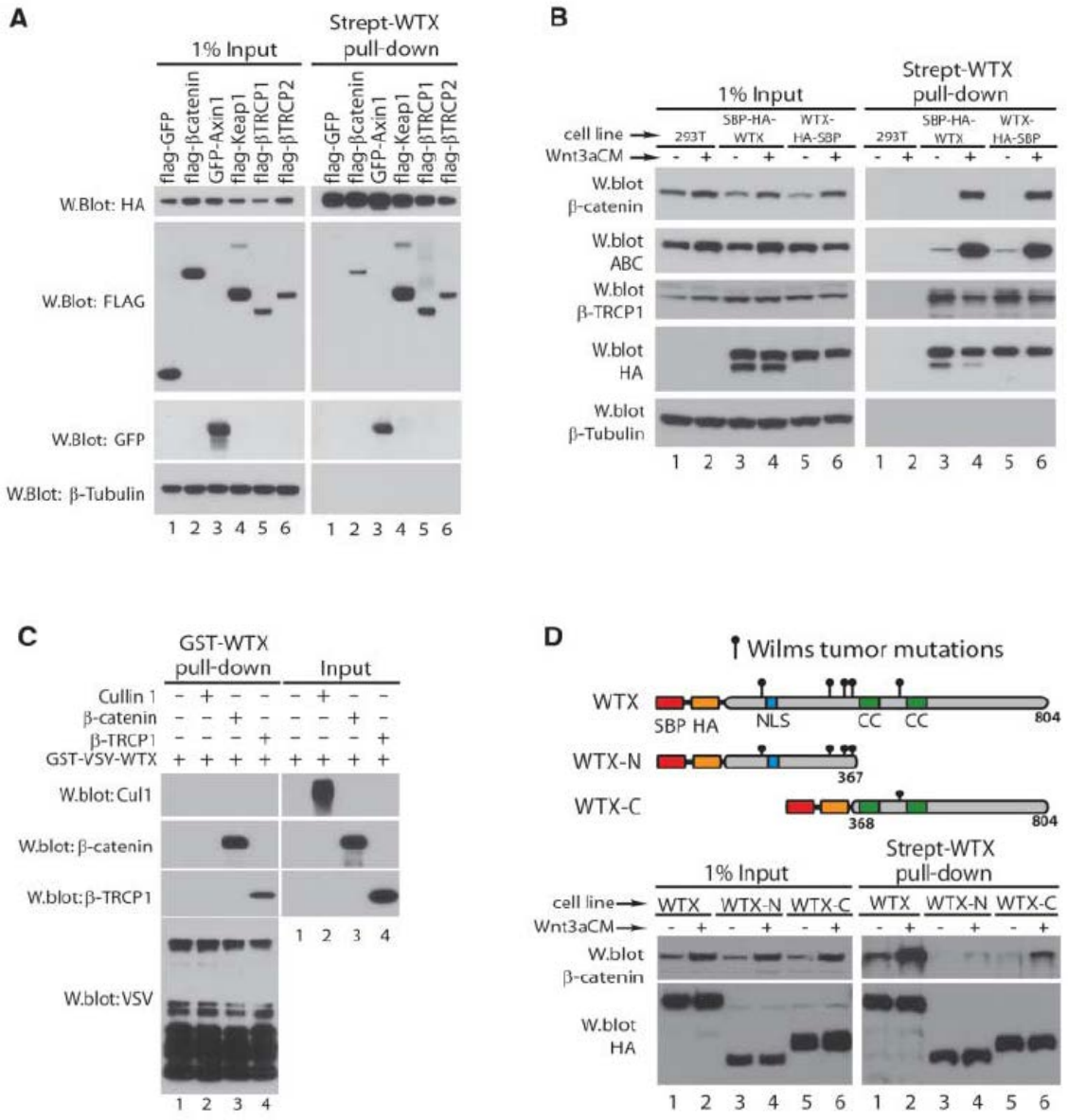

Figure 5. WTX directly binds the β-catenin destruction complex. (A) WTX associates with ectopically expressed β-catenin, AXIN1, βTRCP1, βTRCP2 and Keap1. FLAG-tagged proteins were transiently expressed in HEK293T cells stably expressing SBP-HA-WTX. Protein lysates were subjected to streptavidin affinity pulldown followed by western blot analysis. **(B)** WTX associates with endogenous β-catenin and βTRCP1. Parental HEK293T cells or HEK293T cells stably expressing N-terminal or C-terminal pGlue-WTX were treated with Wnt3a conditioned media (CM) for 2 hours prior to lysis, streptavidin affinity pulldown and western blot analysis (ABC; active β-catenin). **(C)** WTX directly binds β-catenin and βTRCP1. GST-VSV-WTX recombinant protein was incubated with recombinant CUL1, β-

catenin or βTRCP at equal molar ratios. Following 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. The cartoon above 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 pulldown and western blotting were performed as in (B).

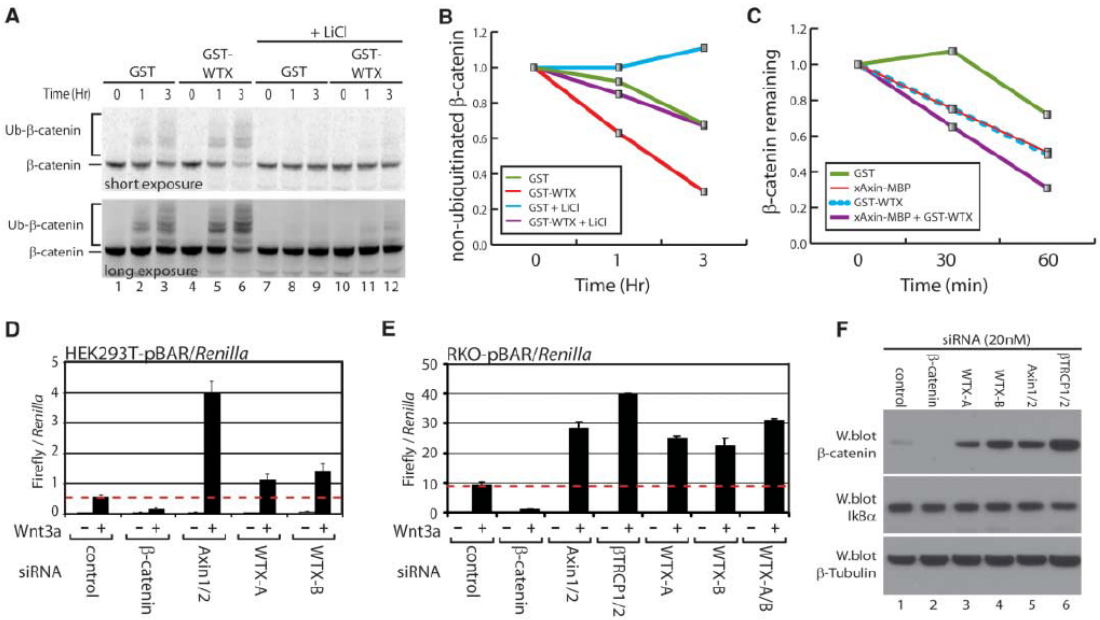

Figure 6. WTX promotes β-catenin ubiquitination and degradation. **(A)** A cell-free system of *Xenopus* egg extracts was used to monitor β-catenin ubiquitination as a function of time. *In vitro* transcribed and translated ^{35}S-β-catenin was added to *Xenopus* egg extracts in the presence of methylated ubiquitin (MeUb) and either purified GST or GST-WTX protein. The extent of ^{35}S-β-catenin ubiquitination was followed by SDS/PAGE and autoradiography. As a measure of specificity, LiCl (10 mM) was added to inhibit β-catenin phosphorylation and subsequent ubiquitination. **(B)** Quantitation of non-ubiquitinated ^{35}S-β-catenin levels from (A). **(C)** Recombinant GST-WTX and MBP-Axin1 synergize to degrade ^{35}S-β-catenin in *Xenopus* egg extracts. Graphical representation of ^{35}S-β-catenin degradation as a function of time; note absence of meUb in this experiment, and 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, a βTRCP substrate induced by tumor necrosis factor-α stimulation, and β-Tubulin demonstrate equal protein loading in the blots.

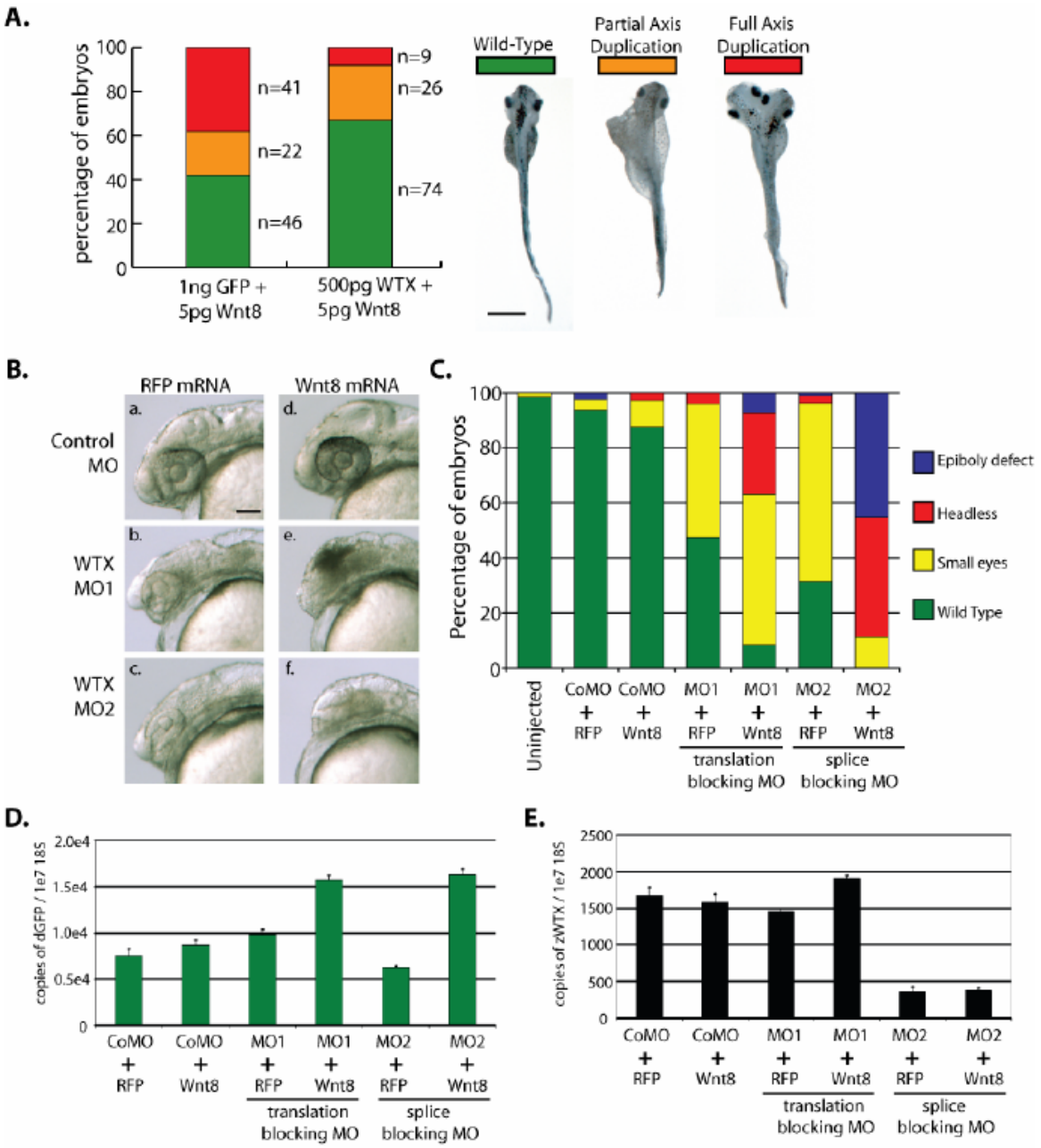

Figure 7. WTX negatively regulates Wnt signaling in *Xenopus* and zebrafish. (A) WTX inhibits Wnt8-induced axis duplication in *Xenopus* embryos. *GFP* or *WTX* mRNA was microinjected into ventral blastomeres at the 4-cell stage, allowed to develop to the 16 cell stage, and then a single ventral cell 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 (3 ng, blocks

translation) and WTX-MO2 (3 ng, 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 (10 pg) and either WTX-MO1 or WTX-MO2 results in complete loss of anterior structures. *RFP* mRNA (10 pg) was used as a negative control. The scale bar represents 100 μM. **(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.

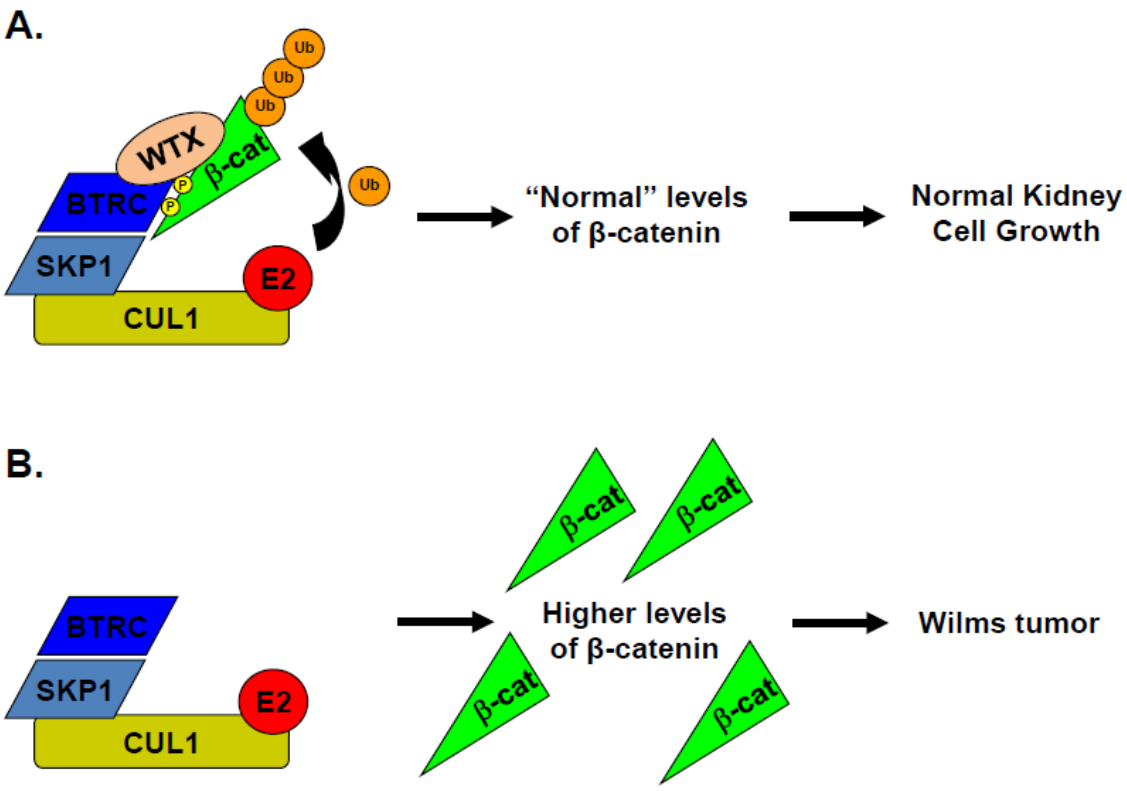

Figure 8. Proposed mechanism of WTX in the Wnt/β-catenin pathway and Wilms tumor. (A) In the presence of WTX, phosphorylated β-catenin is efficiently ubiquitinated by the SCF^{BTRC} E3 ubiquitin ligase complex. We propose that WTX is required for maintaining appropriate levels of β-catenin required for normal Kidney development. **(B)** In the absence of WTX, the ubiquitination of β-catenin is no longer regulated by the SCF^{BTRC} complex, leading to elevated levels of β-catenin that lead to abnormal Kidney development and the onset of Wilms tumor.

Table I. Representative mass spectrometry results. The total number of peptides 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.

CHAPTER 3

Introduction to KEAP1/NRF2 signaling

A brief history of NRF2

Cells are continuously bombarded by environmental insults such as reactive oxygen species (ROS) and xenobiotics. Without the ability to respond to and eliminate these insults, cells would be defenseless against agents that damage DNA, protein, and lipids. One important mechanism by which cells respond to environmental insults is through induction of what are collectively termed "phase II" detoxification enzymes. These enzymes, including NQO1, HMOX1, and multiple GST homologues, neutralize oxidants, clear xenobiotics, and degrade damaged and misfolded proteins (Kensler, Wakabayashi et al. 2007).

One commonality among the phase II detoxification genes is the presence of an Antioxidant Response Element (ARE) in their respective promoter regions. Early studies defined the ARE as a 24 base-pair region in the promoter region of *NQO1* that is required for induction of phase II enzymes by β-napthoflavone (β-NF), *tert*-butylhydroquinone (*t*BHQ), and hydrogen peroxide (Li and Jaiswal 1992; Jaiswal 1994; Xie, Belinsky et al. 1995). A subsequent study by Venugopal and Jaiswal was predicated on the observation that the NF-E2 (Nuclear Factor – Erythroid 2) related transcription factors, NRF1 and NRF2, bind to similar sequences as the ARE (Chan, Han et al. 1993; Moi, Chan et al. 1994). This seminal study revealed that NRF1 binds to the ARE and overexpression of

either NRF1 or NRF2 leads to induction of ARE-mediated transcription (Venugopal and Jaiswal 1996).

Subsequent studies determined that NRF1 contributes to the regulation of basal and expression levels of certain cytoprotective enzymes (Kwong, Kan et al. 1999; Leung, Kwong et al. 2003). NRF2, however, was shown to be an essential transcription factor in response to cytotoxic stress *in vivo* (Itoh, Chiba et al. 1997). Whereas administration of the phenolic antioxidant butylated hydroxyanisole (BHA) to wild-type mice results in an induction of several GST subunits and NQO1, this response is almost completely absent in *Nrf2*-null mice. Additional studies utilizing *Nrf2*-null mice have demonstrated the importance of Nrf2 in the response to a variety of insults (Ishii, Itoh et al. 2000; McMahon, Itoh et al. 2001).

Discovery of KEAP1, a negative regulator of NRF2

NRF2 contains 6 highly conserved NRF2-ECH homology (Neh) domains. Deletion of the N-terminal Neh2 domain results in increased transcriptional activity (Itoh, Wakabayashi et al. 1999). This implies that the Neh2 domain is important for negatively regulating NRF2 activity, possibly through recruitment of another protein. In the same study, Itoh et al. performed a yeast-two hybrid screen for interactors of the Neh2 domain and identified a potential NRF2 repressor, which they termed Kelch-like ECH associating protein 1, or KEAP1.

KEAP1 has three well-defined domains, an N-terminal Bric-a-brac, tramtrack, broad-complex (BTB) domain, an intervening region (IVR), and a C-terminal region containing 6 KELCH repeats (named after similar repeats initially identified in *Drosophila* Kelch protein). The KELCH repeats form a β-propeller structure that directly interacts with NRF2. The N-terminal Neh2 domain of NRF2 contains two highly conserved regions, one bearing an LxxQDxDLG motif (McMahon, Thomas et al. 2004; Katoh, Iida et al. 2005) and the other bearing a DxETGE motif (Kobayashi, Itoh et al. 2002). Both motifs interact with the same site in the KELCH repeats of KEAP1, although the DxETGE motif binds with approximately 100-fold higher affinity (Tong, Katoh et al. 2006). Multiple studies have shown that KEAP1 forms a homodimer through its BTB domain (Zipper and Mulcahy 2002; Ogura, Tong et al. 2010), but it is unclear if the homodimer binds a single NRF2 molecule through both LxxQDxDLG and DxETGE motifs and/or binds two NRF2 molecules via their DxETGE motifs.

Several observations provided a molecular mechanism by which KEAP1 negatively regulates NRF2 transcription. First, *nrf2* transcript levels are abundant and easily detectable, yet protein levels are undetectable under normal conditions. Second, proteosome inhibitors (i.e. MG132) robustly increase steady-state levels of NRF2, suggesting it is constitutively degraded by the ubiquitin-proteosome pathway in the absence of inducers. In 2003, several groups determined that the CUL3 ubiquitin ligase complex interacts with BTB domain-containing substrate adaptors, which recruit substrates to the complex (Furukawa, He et al. 2003; Geyer, Wee et al. 2003; Pintard, Willis et al. 2003; Xu, Wei et al. 2003). Given that KEAP1 contains a BTB domain and

binds NRF2, it was not surprising that KEAP1 was shown to interact with CUL3 and recruit NRF2 to the CUL3 ubiquitin ligase complex, facilitating NRF2 polyubiquitination and proteasome-mediated degradation (Cullinan, Gordan et al. 2004; Kobayashi, Kang et al. 2004; Zhang, Lo et al. 2004).

Activation of NRF2 by phase II inducers

KEAP1-dependent degradation of NRF2 is inhibited by small molecules known as phase II inducers (such as *t*BHQ). Phase II inducers are classified into nine structurally diverse chemical groups (Dinkova-Kostova, Massiah et al. 2001). One common property of these inducers is the ability to modify sulfhydryl groups by alkylation, oxidation, or reduction. This suggests that these molecules may modify KEAP1, resulting in changes in the ubiquitination of NRF2. KEAP1 contains 25 highly conserved cysteine residues, several of which are reactive to phase II inducers (Dinkova-Kostova, Holtzclaw et al. 2002). The majority of reactive cysteines in KEAP1 lie in the central IVR domain, and the importance of these residues in the response to phase II inducers was first demonstrated *in vitro*. The electrophiles 15d-PGJ_2, 15-A_{2t}-isoprostane, and 4-hydroxy-2-nonenal directly bind KEAP1 and activate NRF2 (Itoh, Mochizuki et al. 2004; Levonen, Landar et al. 2004; Hosoya, Maruyama et al. 2005). When 7 cysteines were mutated in the IVR domain, 15d-PGJ_2 lost the ability to interact with KEAP1 (Levonen, Landar et al. 2004).

The previously mentioned studies provided the groundwork for analyzing the importance of the cysteine residues in KEAP1. Mutation analysis unveiled at least three

critical Cysteine residues in KEAP1 that are required for its function. Cys273 and Cys288 in the IVR domain are required for KEAP1-dependent ubiquitination of NRF2 (Zhang and Hannink 2003; Wakabayashi, Dinkova-Kostova et al. 2004). As the IVR domain is important for the interaction between KEAP1 and CUL3, a likely hypothesis is that modification of these cysteines leads to dissociation of KEAP1 from CUL3. However, mutation of these cysteines to alanine did not disrupt the interaction between KEAP1 and CUL3 (Zhang and Hannink 2003; Wakabayashi, Dinkova-Kostova et al. 2004). The third critical residue, Cys151, lies in the BTB domain of KEAP1. Although Cys151 is also required for electrophile-induced inhibition of NRF2 ubiquitination, it is not important for the interaction between KEAP1 and CUL3 (Zhang and Hannink 2003). Importantly, the significance of the three critical cysteine residues has been verified *in vivo* as mice harboring these mutations are unresponsive to phase II inducers and have elevated levels of NRF2(Yamamoto, Suzuki et al. 2008).

The mechanism by which modification of the critical cysteine residues inhibits NRF2 ubiquitination is not known. One possibility is that these modifications confer structural changes in KEAP1 that render it unable to promote NRF2 ubiquitination. Under normal, unstressed conditions, KEAP1 interacts with a zinc atom. Zinc is released from KEAP1 in the presence of phase II inducers (Dinkova-Kostova, Holtzclaw et al. 2005). Interestingly, when Cys273 and Cys288 are mutated to alanine, KEAP1 binds zinc with lower affinity. Combined, these observations suggest that phase II inducers alter the structure of KEAP1, rendering it unable to promote ubiquitination of NRF2 while preserving NRF2 and CUL3 interactions.

Although KEAP1 is most likely the key target of phase II inducers, several studies have implicated protein kinases as sensors of electrophilic and oxidative stresses. Protein kinase C (PKC; (Huang, Nguyen et al. 2002; Bloom and Jaiswal 2003; Numazawa, Ishikawa et al. 2003)) and PKR-like endoplasmic reticulum kinase (PERK; (Cullinan, Zhang et al. 2003; Cullinan, Gordan et al. 2004)) are activated in the presence of phase II inducers. Although several other kinases are activated in the presence of phase II inducers, PKC and PERK can phosphorylate NRF2 both *in vitro* and *in vivo* (Huang, Nguyen et al. 2000; Huang, Nguyen et al. 2002; Cullinan, Zhang et al. 2003).

Using a biochemical approach, it was determined that PKC phosphorylates serine residue 40 on NRF2 located between the DLG and ETGE motifs that interact with KEAP1. Upon phosphorylation, NRF2 dissociates from KEAP1 and is no longer degraded. Although the actual site of phosphorylation on NRF2 by PERK is not known, PERK-dependent phosphorylation of NRF2 also disrupts the interaction between NRF2 and KEAP1. Thus, it appears that multiple proteins are sensors of electrophilic and oxidative stress that function to regulate NRF2 stability.

Activation of NRF2 through protein-protein interactions

Although many studies have reported regulation of NRF2 stability and function by small molecules and stressors, relatively little is known about regulation of KEAP1 and NRF2 through protein-protein interactions. Recently, the cyclin-dependent kinase inhibitor p21, a p53-regulated gene with pro-survival properties, was shown to bind the

DLG motif of NRF2 and inhibit its interaction with KEAP1, resulting in elevated NRF2 levels under both basal and chemically induced conditions (Chen, Sun et al. 2009). Another recent study identified p62 as a novel interactor of KEAP1 (Komatsu, Kurokawa et al. 2010). p62 is an important protein in the autophagy process that directs ubiquitinated proteins to degradation by the lysosome. By binding to KEAP1 through an ETGE-like motif similar to that of NRF2, p62 targets KEAP1 for autophagic degradation, thus contributing to NRF2 stabilization. Importantly, both studies were verified *in vivo* (Chen, Sun et al. 2009; Komatsu, Kurokawa et al. 2010). Combined with the many studies on the mechanisms of phase II inducers and stresses, these examples highlight the complex regulation of NRF2 degradation and its importance in both homeostasis and response to cytotoxic stress.

KEAP1 and NRF2 in disease

Nrf2-null mice are viable and fertile. However, when exposed to electrophilic xenobiotics or chemicals that generate intracellular oxidative stress, Nrf2-null mice display increased tissue damage and prolonged inflammation, high amounts of DNA, lipid, and protein oxidation; and increased incidence of cancer (Aoki, Sato et al. 2001; Enomoto, Itoh et al. 2001; Ramos-Gomez, Kwak et al. 2001; Ramos-Gomez, Dolan et al. 2003). These observations support the central hypothesis that NRF2 is critical for defending the body against external insults. Indeed, small molecules that selectively target NRF2 are currently under investigation as novel therapies in Chronic obstructive pulmonary disease (COPD), a lung disease caused by exposure to toxic fumes or smoke (Harvey, Thimmulappa et al. 2011; Malhotra, Thimmulappa et al. 2011). In addition,

several classes of NRF2 activating drugs are effective in mouse models of carcinogenesis and these effects are partially or completely abolished in *Nrf2*-null mice, indicating that their effects are mediated through induction of Nrf2 (Ramos-Gomez, Kwak et al. 2001; Shen, Xu et al. 2006; Xu, Huang et al. 2006; Yates, Kwak et al. 2006).

Small molecules targeting NRFs may also prove beneficial for the treatment of neurodegenerative disease. Oxidative stress in the nervous system is associated with neuronal cell death, a key step in the pathogenesis of multiple neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) (van Muiswinkel and Kuiperij 2005; de Vries, Witte et al. 2008). Neurodegeneration leads to progressive functional impairment and ultimate mortality, for which effective treatments are lacking. The NRF2 pathway is critical for the protection of neurons from oxidative insults in cell-culture sytems (Kraft, Johnson et al. 2004). Furthermore, genetic and pharmacological activation of NRF2 has been demonstrated to be protective in mouse models of AD, PD, HD, and ALS (Burton, Kensler et al. 2006; Kanninen, Malm et al. 2008; Vargas, Johnson et al. 2008; Yang, Calingasan et al. 2009). Combined, these studies highlight the importance of NRF2 in neurodegenerative disease and the intriguing possibility that manipulation of this pathway can be exploited for therapeutic value.

Paradoxic role of NRF2 in cancer

Mutations in KEAP1 and NRF2 that result in constitutive activation of NRF2 are relatively frequent in human cancers (Hayes and McMahon 2009). In their study on the

molecular mechanisms of the interaction between KEAP1 and NRF2, Padmanabhan et al. characterized the critical residues within KEAP1 that interact with NRF2 and identified somatic mutations in KEAP1 present in human lung cancer cell lines (Padmanabhan, Tong et al. 2006). Interestingly, mutant KEAP1 proteins were unable to repress NRF2 activity and, consequently, NRF2 was constitutively activated in the cancer cells. Subsequent studies identified additional high-frequency somatic mutations in KEAP1 in various lung cancer cell lines and non-small cell lung cancer samples (Singh, Misra et al. 2006). These cell lines have elevated levels of NRF2 and are resistant to chemotherapeutic reagents. Although it is unclear whether these mutations promote carcinogenesis, these mutations may explain why NSCLC patients are refractory to standard chemotherapeutics (Ohta, Iijima et al. 2008; Singh, Boldin-Adamsky et al. 2008).

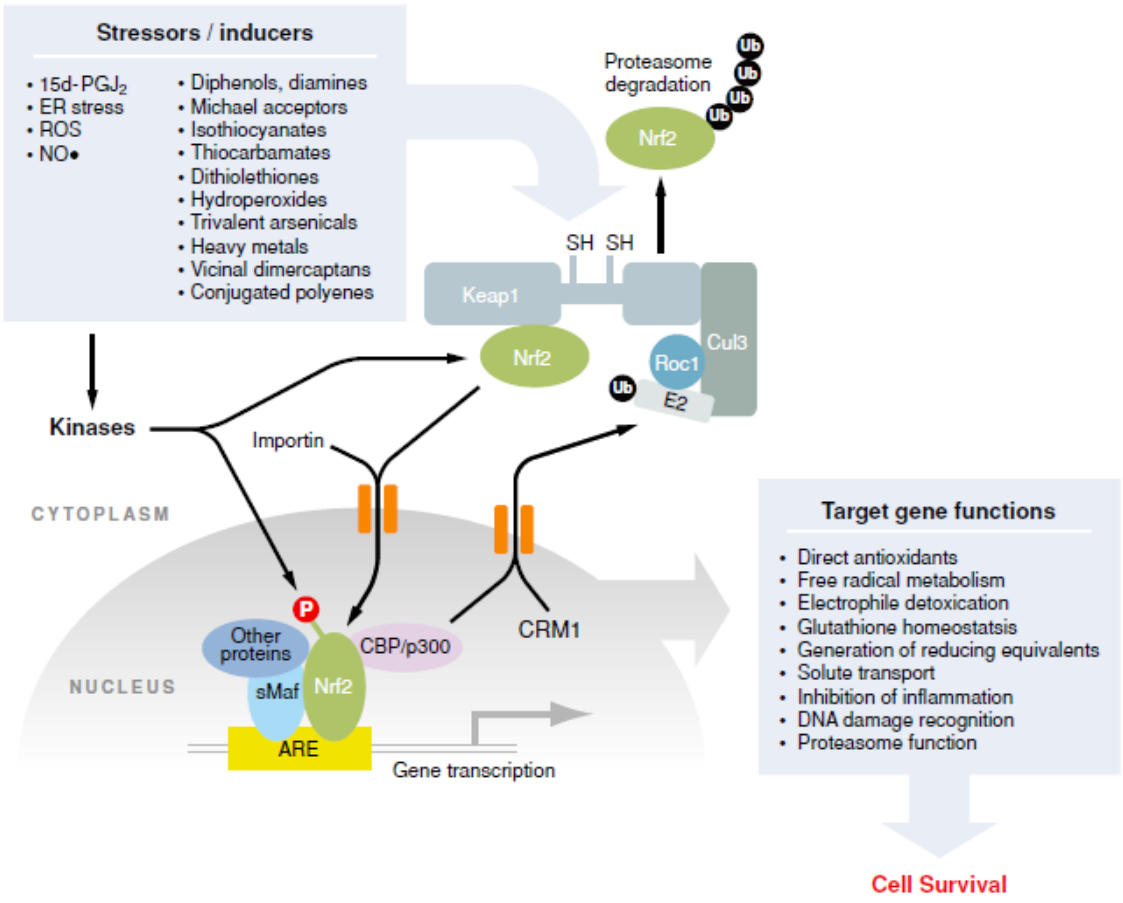

Figure 9. Schematic representation of the KEAP1/NRF2 signaling pathway. Small molecules of endogenous and exogenous origin modify critical cysteine residues on KEAP1 that disrupt the conformation of the KEAP1/NRF2 complex, leading to decreased ubiquitination of NRF2 and nuclear accumulation. In the nucleus, NRF2 interacts with several transcription factors and drives transcription of genes involved in the antioxidant response. This results in an adaptive response that enhances the resistance of cells to environmental stresses mediated by electrophiles and free radicals. Taken from Kensler et al. (Kensler, Wakabayashi et al. 2007).

CHAPTER 4

WTX inhibits the degradation of NRF2 through competitive binding to KEAP1

INTRODUCTION

The *FAM123B/WTX/AMER1* gene (hereafter referred to as *WTX*) is located on the X chromosome and encodes a tumor suppressor protein that is completely lost or mutated in up to 30% of cases of Wilms tumor, the most common pediatric kidney cancer (Rivera, Kim et al. 2007; Perotti, Gamba et al. 2008; Ruteshouser, Robinson et al. 2008). Recently, germline mutations in WTX were also discovered in families suffering from Osteopathia Striata Congenital with Cranial Sclerosis (OSCS), a debilitating skeletal dysplasia that is often accompanied by developmental abnormalities and fatality in males (Jenkins, van Kogelenberg et al. 2009; Perdu, de Freitas et al. 2009).

We previously reported that WTX regulates the stability of β-catenin, a key control point in the Wnt/β-catenin signaling pathway (Major, Camp et al. 2007). In the absence of WNT ligand, β-catenin is phosphorylated by a multi-protein complex often called the "destruction complex" and subsequently recognized by the SCF^{BTRC} (Skp, Cullin, F-box) ubiquitin ligase complex where it is ubiquitinated and targeted for proteasomal degradation (Yost, Torres et al. 1996; Liu, Kato et al. 1999; Winston, Strack et al. 1999). In the presence of WNT ligand, phosphorylation of β-catenin is attenuated, resulting in the nuclear accumulation of β-catenin and the regulation of transcription. Through proteomic and functional dissection of the Wnt/β-catenin signaling pathway, we

discovered that WTX associates with β-catenin and proteins in the destruction complex, including Adenomatous Polyposis Coli (APC), AXIN1, BTRC (commonly referred to as β-TrCP) and FBXW11 (commonly referred to as β-TrCP2) (Major, Camp et al. 2007). WTX promotes the ubiquitination and degradation of β-catenin, through an unknown molecular mechanism.

In addition to regulating the stability of β-catenin, WTX also regulates WNT signal transduction at the membrane (Tanneberger, Pfister et al. 2011), cell-cell adhesion through interactions with APC at the plasma membrane (Grohmann, Tanneberger et al. 2007), and Wilms Tumor 1 (WT1) transcription factor activity in the nucleus (Grohmann, Tanneberger et al. 2007; Rivera, Kim et al. 2009). Thus, aberrant WTX function may cause disease by disrupting multiple distinct signaling mechanisms in a variety of locations within the cell.

We previously used mass-spectrometry proteomics to identify an association between WTX and KEAP1 (Major, Camp et al. 2007). KEAP1 is a substrate recognition module for the CUL3-based E3 ubiquitin ligase that constitutively ubiquitinates the transcription factor NRF2 (NF-E2-related factor-2; NFE2L2) (Cullinan, Gordan et al. 2004; Kobayashi, Kang et al. 2004; Zhang, Lo et al. 2004). In the presence of cytotoxic stress such as xenobiotics and antioxidants, KEAP1 is inhibited and NRF2 is no longer targeted for ubiquitination and degradation. NRF2 then accumulates in the nucleus where it regulates transcription of genes involved in the “phase II” antioxidant response

(Venugopal and Jaiswal 1996; Kensler, Wakabayashi et al. 2007; Hayes and McMahon 2009).

Although many studies have reported that small molecules and stressors regulate NRF2 stability and function, relatively little is known about regulation of KEAP1 and NRF2 through protein-protein interactions. Here, we report that WTX directly binds to KEAP1. Using gain-of-function and loss-of-function approaches, we found that WTX stabilizes NRF2 and positively regulates its transcriptional activity. We identified an ETGE motif within the KEAP1 interacting domain of WTX that is nearly identical to that of NRF2. We show that this motif is not only required for the interaction of WTX with KEAP1, but is also required for the ability of WTX to regulate NRF2 stability and activity. Our observations support a model whereby WTX competes with NRF2 for binding to KEAP1, thereby promoting the NRF2-mediated antioxidant response.

EXPERIMENTAL PROCEDURES

Tissue culture, transfections, and small interfering RNAs

All cell lines were grown in DMEM supplemented with 10% fetal bovine serum in a 37°C humidified incubator with 5% CO2. Selection and passage of stable cell lines was performed with 1.5 μg/ml puromycin until cell death was no long apparent. Expression constructs were transiently transfected in HEK293T cells with Lipofectamine 2000 as directed by the manufacturer (Invitrogen, Carlsbad, CA). Transient transfection of siRNA was performed with Lipofectamine RNAiMAX, as directed by the manufacturer (Invitrogen). Sequences of the siRNA sense strands are as follows: WTX-A

(CCU GGA GAU GAC UGC CUU U dTdT), WTX-B (UAU GCC AGG GAG GCC CAC A dTdT), NRF2 (GUA AGA AGC CAG AUG UUA A dTdT), and KEAP1 (GGG CGU GGC UGU CCU CAA U dTdT). Control siRNA was acquired from Ambion.

Plasmids and Expression Vectors

NRF2, WTX, KEAP1, CUL3 and GFP cDNAS were created with standard PCR-based cloning strategies. The reporter gene fusion construct for human NQO1-ARE (hNQO1-ARE-luciferase) was a kind gift from Jeffrey Johnson.

WTX antibody production

Amino acids 212-438 of WTX were expressed as a GST-fusion protein in *Escherichia coli*. Purified GST-tagged WTX fragment was used as an immunogen in rabbits, following established protocols by Cocalico Biologicals, Inc. Prior to use in Western blot analysis or immunostaining, the antibody was affinity purified over protein/G beads.

Affinity pull-downs and Western blotting

For streptavidin affinity purification, cells were lysed in radioimmunoprecipitation 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) containing 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 with lysis buffer and eluting in NuPAGE loading buffer

(Invitrogen). Detection of proteins by Western blot was performed using the following antibodies: anti-NRF2 (H-300) polyclonal (sc-13032, Santa Cruz Biotechnology), anti-KEAP1 polyclonal (10503-2-AP, Proteintech), anti-HMOX1 monoclonal (ab13248; Abcam), anti-FLAG M2 monoclonal (Sigma, St Louis, MO), anti-HA polyclonal (1867423; Roche), anti-CTNNB1 polyclonal (9562; Cell Signaling Technology), anti-GFP polyclonal (ab290, Abcam), and anti-TUBB1 monoclonal (T7816; Sigma).

In vitro binding experiments

Human GST-VSV-WTX was purified from *Escherichia coli* and mixed with purified CUL3 or KEAP1 in buffered 150 mM NaCl. Following incubation for 30 min at 4°C, complexes were washed with 5 bed-volumes of 350 mM buffered NaCl before elution and Western blot.

ARE-luciferase quantification

DNA: HEK293T cells were transfected with expression constructs, ARE-luciferase (firefly), and a control plasmid containing *Renilla* luciferase driven by a constitutive cytomegalovirus (CMV) promoter for normalization. Approximately six h post-transfection, cells were treated with 100 μM tBHQ and incubated for an additional 12-16 h. Activation was measured as the ratio of firefly to *Renilla* luciferase activity. siRNA: HEK293T cells stably expressing the ARE-luciferase and *Renilla* control reporters were transfected with siRNA. Approximately 48 h later, 100 μM tBHQ was added and cells were incubated for an additional 16 h. Activation was then measured as described above.

NQO1-GFP quantification

H1299 cells containing an YFP fragment retrovirally inserted into intron 1 of the *NQO1* gene (170407PL1A2-NQO1) were a gift from Uri Alon and the Kahn Protein Dynamics group (www.dynamicproteomics.net). Ten thousand cells were seeded into each well of a 96-well clear bottom white plate. Following an 18 h incubation period, cells were transfected with siRNA. After an additional 24 h, 100 μM tBHQ was added and cells were incubated for an additional 24 h. Cells were lysed in RIPA buffer and fluorescence at λ_{ex}= 485 nm was determined.

Cell Viability

HEK293T cells were transfected with siRNA as described above in 6-well tissue-culture plates. Twenty h post-transfection, 30,000 cells were seeded into each well of a 96-well plate. Approximately eight h later, cells were treated with either DMSO (vehicle) or Etoposide. The MTT Cell Proliferation assay was performed 36 h post-treatment as described by the manufacturer (ATCC, Catalog No. 30-1010K-A).

Immunohistochemistry

Formalin fixed, paraffin embedded sections were deparaffinized and rehydrated with standard techniques. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 3 minutes followed by antigen retrieval (NRF2: 50mM Tris, pH 9 for 3 minutes in a pressure cooker, WTX: 10mM Tris, pH 9 for 3 minutes in a pressure cooker, WT1 and KEAP1: 50mM Citrate, pH 6 in a vegetable steamer). After blocking, sections were

incubated with anti-NRF2 polyclonal (sc-722, Santa Cruz Biotechnology, 1:50, 2 hours), anti-WTX polyclonal (1:50, 2 hours), anti-WT1 monoclonal (6F-H2, Dako, 1:100, O/N), or KEAP1 (1:50, O/N). Detection was performed with the Dako Envision + Staining kit (DAKO).

RNA isolation, reverse transcription, and semi-quantitative real-time PCR

Total RNA from cells was harvested in Trizol (Invitrogen) reagent according to the manufacturer's instructions. RNA was quantified by UV spectrophotometry, and cDNA was created using the RevertAid First Strand cDNA Synthesis Kit (Fermentas). PCR was performed in duplicate with the LightCycler FastStart DNA SyBr Green kit (Roche) using the Roche LightCycler 480 instrument (Roche). The PCR conditions are as follows: 35 cycles of amplification with 1 second denaturation at 95 ^{0}C, and five second annealing at 58 ^{0}C. A template free negative control was included in each experiment. Quantitative light cycler PCR primers used are as follows: *WTX* (GAC CCA AAA GGA TGA AGC T; and reverse CCC CTC CAA AGA AAC TAG GC) and *β-actin* (AGA GCA AGA GAG GCA TCC TC; and reverse CTC AAA CAT GAT CTG GGT CA).

Cellular extract degradation

HEK293T cells were transfected with either *WTX*-A or Control siRNA. Forty-eight h post-transfection, cells were resuspended in swelling buffer (20 mM HEPES pH 7.7, 5 mM $MgCl_2$, 5 mM KCl, 1 mM DTT, 0.2 mM ATP, and protease inhibitor) at a ratio of 4:3. Resuspended pellets were subjected to two rounds of freeze/thaw and passed

through a chilled 27 gauge needle two times. Lysates were then centrifuged at 2300 x *g* for five min, and supernatant was transferred to a new tube and centrifuged for 30 min at 16000 x *g*. The middle layer containing the cytosolic fraction was isolated and used in subsequent NRF2 degradation experiments. *in vitro* transcription and translation of ^{35}S-labeled NRF2 was performed using the coupled transcription-translation T7 system (Promega). Reactions consisted of 0.25 μL of ^{35}S-labeled NRF2, 15 μL of cell extract, and one uL NRG cocktail containing a 1:1:1 ratio of energy mix (150 mM creatine phosphate, 20 mM ATP, 2 mM EGTA, and 20 mM $MgCl_2$), cycloheximide (0.1 μg/mL), and 0.1 μg/mL recombinant ubiquitin. One μL of each reaction was taken at 0, 30, 120, and 240 min and snap frozen in loading buffer. Samples were then run on a 3-12% gradient gel (Invitrogen), dried onto filter paper, and exposed to a phosphor screen overnight. Bands were quantified and percent of NRF2 remaining was determined as the ratio of each time point compared to the zero time point.

In vitro ubiquitination

In vitro transcription/translation of CUL3, NRF2, KEAP1, WTX, and GFP was performed with the TNT® Quick Coupled Transcription/Translation System (Promega). Five μL of each product was incubated in various combinations with recombinant ubiquitin (1.25 μg), UBE1 (12.5 μg), and UBCH5B (20 μg) in the presence of 2 mM ATP, 5 mM $MgCl_2$. The mixtures were incubated at room temperature for 45 min. NRF2 was immunoprecipitated and complexes were analyzed by western blot for Ubiquitin.

Immobilized metal affinity chromatography and mass spectrometry

Affinity purification of GLUE-WTX and FLAG-KEAP1 was performed as previously described (Major, Camp et al. 2007). The precipitated proteins were trypsinized directly off beads following reduction with 5mM DTT and alkylation with 15 mM chloroacetamide. Following "stage-tip" desalting (Rappsilber, Mann et al. 2007), peptides were re-suspended in binding buffer (250 mM acetic acid and 30% acetonitrile) and incubated for 30 min with immobilized metal affinity chromatography (IMAC) gel (#I1408, Sigma-Aldrich). The phosphorylated peptides were washed three times with binding buffer, eluted with 200 mM ammonium phosphate buffer, and processed for mass spectrometry. Raw mass spectrometry data was searched with SEQUEST (Thermofisher) and phosphorylation was queried via specification of a differential modification of 79.6 AMU on Serine, Threonine or Tyrosine. Proteins were scored using the Institute for Systems Biology's trans-proteomic pipeline (Nesvizhskii, Keller et al. 2003), and phosphosite accuracy was evaluated using Ascore (Beausoleil, Villen et al. 2006).

AlphaScreen

Recombinant GST-NRF2 and 6xHIS-hKELCH were purified using standard techniques. Seventy ng of AlphaScreen GSH-Donor and AlphaLISA Nickel-Acceptor beads (Perkin Elmer) were mixed with 100 nM 6xHIS-hKELCH, 20 nM GST-NRF2, and respective peptides in binding buffer containing 20 mM TRIS pH 8, 200 mM NaCl, 1 mM DTT, and 0.05% Tween-20. Reactions were incubated at room temperature for 30 min and AlphaScreen Signal was determined with an Envision plate-reader (Perkin

Elmer). Peptide sequences: NRF2 16mer- AFFAQLQLDEETGEFL, WTX 16mer- ASSLEEPHSPETGEKV, β-catenin 26mer- KAAVSHWQQQSYLDSGIHSGATTTAP.

Statistical Analysis

Student's *t* test was used to assess the statistical significance of the differences between the different groups; a *P* value of <0.05 was considered significant.

RESULTS

WTX directly interacts with KEAP1

The *WTX* gene encodes an 1135 amino acid protein (WTX-WT) characterized by multiple protein-protein interaction domains and an amino-terminal phosphatidylinositol (4,5)-bisphosphate binding domain that mediates its localization to the plasma membrane (Grohmann, Tanneberger et al. 2007). Through in-frame alternative splicing, a shorter isoform (WTX-S) of 858 amino acids is produced that lacks residues 50-326 and does not localize to the plasma membrane (Rivera, Kim et al. 2009). Here, we employ an 804 amino acid variant of WTX, previously reported by us and by Grohmann et al. (2007) that shares perfect identity with WTX-WT through residue 785. This isoform contains the amino-terminal phosphatidylinositol (4,5)-bisphosphate binding domain, as well as the binding domains for KEAP1, β-catenin, APC, AXIN1, BTRC and FBXW11.

We previously observed that KEAP1 co-purifies with WTX protein complexes (Major, Camp et al. 2007) as determined by mass spectrometry-based proteomics. To validate this interaction we engineered HEK293T cells to express a fusion protein

containing an amino-terminal streptavidin binding protein (SBP), calmodulin binding protein (CBP), the hemagglutinin epitope (HA) and WTX (GLUE-WTX). Affinity purification of GLUE-WTX and Western blotting confirmed that endogenous KEAP1 forms a complex with GLUE-WTX **(Figure 10A, compare lane 10 to control lane 7)**. This interaction was independent of the activity of either the KEAP1/NRF2 or the Wnt/β-catenin signaling pathways as neither the KEAP1 antagonist tBHQ (*tert*-butylhydroquinone) nor WNT3A conditioned media affected the interaction of WTX with KEAP1 **(Figure 10A, compare lanes 10, 11, and 12)**.

To determine whether WTX directly interacts with KEAP1, we tested whether recombinant GST-WTX was able to pull-down recombinant KEAP1 *in vitro*. Using this method we determined that WTX directly interacts with KEAP1 but not with the KEAP1-associated CUL3 protein **(Figure 10B, compare lanes 4 and 5)**. Together with our previous work (Major, Camp et al. 2007), these data demonstrate that WTX directly binds the substrate recognition modules of two different E3 ubiquitin ligases, namely BTRC and KEAP1.

KEAP1 has three well-defined domains, an N-terminal BTB domain that binds CUL3 (Cullinan, Gordan et al. 2004), an intervening region (IVR), and the C-terminal KELCH repeats that form a β-propeller fold that binds NRF2 (Padmanabhan, Tong et al. 2006). To determine the domain in KEAP1 that binds WTX, HEK293T cells expressing GLUE-WTX were transfected with FLAG-KEAP1, FLAG-BTB or FLAG-KELCH constructs. Affinity purification of GLUE-WTX revealed an association with full length

KEAP1 and the KELCH repeats, but not with the KEAP1 BTB domain **(Figure 10C, compare lanes 6, 7, and 8).** Taken together, our data suggest that WTX directly associates with the KELCH repeats of KEAP1. Despite its association with the substrate-recognition domain of KEAP1, the steady-state levels of WTX are not regulated by KEAP1 or proteasome-mediated degradation (**Figure 11**). Thus we conclude that WTX is not a substrate of KEAP1.

WTX promotes NRF2-dependent transcription

Based on the physical interaction between WTX and KEAP1, we next tested if WTX regulates NRF2 activity. First, we employed a luciferase reporter containing the Antioxidant Response Element (ARE) of *NQO1*, a NRF2 target gene (Lee, Hanson et al. 2001). Two non-overlapping *WTX*-specific siRNAs decreased both basal and tBHQ-induced activation of this ARE-luciferase reporter in HEK293T cells, similar to the effect of depleting *NRF2* with siRNA **(Figure 12A and B)**. Conversely, over-expression of WTX activated the luciferase reporter compared to control GFP **(Figure 12C)**. Second, because the engineered luciferase reporter may not accurately represent endogenous transcription, we tested whether WTX regulates the expression of endogenous *NQO1*. We utilized the H1299 non-small cell lung cancer (NSCLC) cell line containing an YFP fragment retrovirally inserted into intron 1 of the endogenous *NQO1* genomic locus resulting in an NQO1-YFP product (Cohen, Geva-Zatorsky et al. 2008). Induction of YFP in the presence of tBHQ was significantly lower in cells transfected with *WTX* siRNA compared to control (**Figure 12D**). Taken together, these observations suggest

that WTX is a positive regulator of NRF2-dependent transcription, unlike its role as a negative regulator of β-catenin-dependent transcription (Major, Camp et al. 2007).

KEAP1 and NRF2 are known to play opposing roles in the cellular response to oxidative stress and chemotherapeutics, most notably in NSCLC where constitutive activation of NRF2 promotes resistance to chemotherapeutics (Singh, Misra et al. 2006). In order to determine if WTX plays a similar role, we asked if loss of *WTX* sensitizes cells to the chemotherapeutic reagent etoposide. Compared to control, cells transfected with siRNA targeting *WTX* were more sensitive to cell death in the presence of etoposide as determined by mitochondrial activity **(Figure 12E)**. We conclude from these experiments that WTX and NRF2 promote resistance to apoptotic inducing reagents.

WTX regulates the steady-state levels of NRF2 by inhibiting its ubiquitination

As WTX regulates the stability of β-catenin (Major, Camp et al. 2007), we hypothesized that WTX also regulates the stability of NRF2. To test this, we transfected HEK293T cells with control, *NRF2* or *WTX* siRNAs and monitored the levels of NRF2 and its target gene HMOX1 following treatment with tBHQ. siRNA-mediated silencing of *WTX* in HEK293T cells resulted in lower NRF2 and HMOX1 protein levels compared to control **(Figure 13A, compare lanes 3 and 4 to control lane 1)**. Similar results were also observed in cell lines derived from normal lung (BEAS2B) and lung carcinoma (A549) (**Figure 13B, compare lane 3 to control lane 1 and lane 6 to control lane 4**). Of note, whereas HEK293T and BEAS2B cells have wild-type KEAP1, A549 cells harbor a G333C mutation in the KELCH repeats of KEAP1 that partially impairs the

ubiquitination of NRF2, resulting in relatively high basal levels of NRF2. As a complement to the siRNA-based loss-of-function approach, we tested whether over-expression of WTX results in increased levels of NRF2 protein. In HEK293T cells transiently transfected with a VENUS-WTX expression construct, we observed increased levels of endogenous NRF2 **(Figure 13C, compare lane 4 with control lane 2, and lane 5 with control lane 3)**. Combined, these results suggest that WTX regulates the steady-state levels of NRF2.

We next asked if WTX regulates the steady-state levels of NRF2 by inhibiting the degradation of NRF2 protein. In order to test this we measured the degradation of NRF2 in cell-free extracts generated from HEK293T cells. ^{35}S-labeled NRF2 was less stable in extracts purified from cells transfected with *WTX* siRNA compared to control extracts **(Figure 13D)**. Conversely, when extracts from HEK293T cells were supplemented with recombinant WTX, levels of ^{35}S-labeled NRF2 were relatively stable compared to control, suggesting that WTX inhibits the degradation of NRF2 **(Figure 13E)**. Of note, whereas we observed that the half-life of exogenous NRF2 in cell extracts is approximately six hours, previous reports have demonstrated that exogenous and endogenous NRF2 has a half-life of approximately 20 minutes in intact cells (Nguyen, Sherratt et al. 2003; Kobayashi, Kang et al. 2004). We also observed that NRF2 has a half-life of minutes in intact cells (**Figure 10B**), and attribute the differences in extracts to differences in experimental systems.

As WTX directly binds to KEAP1 and inhibits the degradation of NRF2, we hypothesized that WTX inhibits the ubiquitination of NRF2 by KEAP1. To test this hypothesis, we utilized an *in vitro* assay to monitor the ubiquitination of NRF2. *In vitro* transcribed/translated CUL3, KEAP1, and NRF2 were combined with either mock, GFP, or WTX and incubated with ubiquitination reagents. NRF2 was then immunoprecipitated and ubiquitination was monitored by Western blot. Compared to control, WTX inhibited the ubiquitination of NRF2 **(Figure 13F, compare lane 3 to control lane 1)**. Taken together, these results indicate that WTX stabilizes NRF2 by inhibiting KEAP1-dependent ubiquitination.

WTX and NRF2 localize to similar cell-types in the developing human kidney

If WTX regulates the steady-state levels of NRF2 *in vivo*, we would expect that the levels of NRF2 would be higher in tissues with relatively high levels of WTX. As WTX is expressed in the developing kidney and is mutated in Wilms tumor, we determined the expression patterns of WTX, NRF2, KEAP1 and WT1 (Wilms Tumor 1) by immunohistochemistry in 18-week old human gestational kidney. As previously reported, we detected WT1 expression in metanephric mesenchyme and comma and S-shaped bodies with increasing intensity in differentiating glomeruli/glomerular podocytes (**Figure 14**) (Rivera, Kim et al. 2007). Weak expression of WTX was detected in mesenchyme and comma and S-shaped bodies with increased expression in the differentiating tubules and diminished expression in differentiating glomeruli. NRF2 exhibited a pattern of expression similar to WTX, while KEAP1 expression was present in differentiating tubules and largely absent in mesenchyme or glomeruli. These data

demonstrate similar localizations of WTX, NRF2 and WT1 in condensing mesenchyme, and subsequent divergence with nephron patterning and cell specification. Moreover, the similar expression patterns of NRF2 and WTX provide *in vivo* support for our cell-based discoveries.

WTX interacts with KEAP1 through an ETGE motif

KEAP1 and members of the BTRC family (BTRC and FBXW11) are substrate-adaptor proteins that bind to CUL3 and CUL1-based E3 ubiquitin ligase complexes, respectively (Winston, Strack et al. 1999; Zhang, Lo et al. 2004). In order to gain insight into the mechanism(s) by which WTX regulates these complexes, we wanted to define the domain(s) within WTX that bind KEAP1 and the BTRC family (BTRC and FBXW11). Numerous fragments of the WTX protein were expressed as VENUS-fusions in HEK293T cells engineered to express GLUE-KEAP1 or GLUE-FBXW11. Interestingly, affinity purification and Western blot revealed that WTX binds KEAP1 and FBXW11 through separable domains. Specifically, amino acids 212-367 of WTX were required to bind KEAP1 and amino acids 439-624 were required to bind FBXW11 **(Figure 15A and B, respectively. Results are summarized in Figure 13C)**.

Within the KEAP1 binding domain of WTX, our attention was drawn to amino acids 286-291, containing the sequence SPETGE (**Figure 15C**). KEAP1 directly interacts with a conserved DxETGE motif within NRF2 (Kobayashi, Itoh et al. 2002), raising the possibility that WTX binds KEAP1 through its ETGE motif. To test this, we expressed a WTX construct lacking amino acids 288-291 (VENUS-WTX ΔETGE) in

GLUE-KEAP1 cells. Western blot analysis of KEAP1 affinity purified complexes revealed substantially less WTX ΔETGE compared to wild-type WTX **(Figure 15D, compare lanes 2 and 3)**. Threonine 80 within the ETGE motif of NRF2 makes critical contacts with KEAP1 and is required for interacting with KEAP1 (Lo, Li et al. 2006). Similarly, we observed that mutation of Threonine 289 to Alanine in the ETGE motif of WTX disrupts the interaction with KEAP1 **(Figure 15D, compare lanes 2 and 4)**. Interestingly, Lysine 292, adjacent to the ETGE motif, is mutated to Asparagine in several reported Wilms tumor cases and a single instance of Acute Myeloid Leukemia (AML) (Rivera, Kim et al. 2007; Owen, Virappane et al. 2008; Ruteshouser, Robinson et al. 2008). Compared to wild-type WTX, KEAP1 pulled down less WTX K292N, although more than WTX ΔETGE **(Figure 15D, compare lanes 2, 3, and 5)**. Combined, these results suggest that WTX binds to KEAP1 through a similar motif as NRF2.

One notable difference between the KEAP1 interaction domains of NRF2 and WTX is the two amino acids upstream of their respective ETGE motifs. The human NRF2 motif contains a conserved Aspartic Acid that interacts with two water molecules that in turn hydrogen bond with two Arginine residues in KEAP1 (Lo, Li et al. 2006). In place of the Aspartic Acid, WTX contains a Serine (residue 286). Interestingly, mutation of Serine 286 to Alanine inhibited the ability of KEAP1 to pull down WTX **(Figure 15D, compare lanes 2 and 6)**. Based on the crystal structure of the KELCH repeats and an NRF2 peptide (Lo, Li et al. 2006; Padmanabhan, Tong et al. 2006), we reasoned that mutation of Serine 286 to an acidic residue would enhance the interaction between WTX and KEAP1. Although we only observed a modest increase in the interaction between

KEAP1 and WTX S286E, WTX S286D was significantly enriched relative to wild-type WTX in KEAP1 pull-downs **(Figure 15D, compare lanes 2, 7, and 8)**. We conclude from these experiments that in addition to the ETGE motif, Serine 286 is also important in the KEAP1 interacting domain of WTX.

Serine 286 is phosphorylated in vivo

Given that mutation of Serine 286 to Alanine and Aspartic Acid differentially affected the ability of KEAP1 to pull down WTX, we hypothesized that phosphorylation of WTX could affect its interaction with KEAP1. To directly test whether WTX is phosphorylated *in vivo,* we trypsinized GLUE-WTX complexes from HEK293T cells and enriched for phosphorylated peptide fragments by IMAC (Immobilized Metal Affinity Chromatography). Peptides were then analyzed for the presence of phosphorylation by mass spectrometry. We identified sixteen unique phosphorylation sites in sixteen unique mono- or di-phosphorylated peptides (**Table II**). Of the sixteen unique peptides, two were phosphorylated at Serine 286, including one with a second phosphorylated site at Serine 280. The phosphorylation at Serine 286 is considered definitive by the Ascore phosphorylation site algorithm, where any score above nineteen has a greater than ninety-nine percent chance of certainty (Beausoleil, Villen et al. 2006).

Finally, we wanted to determine if endogenous WTX is phosphorylated on Serine 286 when associated with KEAP1. IMAC enriched phospho-peptides from FLAG-KEAP1 affinity-purified protein complexes were identified by mass spectrometry. Within FLAG-KEAP1 complexes, we identified six phosphorylation sites on endogenous WTX,

including Serine 286 (**Table III**). Although these data do not rule out the possibility that unphosphorylated WTX interacts with KEAP1, they confirm that endogenous WTX is phosphorylated at Serine 286 and interacts with KEAP1.

WTX competes with NRF2 for binding to KEAP1

Our observation that WTX interacts with KEAP1 through a similar motif as NRF2 raises the possibility that WTX and NRF2 compete for the same binding site in KEAP1. In order to directly test this, we utilized an AlphaScreen approach to ask if a WTX peptide containing the SPETGE sequence could inhibit the interaction between NRF2 and KEAP1. We observed a strong interaction between GST-NRF2 and 6xHIS-hKELCH (KELCH repeats of KEAP1 that directly interact with NRF2) as indicated by a robust AlphaScreen signal and the specificity of the interaction was validated as a peptide containing the DxETGE motif of NRF2 inhibited the signal in a dose-dependent manner (**Figure 16A**). Whereas a control β-catenin peptide had no effect, a peptide containing the SPETGE motif of WTX (WTX 16mer) inhibited the interaction between NRF2 and the KELCH repeats of KEAP1 in a dose-dependent manner (**Figure 16A**). These results suggest that WTX and NRF2 compete for binding to KEAP1.

If WTX regulates NRF2 steady-state levels through direct competition with KEAP1, we would expect that mutations in WTX that inhibit its interaction with KEAP1 should impair its ability to regulate the steady-state levels of NRF2. In order to test this, we assessed the ability of VENUS-WTX ΔETGE to stabilize NRF2. Whereas over-expression of wild-type WTX robustly stabilized NRF2 in a dose-dependent manner,

WTX ΔETGE only modestly stabilized NRF2 at significantly higher levels of expression **(Figure 16B, compare lane 4 to lanes 6 and 7)**. In addition to regulating the steady-state levels of NRF2, the interaction of WTX with KEAP1 should also regulate NRF2-dependent transcription. To test this, we examined whether the various WTX mutants could regulate NRF2-dependent transcription in the presence of exogenous KEAP1. Over-expression of FLAG-NRF2 activated an ARE-luciferase reporter and was partially inhibited by over-expression of KEAP1. Concomitant expression of the various WTX mutants rescued FLAG-NRF2 activity correlating with the ability of the WTX mutants to bind KEAP1 **(Figure 16C)**. We conclude from these experiments that the ability of WTX to regulate the steady-state levels of NRF2 and NRF2-dependent transcription directly correlates with its ability to bind KEAP1. Taken together, our data suggest that WTX regulates NRF2 steady-state levels and NRF2-dependent transcription by competing for binding to KEAP1.

DISCUSSION

Here we expand on the knowledge of the molecular mechanisms of action of WTX with the observation that WTX regulates the steady-state levels of NRF2 and NRF2-dependent transcription by competing with NRF2 for binding to KEAP1. KEAP1 forms a homodimer and interacts with CUL3 through its BTB domain (Zipper and Mulcahy 2002; Ogura, Tong et al. 2010) and interacts with the N-terminal Neh2 domain of a single NRF2 molecule through its KELCH repeats. The Neh2 domain of NRF2 contains two highly conserved regions, one bearing an LxxQDxDLG (DLG) motif (McMahon, Thomas et al. 2004; Katoh, Iida et al. 2005), and the other bearing a

DxETGE motif (Kobayashi, Itoh et al. 2002). These motifs interact with a separate KEAP1 molecule in the KEAP1 homodimer, although the DxETGE motif binds with approximately 100-fold higher affinity (Tong, Katoh et al. 2006). WTX contains a similar SPETGE motif that can directly inhibit the interaction between NRF2 and the KELCH repeats of KEAP1 *in vitro*. As WTX does not contain a DLG motif similar to NRF2, it is possible that NRF2 can still interact with a KEAP1 dimer through this low affinity interaction motif. We hypothesize that WTX enhances NRF2 steady-state levels by disrupting the conformation of the E3 ubiquitin ligase complex, resulting in lower ubiquitination of NRF2 (**Figure 17**). In support of our findings, expression of the NRF2 target genes NQO1 and HMOX1 was significantly enhanced in HEK293T cells over-expressing a fragment of WTX containing the KEAP1 interaction domain (Kim, Min et al. 2011).

In addition to the ETGE motif, we determined that Serine 286 is also required for the ability of WTX to interact with KEAP1 and regulate NRF2. Interestingly, mutation of Serine 286 to either Glutamic or Aspartic Acid enhanced the functional effects of WTX. Although these mutations may only present conformational changes in WTX that make it more suitable for binding to KEAP1, both Glutamic and Aspartic Acid resemble phosphorylated Serine. Thus, phosphorylation of WTX at Serine 286 may increase its affinity for KEAP1. Furthermore, we found that WTX is phosphorylated *in vivo*, and KEAP1 interacts with endogenous WTX that is phosphorylated at Serine 286, raising the intriguing possibility that the interaction between WTX and KEAP1 is regulated by a yet to be identified kinase(s).

Under normal conditions, NRF2 is constitutively ubiquitinated through its association with KEAP1 (Cullinan, Gordan et al. 2004; Kobayashi, Kang et al. 2004; Zhang, Lo et al. 2004). In the presence of oxidative stress, Cysteine residues in KEAP1 are modified resulting in a conformational change that disrupts the ubiquitination of NRF2 and NRF2 accumulates in the nucleus where it regulates gene transcription (Zhang, Lo et al. 2004; Yamamoto, Suzuki et al. 2008). In addition to our findings, several recent studies have also revealed that NRF2 signaling is regulated through protein-protein interactions. The cyclin-dependent kinase inhibitor p21, a p53-regulated gene with pro-survival properties, was recently shown to bind the DLG motif of NRF2 and inhibit its interaction with KEAP1, resulting in elevated NRF2 levels under both basal and chemically induced conditions (Chen, Sun et al. 2009). Another recent study identified p62 as a novel interactor of KEAP1 (Komatsu, Kurokawa et al. 2010). During autophagy, p62 directs ubiquitinated proteins to degradation by the lysosome. By binding to KEAP1 through an ETGE-like motif similar to that of NRF2 and WTX, p62 targets KEAP1 for autophagic degradation, thus contributing to the stabilization of NRF2. Combined with WTX, these examples highlight the complex regulation of NRF2 degradation and its importance in both homeostasis and response to cytotoxic stress.

The observation that WTX regulates the NRF2-mediated antioxidant response supports further study of its role in disease beyond Wilms tumor and OSCS. One of the best-studied diseases with altered KEAP1/NRF2 activity is NSCLC, where mutations in KEAP1 lead to constitutive NRF2-mediated transcription. Consequently, cultured lung

cancer cell lines with constitutive NRF2 activity are resistant to cell death induced by etoposide (Singh, Misra et al. 2006). Similarly, silencing *NRF2* restores resistance to chemotherapeutics in KEAP1 mutant cells (Homma, Ishii et al. 2009). We find that silencing *WTX* sensitizes HEK293T cells to death induced by etoposide, similar to loss of *NRF2*. Interestingly, while *WTX* expression is diminished in adult mouse brain and kidney compared to embryonic tissues, expression levels remain high in the lung, suggesting it may contribute to lung cell homeostasis in the adult (Rivera, Kim et al. 2007). Coupled with the established roles of WTX in Wilms tumor and KEAP1/NRF2 in lung cancer, our data supports future investigations into a functional role for WTX in lung cancer and other disease associated with aberrant NRF2 activity.

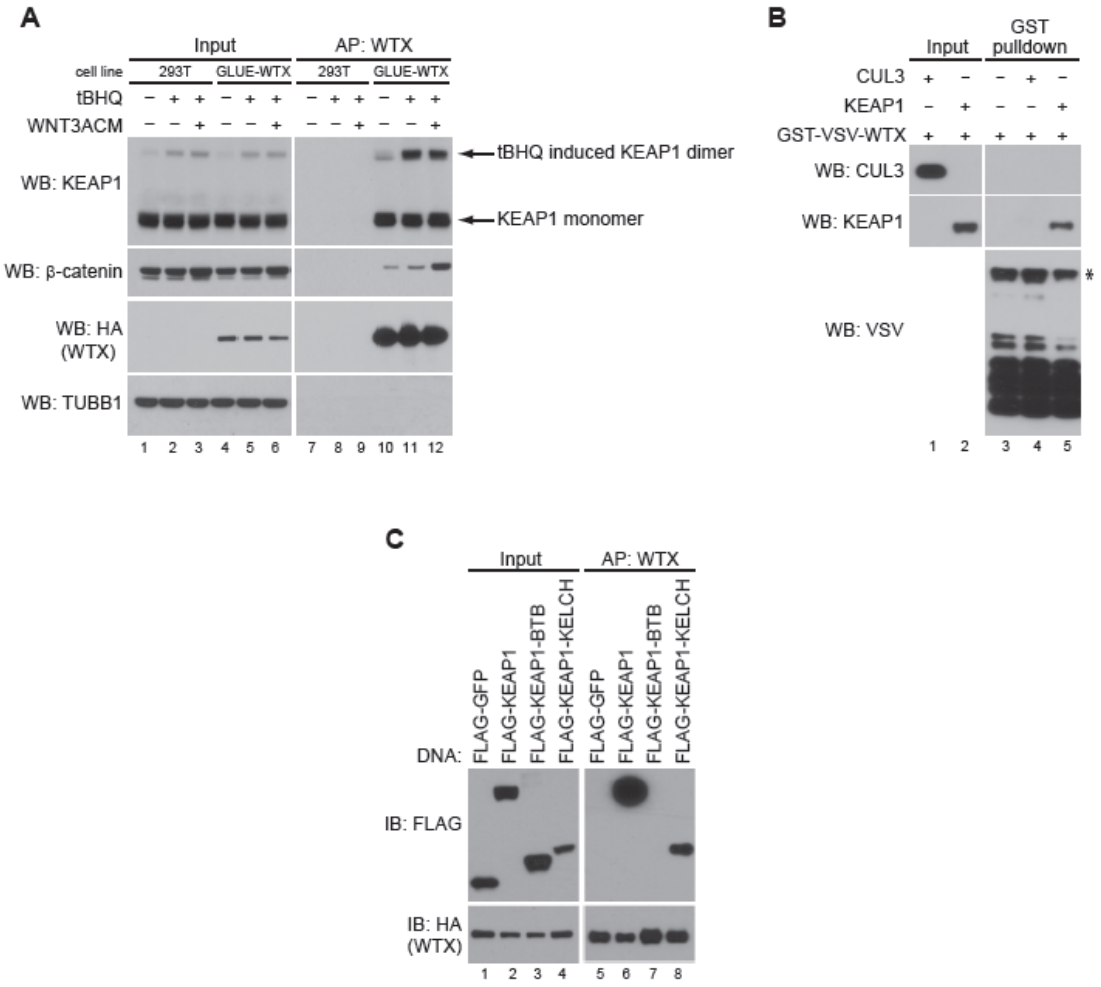

Figure 10. WTX directly interacts with KEAP1. (A) GLUE-WTX interacts with endogenous KEAP1. HEK293T parental or HEK293T cells stably expressing GLUE-WTX were treated with DMSO, 100 μM tBHQ, or 100 μM tBHQ and WNT3A conditioned media (WNT3ACM) for 2 hours. Lysates were subjected to streptavidin affinity pull-down (AP) followed by Western blot analysis. **(B)** WTX directly binds KEAP1. Recombinant GST-VSV-WTX was incubated with recombinant CUL3 or KEAP1. After GST affinity purification, complexes were washed and associated proteins were resolved by Western blot. * denotes full length GST-VSV-WTX. **(C)** WTX interacts with the KELCH repeats of KEAP1. HEK293T cells stably expressing GLUE-WTX were transfected with FLAG-tagged KEAP1 constructs. Lysates were subjected to streptavidin affinity pull-down followed by Western blot analysis.

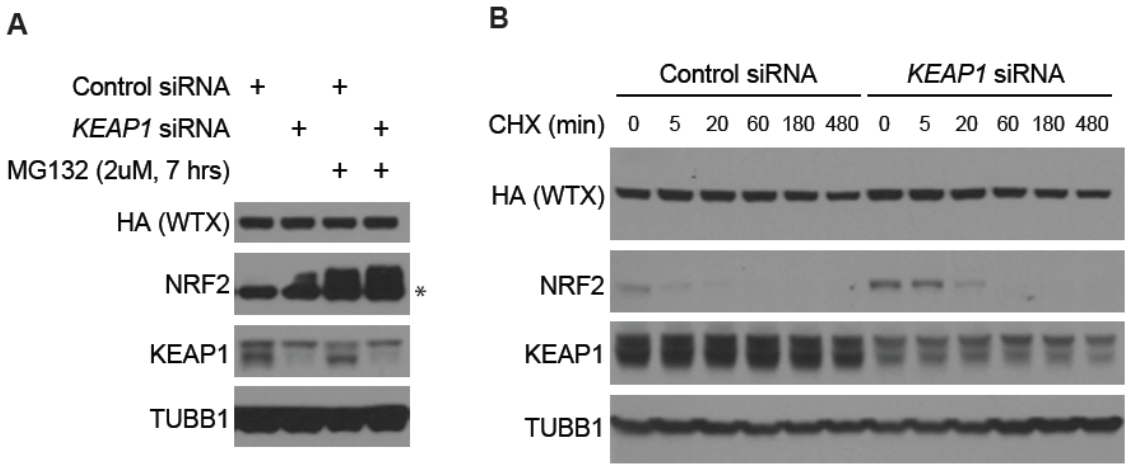

Figure 11. The steady-state levels of WTX are not regulated by KEAP1. (A) HEK293T cells stably expressing GLUE-WTX were transfected with either control siRNA or siRNA targeting *KEAP1*. Approximately 48 hours post transfection, cells were treated with MG132 for 7 hours. Cells were lysed in RIPA buffer and lysates were analyzed by Western blot. * indicates a non-specific band. **(B)** HEK293T cells stably expressing GLUE-WTX were transfected with either control siRNA or siRNA targeting *KEAP1*. Approximately 48 hours post transfection, cells were treated with 10 µg/mL Cycloheximide (CHX) as indicated. Cells were lysed in RIPA buffer and lysates were analyzed by Western blot.

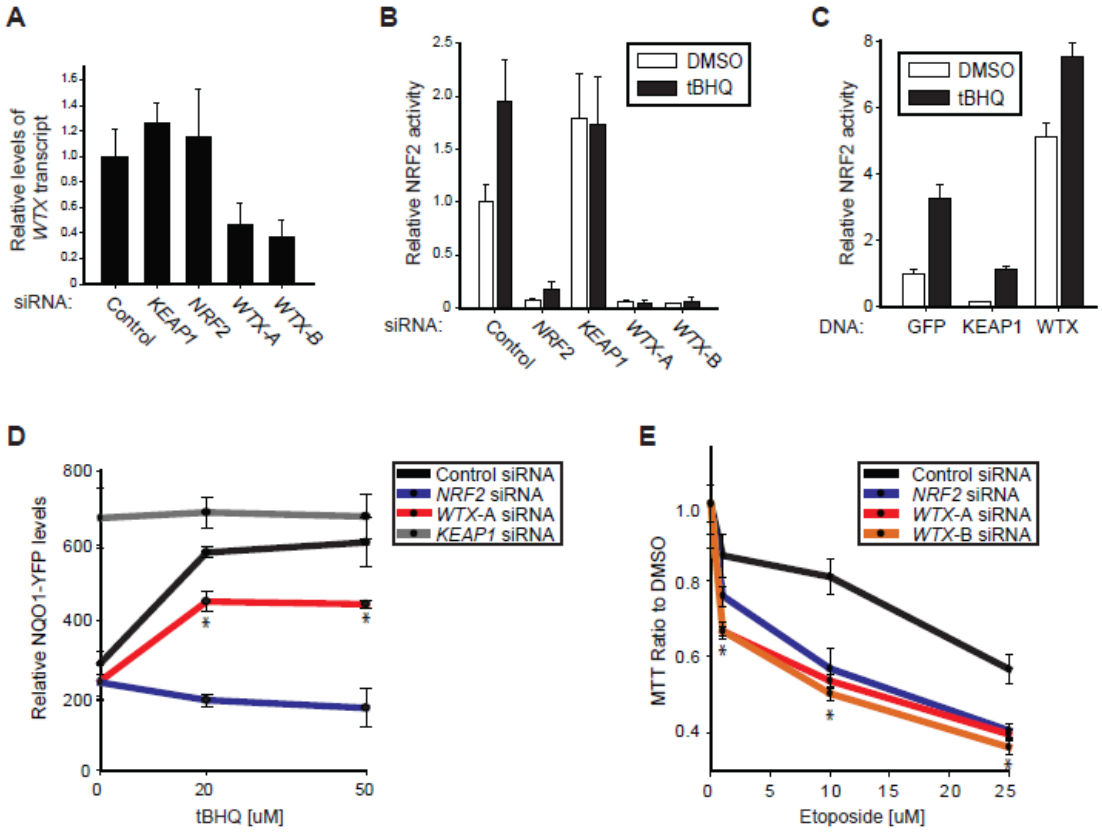

Figure 12. WTX promotes NRF2-dependent transcription. **(A)** 2 non-overlapping siRNAs reduce *WTX* transcript levels. HEK293T cells were transfected with siRNA. 48 h after transfection, total RNA was isolated and relative transcript levels were quantified as the ratio compared to *β-actin* transcripts. **(B)** *WTX* is required for activation of an ARE-luciferase reporter. HEK293T cells stably expressing an ARE-driven firefly-luciferase reporter and a control CMV-driven *Renilla* luciferase reporter were transfected with siRNAs and treated with either DMSO or tBHQ. Relative levels of ARE-driven firefly luciferase compared to control CMV-driven *Renilla* luciferase are plotted. Error bars represent standard deviation from the mean of 3 technical replicates. Data are representative of 3 biological replicates. **(C)** Overexpression of WTX activates an ARE-luciferase reporter. HEK293T cells were transfected with the ARE-firefly luciferase reporter, a control CMV *Renilla* luciferase control reporter, and the indicated FLAG-tagged DNA constructs. Cells were treated with tBHQ and luciferase levels were quantified as in B. **(D)** Loss of *WTX* inhibits transcription of endogenous *NQO1*. H1299 cells expressing an NQO1-YFP fusion protein under control of the endogenous promoter were transfected with siRNA and treated with either DMSO or tBHQ. Relative YFP fluorescence is shown. Error bars represent standard deviation from the mean of 4 technical replicates. Data are representative of 3 biological replicates. *, $P < 0.02$ when comparing *WTX* siRNA to control. **(E)** Loss of *WTX* increases the cellular sensitivity to Etoposide. HEK293T cells were transfected with siRNA and treated with Etoposide. Cell viability was determined by the MTT assay. *, $P < 0.03$ when comparing *NRF2* and *WTX* siRNAs to control

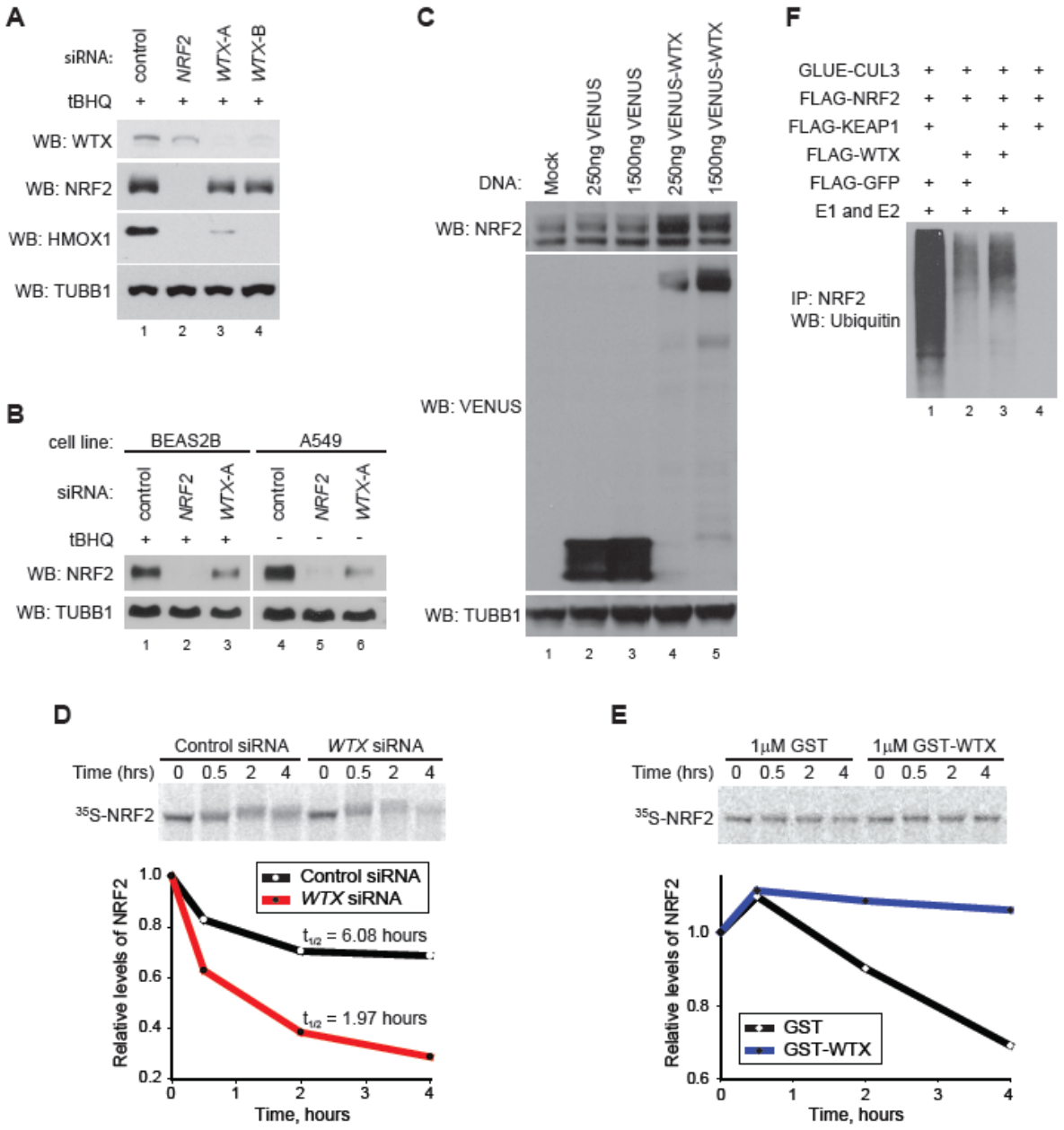

Figure 13. WTX regulates the steady-state levels of NRF2 by inhibiting its ubiquitination. (A and B) Knockdown of *WTX* inhibits stabilization of NRF2. **(A)** HEK293T cells were transfected with the indicated siRNAs and treated with tBHQ (100 μM, 4 hours) preceding lysis and Western blot analysis. **(B)** BEAS2B and A549 cell lines were transfected with the indicated siRNAs. BEAS2B cells were treated with tBHQ preceding lysis and Western blot analysis. **(C)** Overexpression of WTX stabilizes NRF2. HEK293T cells were transfected with the indicated constructs and analyzed as in A and B. **(D and E)** WTX regulates NRF2 steady-state levels. **(D)** HEK293T cells were transfected with the indicated siRNAs and cellular extracts were purified. ^{35}S-radio-labeled NRF2 was added to

cellular extracts and degradation was monitored over time by autoradiography. A representative autoradiograph and quantification from three independent experiments is shown. **(E)** ^{35}S-NRF2 and recombinant GST or GST-WTX were added to HEK293T cellular extracts. ^{35}S-NRF2 degradation was monitored and quantified as in E. **(F)** *In vitro* transcribed and translated CUL3, NRF2, KEAP1, WTX, and GFP were mixed as indicated. NRF2 was immunoprecipitated from each reaction and ubiquitination was observed by Western blot analysis.

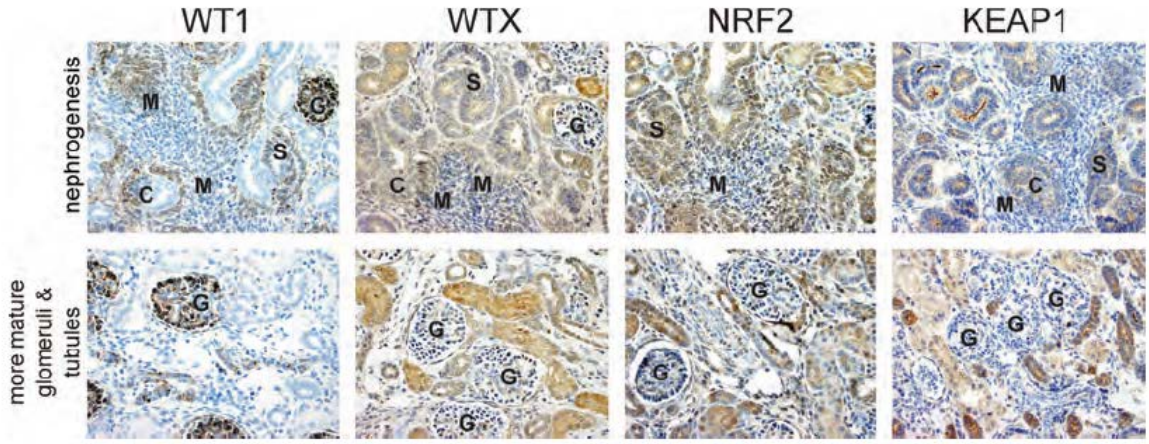

Figure 14. WTX and NRF2 localize to similar cell-types in the developing human kidney. 18-week-old human embryonic kidney sections were stained with antibodies against WT1, WTX, KEAP1, and NRF2. Images were taken at 40X magnification. M = nephrogenic mesenchyme, C = comma-shaped bodies, S = s-shaped bodies, and G = glomeruli.

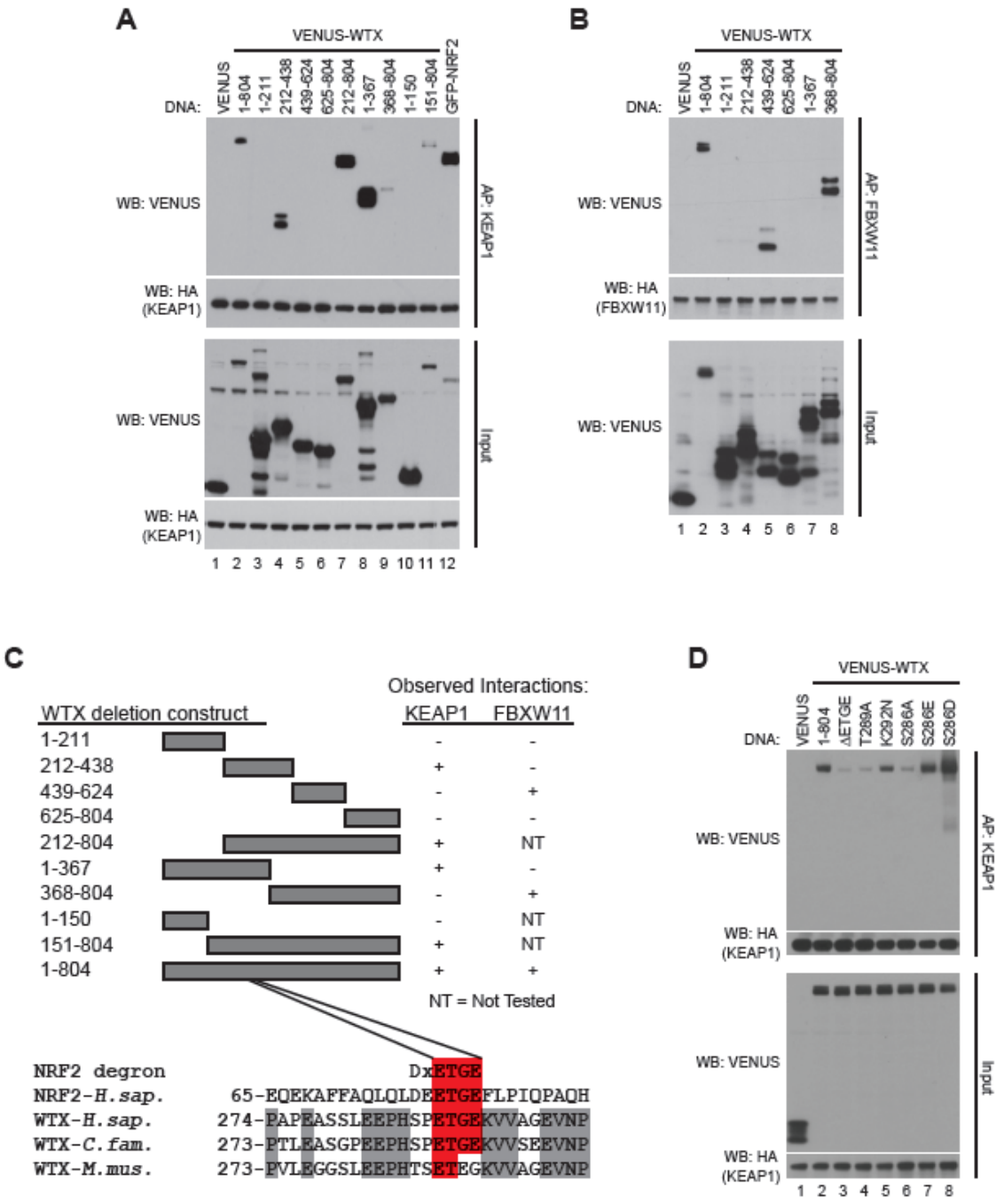

Figure 15. WTX interacts with KEAP1 through an ETGE motif. (A and B) WTX associates with KEAP1 and FBXW11 through separable domains. **(A)** HEK293T cells stably expressing GLUE-KEAP1 were transfected with the indicated VENUS-WTX deletion constructs. Lysates were subjected to streptavidin affinity pull-down (AP) followed by Western blot analysis. **(B)** HEK293T cells stably expressing GLUE-FBXW11 were transfected with the indicated VENUS-WTX deletion constructs. Lysates were analyzed as in B. **(C)** A schematic representation of the observed interactions in figures 5A and B. Below

the schematic, conserved residues of WTX are indicated by gray shading. Conserved residues that are identical to the NRF2 degron are indicated by red shading. **(D)** The interaction between WTX and KEAP1 is mediated through an ETGE motif. The indicated VENUS-WTX mutation constructs were transfected into GLUE-KEAP1 cells. Complexes were purified and analyzed as in A and B.

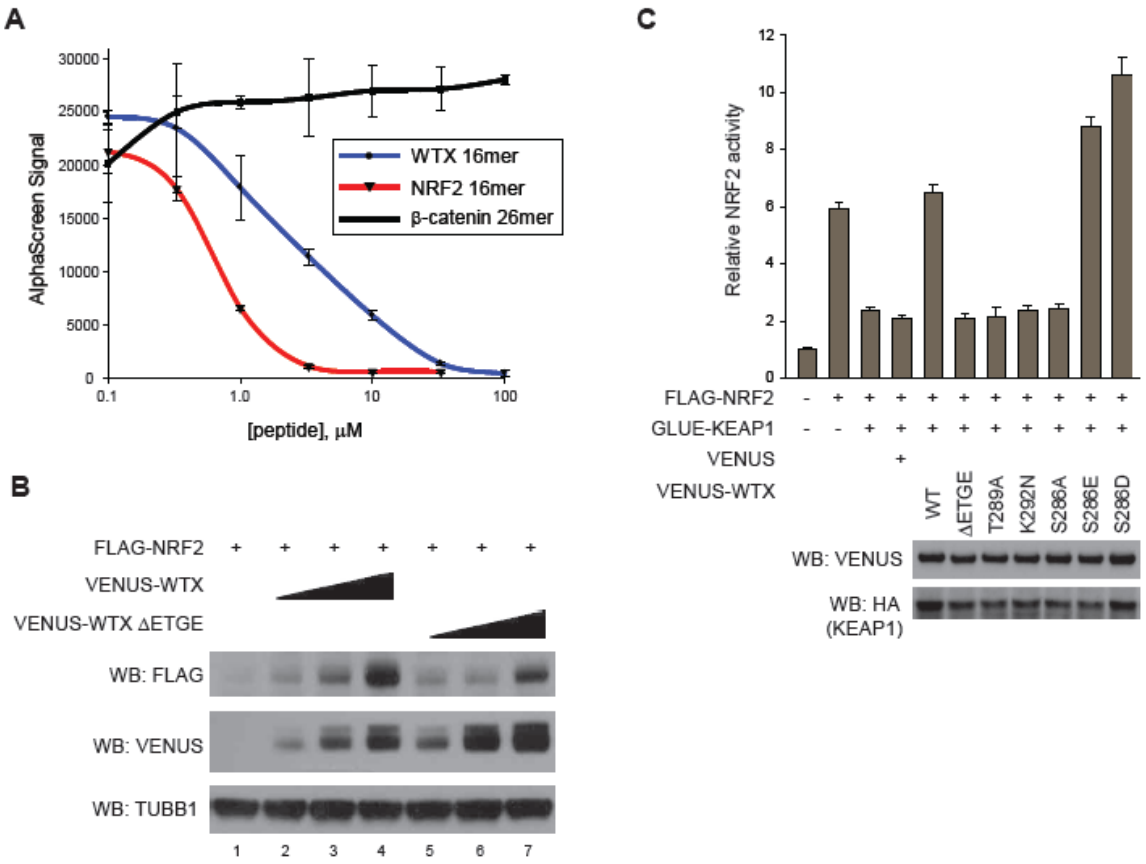

Figure 16. WTX competes with NRF2 for binding to KEAP1. (A) A WTX peptide containing the ETGE motif inhibits the interaction between KEAP1 and NRF2. A fixed concentration of GST-NRF2 and 6xHIS-hKELCH was mixed with increasing amounts of the indicated peptides in the presence of GSH-donor and Nickel-acceptor AlphaScreen beads. The interaction between GST-NRF2 and 6xHIS-hKELCH is represented as AlphaScreen signal. Error bars represent standard deviation from the mean of 3 technical replicates. Data are representative of 3 biological replicates. **(B)** The ETGE motif is required to stabilize NRF2. HEK293T cells were transfected with the indicated expression constructs. Proteins were analyzed by Western blot. **(C)** The ETGE motif is required to regulate NRF2 transcriptional activity. HEK293T cells in a 48-well tissue culture plate were transfected with 5ng of an ARE-luciferase reporter, 10ng of a control CMV *Renilla* luciferase control reporter, and the indicated DNA (2ng FLAG-NRF2, 10ng GLUE-KEAP1, and 10ng VENUS constructs). The relative levels of ARE-driven firefly luciferase compared to control CMV-driven *Renilla* luciferase are plotted. Error bars represent standard deviation from the mean for 3 replicates. Data are representative of 3 independent experiments. Lysates were also analyzed by Western blot analysis for relative WTX and KEAP1 expression.

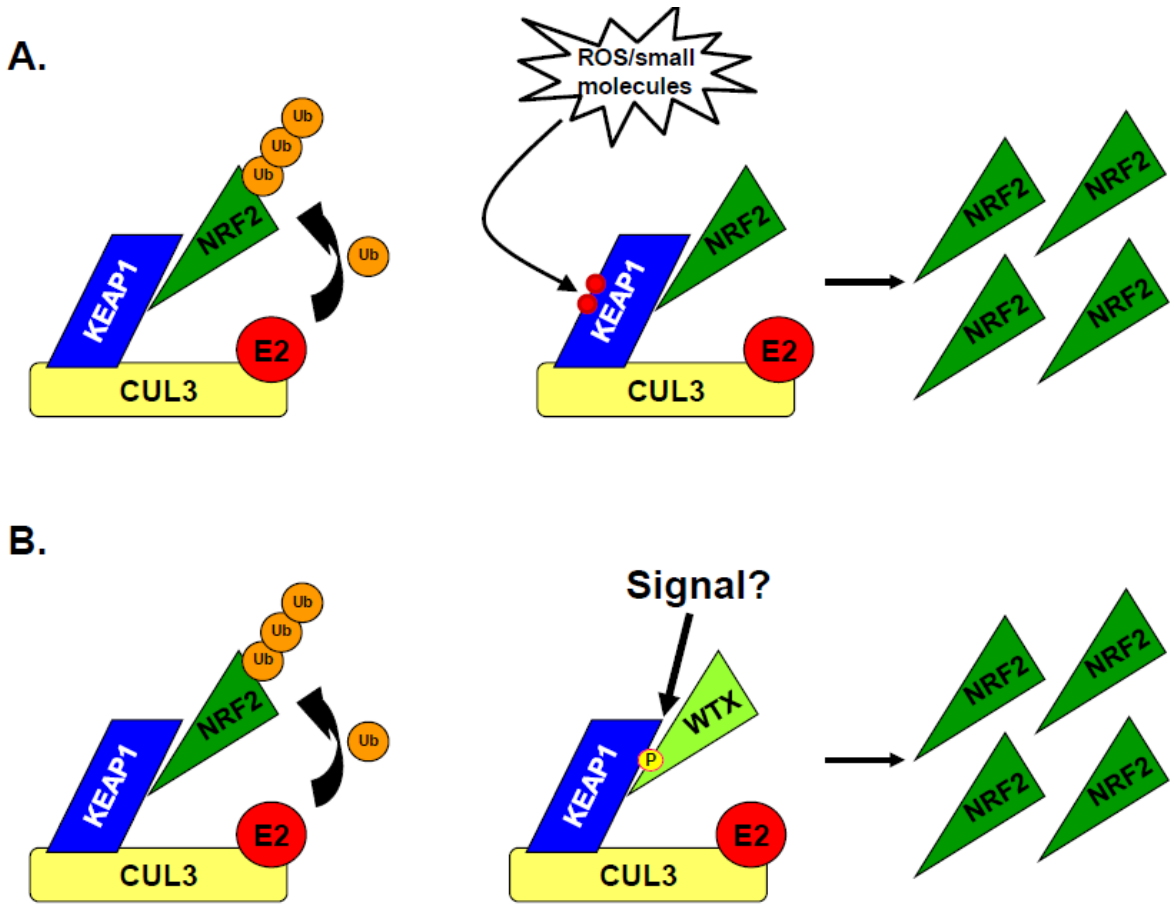

Figure 17. Proposed mechanism of WTX in the KEAP1/NRF2 antioxidant response. (A) Traditional model of activation of the NRF2-dependent antioxidant response by oxidants. Left: In the absence of oxidants, NRF2 is constitutively ubiquitinated by the KEAP1/CUL3 E3 ubiquitin ligase complex. Right: in the presence of oxidants or various small molecules, Cysteine residues on KEAP1 are modified and NRF2 is no longer ubiqutinated. **(B)** Proposed model of activation of the NRF2-dependent antioxidant response by WTX. Upon an as yet to be determined signal, WTX is phosphorylated and competes with NRF2 for binding to KEAP1.

Table II. Phosphorylated WTX peptides identified by mass spectrometry

Unique Peptide Sequence	Peptide Probability	Index	Ascore1	Ascore2
KENANPQDAPGPKVS*PTPEPSPPATEK	.99	240	32.48	N/A
VSPT*PEPSPPATEK	.99	242	6.46	N/A
VSPTPEPS*PPATEK	.99	246	33.57	N/A
KENANPQDAPGPKVS*PTPEPS*PPATEK	.99	240, 246	32.48	33.57
VSPT*PEPS*PPATEK	.99	242, 246	6.46	33.57
PAPEASS*LEEPHSPETGEK	.89	280	9.7	N/A
PAPEASSLEEPHS*PETGEK	.99	286	46.85	N/A
PAPEASS*LEEPHS*PETGEK	.91	280, 286	9.7	46.85
T*SLKSFDSLTGCGDIIAEQDMDSMTDSMASGGQR	.99	317	0	N/A
FDS*LT*GCGDIIAEQDMDSMTDSMASGGQR	.29	324, 326	11.63	12.28
PNMNLGYHPTT*SPGHHGYMLLDPVR	.99	429	0	N/A
PNMNLGYHPTTS*PGHHGY	.57	430	0	N/A
DSYSGDALYEFYEPDDSLENS*PPGDDCLY*DLH	.85	518, 526	0	0
SSEMFDPFLNFEPFLSS*RPPGAMET*EEE	.80	548, 556	2.99	5.45
ITS*AFPTTASSEPDWR	.21	683	0	N/A
RQVTQACGT*W	.99	803[†]	69.97	N/A

* Indicates phosphorylated residue

[†] Threonine 803 is absent in the full-length isoform of WTX (1-1135)

Table III. Phosphorylated WTX peptides associated with KEAP1

Unique Peptide Sequence	Peptide Probability	Index	Ascore1	Ascore2
KENANPQDAPGPKVS*PTPEPS*PPATEK	.81	240, 246	1	7.23
VSPT*PEPS*PPATEK	.99	242, 246	13.7	38.5
VSPTPEPS*PPATEK	.99	246	20.22	N/A
PAPEASSLEEPHS*PETGEK	.99	286	7.79	N/A
AYPTYS*PPEDPEEEEVEK	.99	749	19.85	N/A
FYQGLPWGVSS*LPR	.99	868	7.53	N/A

* Indicates phosphorylated residue

CHAPTER 5

Conclusions

The *WTX* gene encodes a tumor suppressor protein; it is located on the X chromosome and is somatically lost or mutated in 7-30% of cases of Wilms tumor (Rivera, Kim et al. 2007; Perotti, Gamba et al. 2008; Ruteshouser, Robinson et al. 2008). Germline mutations in WTX give rise to OSCS, a debilitating and fatal disease that largely affects the skeletal system (Jenkins, van Kogelenberg et al. 2009; Perdu, de Freitas et al. 2009). Although these diseases appear markedly different, their respective phenotypes are consistent with elevated β-catenin activity. Given that loss of WTX leads to increased levels of β-catenin, it is likely that increased β-catenin levels drive these diseases.

As the discovery of WTX and its mutations in human disease is relatively new, the molecular function(s) and developmental or homeostatic consequences of its loss is only beginning to be unraveled. In addition to its role as a regulator of β-catenin at the level of the destruction complex, Jurgen Behrens' group identified WTX as a novel interactor of APC that regulates the localization of APC at the membrane (Grohmann, Tanneberger et al. 2007). Besides its role in regulating the degradation of β-catenin, APC also regulates cell adhesion and migration by interacting with the plasma membrane and the microtubule cytoskeleton (Aoki and Taketo 2007). Behrens' group identified WTX as a novel interactor of APC in parallel with our studies, and went on to show that WTX

interacts with the membrane and regulates the subcellular distribution of APC between membrane- and microtubule-associated pools, and thus may regulate APC-dependent morphogenesis, cell-migration, and adhesion.

The same group also went on to show that WTX promotes WNT signal transduction at the membrane (Tanneberger, Pfister et al. 2011). LRP6 becomes phosphorylated when a WNT ligand binds to Frizzled and LRP6 (Tamai, Zeng et al. 2004). The precise details are unclear, but it is thought that in the presence of WNT, DVL becomes associated with Frizzled, and in turn recruits Axin and GSK3β. At the membrane, GSK3β and CK1α phosphorylate LRP6 at 5 conserved PPPSPxS motifs and adjacent S/T clusters, creating docking sites for AXIN. This complex of LRP6/FRZ/AXIN/GSKβ is called a "signalosome" and is suggested to be required for signaling, perhaps by sequestering GSK3β and AXIN such that they can no longer promote phosphorylation of β-catenin in the cytosol (Bilic, Huang et al. 2007). Behrens' group showed that WTX is required for both phosphorylation of LRP6 and internalization of signalosomes. Mechanistically, it is thought that WNT promotes phosphatidyl inositol phosphate generation that promotes WTX recruitment to the membrane, which in turn helps recruit AXIN and GSK3β to the membrane. Thus, in addition to promoting the destruction of β-catenin, WTX also promotes WNT signaling at the membrane.

Common mutations in Wilms tumors include WT1, β-catenin (activating), and WTX. Recently, it was shown that in addition to β-catenin, WTX also promotes the transcriptional activity of WT1 (Rivera, Kim et al. 2009). The C-terminus of WTX interacts with WT1 and can regulate WT1 target genes such as amphiregulin. Interestingly, it may be isoform specific. WTX exists in 2 isoforms, the short isoform lacking residues 50-326. Whereas the full-length isoform localizes to the plasma membrane, the short isoform localizes to a specific intranuclear compartment. These two observations may suggest that the short isoform specifically regulates WT1 in the nucleus, although this remains to be seen.

In the preceding chapters I have described our studies on WTX, which suggest that it is an important regulator of ubiquitination in the Wnt/β-catenin and KEAP1/NRF2 pathways. While WTX inhibits the ubiquitination of NRF2, we also observed that WTX promotes the ubiquitination of β-catenin by the SCF^{BTRC} ubiquitin ligase complex. To our knowledge, WTX is the first described protein to interact with two E3 ubiquitin ligase adaptors (BTRC and KEAP1) and have opposite regulatory effects on their respective substrates. How is this possible? WTX interacts with the KELCH repeats that form the β-propeller fold of KEAP1, and likely inhibits the formation of a functional CUL3/KEAP1/NRF2 complex. Through a separable domain, WTX interacts with both BTRC and β-catenin, suggesting that it binds to an intact SCF^{BTRC}/substrate complex (Major, Camp et al. 2007). BTRC also employs a β-propeller fold for substrate capture

(Wu, Xu et al. 2003), but it is unlikely that WTX interacts with the β-propeller fold of BTRC in a similar fashion as KEAP1 as this would inhibit the formation of a functional CUL1/SKP1/BTRC/β-catenin complex and result in elevated β-catenin levels. Interestingly, a recent study demonstrated that NRF2 is phosphorylated by GSK3β in the central Neh6 domain (DSGIS, residues 334-338), creating an SCF^{BTRC} destruction motif similar to that of β-catenin (Rada, Rojo et al. 2011). This phosphorylated form of NRF2 is recognized by BTRC. Additionally, KEAP1 and BTRC have been identified in the same complex by mass spectrometry (Sowa, Bennett et al. 2009). This raises the possibility that multiple E3 ubiquitin ligase complexes consisting of a KEAP1 homodimer, a BTRC homodimer, or a KEAP1/BTRC heterodimer regulate the ubiquitination of NRF2. As WTX interacts with BTRC and KEAP1 through separable domains, we hypothesize that WTX coordinates the adaptors in the E3 ubiquitin ligase complexes, resulting in variable ubiquitination of substrates.

As described above, compelling data from numerous research groups have described functions for WTX in controlling WNT/β-catenin signaling, cell-cell adhesion, WT1-dependent transcription, and the KEAP1/NRF2 antioxidant response (Grohmann, Tanneberger et al. 2007; Major, Camp et al. 2007; Rivera, Kim et al. 2007; Rivera, Kim et al. 2009; Tanneberger, Pfister et al. 2011). Given its role in multiple pathways and cellular processes, whether or not β-catenin independent mechanisms contribute to WTX-associated disease is of interest. Although most commonly lost through gene-

encompassing deletions, a small percentage of WTX mutations identified in Wilms tumor yield single amino acid substitutions and truncated proteins (Rivera, Kim et al. 2007). Conversely, the majority of WTX mutations identified in OSCS yield truncated proteins (Jenkins, van Kogelenberg et al. 2009). Aligning the location of these mutations with the WTX protein interaction domains suggests relationships of these diseases with specific binding interfaces (**Figure 17**). The KEAP1 interaction domain lies amino-terminal to the domains that bind β-catenin/BTRC/APC and WT1 and remains intact in 9 out of the 20 reported mutation products. Of note, we determined that the K292N substitution inhibited the association of WTX and KEAP1. The β-catenin/BTRC/APC interacting domains encompass residues 280 through 839. Of the 20 reported mutations, 11 are predicted to alter these binding activities. As the C-terminus of WTX binds WT1, this interaction is lost in all truncation products derived from mutations in WTX. These correlations suggest that WT1 is central to WTX-associated diseases, and that the WTX-KEAP1 and WTX-β-catenin/BTRC/APC functional relationships may contribute to a subset of Wilms tumor and OSCS, perhaps accounting for variability in disease onset or progression.

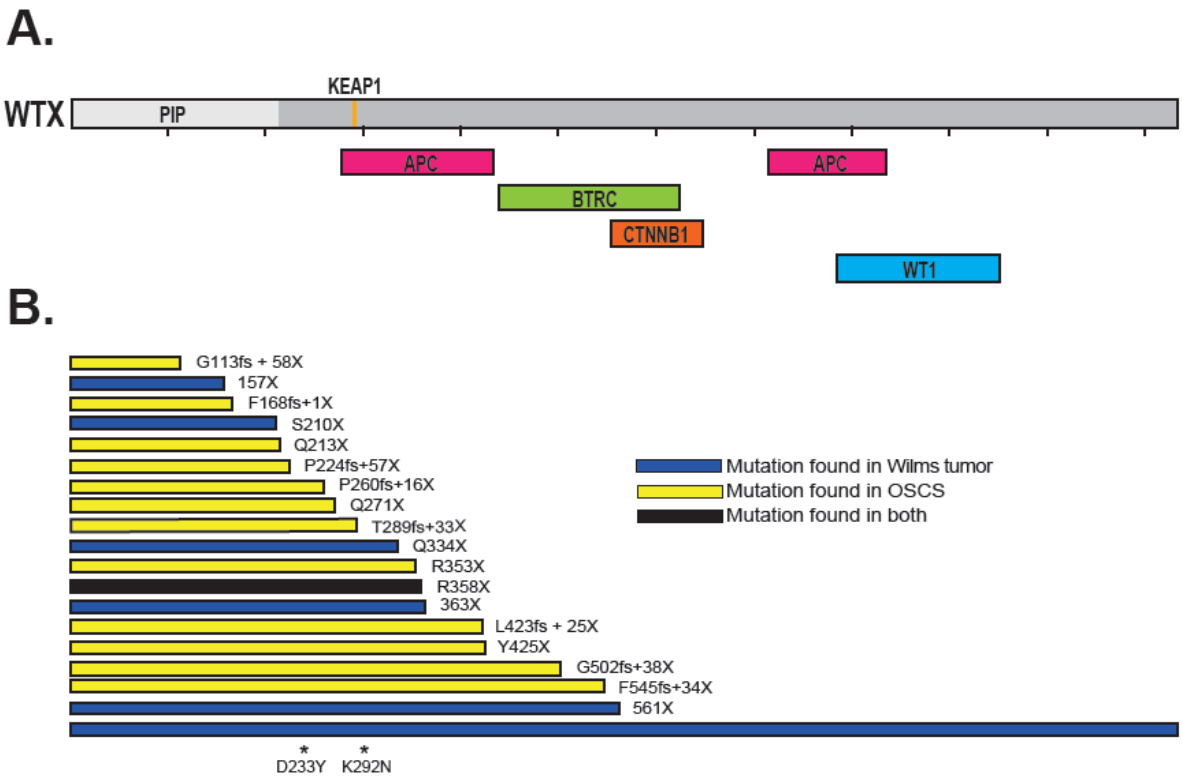

Figure 18. Schematic representation of WTX and its Protein interaction domains. (A) WTX contains an N-terminal PtdIns(4,5)P_2 binding domain (PIP) in addition to its known protein interaction domains. **(B)** Alignment of the known WTX mutations in Wilms tumor and OSCS suggests that the KEAP1 interaction domain is lost in approximately half of all truncation products whereas the β-catenin (CTNNB1) interaction domain is lost in all.

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Curriculum Vitae
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Travis L. Biechele, **Nathan Camp**, Dan Fass, Stephen Haggarty and Randall T. Moon. Molecular activators of the Wnt/Beta-catenin pathway. Provisional filed Dec 24, 2008. Application # 61140655

Travis L. Biechele, **Nathan Camp**, Dan Fass, Stephen Haggarty and Randall T. Moon. Molecular inhibitors of the Wnt/Beta-catenin pathway. Provisional filed Dec 22, 2008. Application # 61139750

Publications

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