MOLECULAR AND CELLULAR BIOLOGY, May 2011, p. 2053–2065 Vol. 31, No. 10
0270-7306/11/$12.00 doi:10.1128/MCB.01094-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

The Ubiquitin-Specific Protease USP34 Regulates Axin Stability and Wnt/β-Catenin Signaling

Tony T. H. Lui,[1] Celine Lacroix,[1] Syed M. Ahmed,[1] Seth J. Goldenberg,[2] Craig A. Leach,[2] Avais M. Daulat,[1] and Stephane Angers[1,3]*

Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, Toronto, Ontario, Canada[1]; Division of Research and Development, Progenra, Inc., Malvern, Pennsylvania 19355[2]; and Department of Biochemistry, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada[3]

Received 16 September 2010/Returned for modification 7 October 2010/Accepted 13 February 2011

Wnt proteins control multiple cell behaviors during development and tissue homeostasis. However, pathological activation of Wnt signaling is the underlying cause of various human diseases. The ubiquitin-proteasome system plays important regulatory functions within the Wnt pathway by regulating the activity of several of its core components. Hence, multiple E3 ubiquitin ligases have been implicated in its regulation. Less is known, however, about the role of ubiquitin-specific proteases in Wnt signaling. Analysis of purified axin-containing protein complexes by liquid chromatography-tandem mass spectrometry revealed the presence of the ubiquitin protease USP34. Our results indicate that USP34 functions downstream of the β-catenin destruction complex to control the stability of axin and opposes its tankyrase-dependent ubiquitination. Reflecting on the requirement for tight control of axin homeostasis during Wnt signaling, interfering with USP34 function by RNA interference leads to the degradation of axin and to the inhibition of β-catenin-mediated transcription. Given the numerous human diseases exhibiting spurious Wnt pathway activation, the development of USP34 inhibitors may offer a novel therapeutic opportunity.

During embryonic development and tissue homeostasis in adults, the Wnt family of secreted glycoproteins modulates several cell behaviors, including differentiation, proliferation, cell movement, and polarity (32, 37). Malfunctioning Wnt-activated signaling pathways are associated with multiple human diseases, including cancer (10, 38). The etiology of colon carcinoma is a particularly striking example that reflects the critical importance of the integrity of this signaling cascade during intestinal epithelium homeostasis (45). Approximately 80% of all colon cancers are molecularly rooted in mutations of Wnt pathway components. These primarily consist of inactivating mutations in the gene coding for the tumor suppressor adenomatous polyposis coli (APC) (44, 47, 51) but also of activating mutations in the transcription factor β-catenin (39) and loss-of-function mutations in the scaffolding axin protein (22).

APC and axin are the core components of a cellular machinery dubbed the "destruction complex" that promotes the phosphorylation of the cytoplasmic pool of β-catenin (24). Axin, through binding to the destruction complex kinases casein kinase 1α (CK1α) and glycogen synthase kinase 3 (GSK3), orchestrates β-catenin phosphorylation (31). Phospho-β-catenin is in turn recognized by the $SCF^{\beta\text{-TrCP}}$ (Skp1-Cullin1-FBOX) E3 ubiquitin ligase that polyubiquitinates β-catenin and promotes its proteolysis by the proteasome (26, 59). The destruction complex thereby maintains low levels of cytosolic β-catenin in the absence of Wnt stimulation. The recognition of Wnt ligands by the cell surface receptor complex Frizzled-LRP5/6 leads to the activation of Dishevelled (Dsh) (62), which promotes the GSK3- and CK1γ-dependent phosphorylation of the LRP5/6 cytosolic domain (12, 63). The phosphorylated LRP5/6 cytosolic domain acts as a high-affinity binding site for axin (36, 53) that is suspected to inactivate the destruction complex and to lead to β-catenin accumulation. Stabilized β-catenin can then enter the nucleus and cooperate with LEF/TCF transcription factors to regulate Wnt-dependent transcriptional programs in a context-dependent fashion (50).

The ubiquitin-proteasome system (UPS) is emerging as master regulator of Wnt signaling, controlling the pathway at multiple levels. In addition to the well-characterized function of the $SCF^{\beta\text{-TrCP}}$ E3 ligase for β-catenin ubiquitination in the absence of Wnt-driven signals (17, 26, 59), other proteins of the pathway are either targeted for degradation or regulated by the UPS. The ubiquitination of APC (9, 56) and Dishevelled (3, 54), for instance, leads to their proteasome-mediated degradation or to degradation-independent functional regulation. This dual regulation by the UPS depends on whether K48- or K63-linked ubiquitin chains are involved. Although the E3 ubiquitin ligase for APC has not been identified, this process is thought to involve axin, at least for the situation where APC is degraded (56). Another example is the posttranslational control of Dsh stability by the Cullin3-KLHL12 E3 ligase (3). Consistent with roles in both β-catenin-dependent and -independent Wnt pathways for Dsh, the activity of this E3 ligase was shown to impact both pathways in *Xenopus* and zebrafish embryos. Axin has also been postulated to be regulated through the modulation of its stability, which might be a necessary step for the activation of the β-catenin pathway (27, 58). The precise mechanisms regulating the degra-

* Corresponding author. Mailing address: Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College Street, Toronto, M5S 3M2 Ontario, Canada. Phone: (416) 978-4939. Fax: (416) 978-8511. E-mail: stephane.angers@utoronto.ca.

▽ Published ahead of print on 7 March 2011.

dation of axin are, however, not known at present, but its parsylation by tankyrase and its sumoylation have recently been shown to control its ubiquitin-dependent degradation (20, 23).

Due to the multiple roles of the UPS in Wnt signaling, it is likely that members of the ubiquitin-specific proteases (USPs; also termed deubiquitinating enzymes [DUBs]) regulate some of these events and could therefore have important functional roles in Wnt signaling. An estimated 79 USPs are present in humans that function to remove ubiquitin conjugates from target proteins (43). Supporting the possibility that USPs may regulate Wnt signaling, recent report have identified the ubiquitin protease Trabid (56) and USP4 (64) as novel regulators of this pathway. Trabid regulates APC function through the editing of its K63-conjugated chains, whereas USP4 regulates TCF4 (64).

A recurrent theme in Wnt signal transduction is the reutilization of Wnt pathway components in different subcellular compartments, often to perform alternate functions. For example, Dsh has been localized to punctate structures within the cytoplasm (7, 49) or to the plasma membrane upon Wnt activation of the Frizzled-LRP receptor complex (5, 62). However, other studies have shown that Dsh is also translocated to the nucleus, where it performs a required but ill-defined role during Wnt signaling (15, 21). β-Catenin-independent Wnt signaling also likely involves the relocalization of Dsh to additional subcellular structures in order to modulate cytoskeleton-associated processes (4). Likewise, GSK3 acts primarily as a negative regulator of Wnt signaling by promoting the phosphorylation of β-catenin. However, as mentioned above, GSK3 also plays a positive role, at the plasma membrane, via the phosphorylation of the LRP5/6 Wnt coreceptor (12, 63) and has also been found to have nuclear roles (8). Similarly, in addition to its task in the destruction complex, a nuclear role has been proposed for APC in Wnt signaling. Indeed, APC contains bipartite nuclear localization and nuclear export signals that promote its nuclear cytoplasmic shuttling (18, 40, 46). Nuclear APC antagonizes β-catenin-mediated transcription by either the modulation of β-catenin nuclear export (18), the sequestration of β-catenin away from an active transcription complex (41), or its association with transcriptional repressors (16). In contrast, a recent genetic screen in *Drosophila* uncovered a positive functional role for APC homologs in Wg signaling (52). It is therefore a common theme in Wnt signaling that its effectors are reutilized in a context-dependent manner.

Axin, normally associated with the destruction complex, does not escape this trend as it is recruited to the activated and phosphorylated LRP5/6 coreceptor (36, 53) at the plasma membrane. Moreover, axin is also known to shuttle between the nucleus and the cytoplasm (11, 57) and is greatly enriched in the nuclei of cells from diverse cancer cell lines and tissues (1, 29, 48, 60). However, the precise function of nuclear axin in Wnt signaling is not well understood.

Here, by using a proteomic approach, we show that axin associates with ubiquitin-specific protease 34 (USP34). Our results indicate that USP34 controls the levels of axin and positively modulate Wnt signaling by acting downstream of β-catenin stabilization through controlling the nuclear accumulation of axin.

MATERIALS AND METHODS

Plasmids. Human *AXIN1* and *AXIN2* cDNAs were cloned by PCR from a human brain cDNA library into the pGLUE tandem-affinity purification (TAP) plasmid (3) that contains streptavidin (SBP)- and calmodulin-binding peptides (CBP) to generate pGLUE-hAXIN1 and pGLUE-hAXIN2. AXIN1 was also cloned downstream of a cDNA coding for the Venus fluorescent protein in the pIRES-puro vector to generate the pIRES-puro-Venus-hAXIN1 plasmid. The human point mutant β-catenin (pt.mutant-hβCATENIN-CBP-HA-SBP) (34) and human Dishevelled-2 (pGLUE-hDSH2) (3) were described previously. USP34 core (residues 1892 to 2241) was expressed and purified as a His-tagged protein from *Escherichia coli*. USP2 core was expressed and purified as previously described (42). All PCR-amplified regions were sequence validated. Detailed description of plasmid maps and sequences will be provided upon request and are posted on the lab web site (http://phm.utoronto.ca/angers/).

Reagents, tissue culture, and transfection. Human HEK293T, RKO colon carcinoma (ATCC CRL-2577), SW480 colorectal adenocarcinoma (CCL-228), HCT116 colorectal carcinoma (CCL-247), and mouse L cells (CRL-2647/CRL-2648) were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO) in a 37°C humidified incubator with 5% CO_2. HEK293T stable cell lines were generated by transfection with calcium phosphate, followed by puromycin selection (2 μg/ml). Transient cDNA transfections were performed according to the manufacturer's recommendations using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

For small interfering RNA (siRNA) experiments, cells were transfected with 20 nM siRNA with the recommended amounts of Lipofectamine RNAiMax (Invitrogen). Previously validated siRNAs against β-catenin, AXIN1, and AXIN2 (34) and nontargeting control (Dharmacon, Lafayette, CO) were used, while a set of four siRNAs targeting *USP34* was obtained from Dharmacon (catalog no. LQ-006082-00-0002) and tested by Western blotting. Within this set, the USP34 siRNA A was the most effective, and its target sequence was 5′-GCAGGGAAGUUCUGACGAA-3′. The target sequences of the other USP34 siRNAs were as follows: B, 5′-CAACAGAUCAGUAGUAAUU-3′; C, 5′-GCAGCUAUCCAGUAUAUUA-3′; and D, 5′-CCAUGUGACUGGA GAUUUA-3′.

For the epistasis experiments involving the expression of pt.mutant-βCATENIN or DSH2 with a given siRNA, the siRNAs were first reverse transfected at the time of seeding cells, followed by replacement of the medium 24 h after seeding and cDNA transfection with Lipofectamine 2000. Cells were then assayed 36 h after cDNA transfection using the TopFlash reporter assay. pGIPZ-based shRNAs for USP34 were obtained from Open Biosystems and screened for their efficiency by Western blotting. The target sequence of the most efficient USP34 shRNA was 5′-CCTATGATGGTTGTTCAAATT-3′.

Wnt3A conditioned medium. Mouse L cells expressing Wnt3A (CRL-2647) were cultured until reaching 90% confluence, after which the medium was collected and refreshed every 2 days for a total of 6 days. Medium from different days was assayed by using TopFlash assays to determine the fractions with maximal activity and subsequently used for Wnt stimulation experiments. Conditioned medium from parental mouse L cells not producing Wnt3A (CRL-2648) was also collected to use as a control.

Western blotting and antibodies. Protein lysates were resolved using SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were stained with antibodies indicated in the figure legends and then incubated with horseradish peroxidase-conjugated secondary antibodies and detected by using chemiluminescence. The antibodies used included αβ-catenin (catalog no. 9587; Cell Signaling Technologies); rabbit monoclonal α-AXIN1 (catalog no. 2074; Cell Signaling Technologies); polyclonal α-AXIN1 (obtained from J. Woodgett, Mt. Sinai, Toronto, Ontario, Canada); p44/42 mitogen-activated protein kinase (ERK; catalog no. 9102; Cell Signaling Technologies); α-USP34 (A300-824A; Bethyl Labs); α-lamin-B (sc-6217; Santa Cruz Biotechnology); α-HA (MMS-101P; Covance); α-FLAG (F1804; Sigma); and peroxidase-conjugated secondary anti-goat, anti-rabbit, and anti-mouse antibodies (catalog numbers 705-035-147, 711-035-152, and 715-035-150; Jackson Immunoresearch Laboratories).

Tandem-affinity purification and mass spectrometry. HEK293T cells (~2×10^8 cells) expressing SBP-HA-CBP-tagged AXIN1 or AXIN2 were used for the tandem-affinity purification procedure as previously described (3). Briefly, the cells were lysed with tandem-affinity purification lysis buffer (10% glycerol, 50 mM HEPES-KOH [pH 8.0], 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 2 mM dithiothreitol [DTT], 10 mM NaF, 0.25 mM $NaOVO_3$, and protease inhibitors [Sigma]). The lysates were cleared by centrifugation at $16,000 \times g$ for 10 min and then incubated at 4°C with 100 μl of packed streptavidin resin (GE Healthcare). The beads were washed, and protein complexes were then eluted from the

streptavidin resin in calmodulin binding buffer supplemented with 2 mM biotin. The second round of affinity purification was performed with 100 μl of calmodulin resin (GE Healthcare). After several washes, the protein complexes were eluted with two 100-μl elutions with calmodulin elution buffer (50 mM ammonium bicarbonate [pH 8.0], 10 mM EGTA) and directly digested with sequencing-grade trypsin (Promega). The resulting peptide mixture was then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using data-dependent acquisition on a LTQ-XL mass spectrometer (Thermo Scientific). Acquired spectra were searched against a FASTA file containing the human NCBI sequences by using a normalized implementation of SEQUEST. The resulting peptide identifications returned by SEQUEST were filtered and assembled into protein identifications using the transproteomic pipeline software running on a Sorcerer platform (SageNResearch).

TopFlash reporter assays. Lentivirus containing the TopFlash β-catenin-dependent luciferase reporter (firefly luciferase) and *Renilla* luciferase were produced and used to establish stable HEK293T, RKO, SW480, and HCT116 Wnt-reporter lines. Cells were seeded on 24-well plates, followed by cDNA transfection with Lipofectamine 2000 and/or reverse transfection with Lipofectamine RNAiMax for siRNA experiments. For experiments involving Wnt stimulation, the medium was replaced with a 1:1 mix of fresh DMEM-Wnt3A or DMEM-control conditioned medium. The cells were then assayed 24 h after stimulation in accordance with the dual luciferase assay protocol (Promega) using an Envision multilabel plate reader (Perkin-Elmer).

Co-affinity purification. For co-affinity purification of endogenous proteins, HEK293T cells (5×10^6) stably expressing pGLUE-HA-hAXIN1 or pGLUE-HA-RADIL were lysed in tandem-affinity purification lysis buffer (10% glycerol, 50 mM HEPES-KOH [pH 8.0], 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 2 mM DTT, 10 mM NaF, 0.25 mM $NaOVO_3$, and protease inhibitors [Sigma]). Lysates were cleared by centrifugation at 16,000 × *g* for 10 min, and affinity purification was performed using streptavidin resin. Purified protein complexes were then analyzed by Western blotting using the antibodies noted in the figure legends.

K48 ubiquitin chain cleavage. Next, 1 μg of purified K48 chains from Boston Biochem (UC-230) was incubated in USP assay buffer (20 mM Tris [pH 8.0], 2 mM $CaCl_2$, 2 mM β-mercaptoethanol) with 20 nM USP2 core, 100 nM USP34 core, 1 μg of affinity-purified axin complex, or 1 μg of axin complex (USP34 shRNA). The samples were incubated at 37°C for 30 min, and the reaction was stopped by the addition of SDS sample buffer. The appearance of monoubiquitin was monitored by Western blotting with α-ubiquitin antibody (Sigma catalog no. U5379).

UBL-PLA_2 assay. 20 nM USP2 core, 20 nM USP34 core, or 1 μg of total protein from purified axin complexes was mixed with 30 nM Ub-PLA_2 and 20 μM NBD C6HPC (phospholipase A_2 [PLA_2] substrate; Invitrogen) in a total volume of 100 μl/well in a black 96-well-plate (Greiner Bio-One). The data were collected 45 min after the addition of Ub-PLA_2 and NBD C6HPC on a Perkin-Elmer Envision fluorescence plate reader with excitation and emission filters of 475 and 555 nm, respectively. The net relative fluorescence units (RFU) were then used to calculate the signal (isopeptidase or complexes plus reporter)-to-background (reporter) ratio. For UBL selectivity assays, the relative isopeptidase activity against various UBL-PLA_2 fusions was determined by adding the USP34 core to a final concentration of 20 nM in combination with 20 μM NBD C6-HPC and 30 nM concentrations of the individual UBL-PLA_2 reporter constructs and is expressed as a percentage of the control isopeptidase: USP2 core (Ub-PLA_2), Senp1core (SUMO3-PLA_2), Den1 (NEDD8-PLA_2), or PLpro (ISG15-PLA_2). The UBL-PLA_2 assay reagents are available from LifeSensors, Inc., as CHOP reporter kits.

***In vitro* deubiquitination assay.** HEK293T cells stably expressing STREP-HA-AXIN1 were transfected with a plasmid coding for FLAG-ubiquitin. In parallel, two 100-mm petri dishes of HEK293T were transfected with pIRES-puro plasmids expressing STREP-HA-USP34 core domain (amino acids 1696 to 2400) or a catalytically inactive STREP-HA-USP34 core domain (C1903S). At 16 h before lysis, STREP-HA-AXIN1 cells were treated with 1 μM MG132. The cells were lysed separately in TAP lysis buffer supplemented with protease inhibitors in the absence (USP34) or the presence (axin) of 5 mM *N*-ethylmaleimide (NEM). The proteins were affinity purified using streptavidin beads. After extensive washes in TAP lysis buffer and two washes in DUB buffer (50 mM Tris [pH 8], 150 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT), an equivalent amount of AXIN1 was incubated for 1 h at 37°C with the USP34 core domains, as indicated in the figures. Proteins were resolved by SDS-PAGE and blotted with FLAG antibodies to detect ubiquitin conjugates and hemagglutinin (HA) to monitor axin and USP34 core domain expression.

Immunofluorescence. Cells were seeded on poly-D-lysine-treated coverslips and, when indicated, reverse transfected with siRNA. At 48 h after transfection the cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) for 20 min and then permeabilized and blocked with 0.2% Triton X-100 and 10% normal donkey serum-PBS for 20 min. Where indicated, cells were treated with 5 ng of leptomycin B (LMB; LC Laboratories)/ml for 3 h. The cells were then stained for indirect immunofluorescence using polyclonal α-AXIN1 antibodies (provided by J. R. Woodgett) and Alexa 488-conjugated anti-rabbit antibodies. Cells were mounted with Vectorshield (Vector) and examined by using a laser scanning confocal microscope (Zeiss LSM 510).

Cycloheximide chase. HEK293T cells expressing scramble or *USP34* shRNA were seeded onto a 6-well plate. The cells were treated with 3 μM XAV939 for 16 h, washed twice with PBS, and treated with 10 μg of cycloheximide/ml for the indicated times. Then, the cells were lysed using TAP lysis buffer supplemented with protease inhibitors. Equivalent amounts of proteins were resolved by using SDS-PAGE, followed by Western blotting with AXIN1 or tubulin antibodies.

β-Catenin stabilization assay. RKO cells were reverse transfected with siRNA. At 48 h after transfection, the cells were stimulated with control or Wnt3A conditioned medium treatment for different times (0.5 to 6 h). The cells were washed and lysed with radioimmunoprecipitation assay buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) for 15 min and then cleared by centrifugation at 16,000 × *g* for 10 min before being resuspended in SDS sample buffer and resolved by SDS-PAGE. β-Catenin accumulation was monitored by Western blotting.

Axin ubiquitination assay. HEK293T cells stably expressing human AXIN1 (pGLUE-AXIN1) were transfected with FLAG-ubiquitin using calcium phosphate. The cells were lysed 48 h after transfection using TAP lysis buffer supplemented or not with 20 mM NEM (Sigma) and then cleared by centrifugation at 16,000 × *g* for 10 min. Axin was purified by streptavidin affinity chromatography for 1 h. Resin beads were then washed three times with lysis buffer (also supplemented with NEM when indicated), and the protein complexes were eluted using 2× SDS sample buffer, followed by SDS-PAGE electrophoresis and Western blotting with FLAG antibodies to detect ubiquitin-conjugated axin proteins.

Real-time quantitative PCR. Total RNA from SW480 cells treated with control or USP34 siRNAs was purified by using Tri-Reagent (Sigma). After DNase I (Invitrogen) treatment, RNA was reverse transcribed into cDNA by using a high-capacity cDNA reverse transcription kit (Applied Biosystems). The primer sequences used were as follows: *CYCLOPHILIN*, 5′-GGAGATGGCACAGGAGGAA-3′ and 5′-GCCCGTAGTGCTTCAGTTT-3′; *NKD1*, 5′-TGAGAAGAAGATGGAGAGAGTGAGCGA-3′ and 5′-GGTGACCTTGCCGTTGTTGTCAAA-3′; and *TNFRSF19*, 5′-GGAGTTGTCTAAGGAATGTGG-3′ and 5′-GCTGAACAATTTGCCTTCTG-3′. Primer pair efficiencies were validated as previously described (6). Quantitative reverse transcription-PCR (RT-PCR) analysis was carried out in triplicate using an Applied Biosystems Prism 7900HT instrument. Each reaction contained 12.5 ng of cDNA, 150 nM concentrations of each primer, and a Power SYBR green PCR Master Mix (Applied Biosystems). Gene expression analysis was performed by using the comparative cycle threshold (C_T) method, normalized to *CYCLOPHILIN* expression, and the fold changes were calculated relative to control siRNA-treated cells.

RESULTS

Targeted proteomic analysis identifies USP34 as an axin-associated protein. To better understand the regulation of axin and its mechanism of action, we isolated human AXIN1 and AXIN2 protein complexes and analyzed their compositions by using LC-MS/MS. We constructed two expression vectors, pGLUE-AXIN1 and pGLUE-AXIN2, and used them to derive HEK293T human cell lines stably expressing fusion proteins of AXIN1 or AXIN2 harboring streptavidin- and calmodulin-binding peptides, as well as the HA epitope (SBP-HA-CBP) in frame with their N termini. We recently optimized this system to rapidly and efficiently purify protein complexes from mammalian cells by using dual affinity tags for their analysis by a gel-free LC-MS/MS approach (2, 3). The detection of several proteins previously demonstrated to associate with axin, including APC, CK1α, β-catenin, PP2A, GSK3β, and GSK3α, demonstrates the efficiency of our approach (Fig. 1A, green circles, and Table 1). Remarkably, we found in both AXIN1 and AXIN2 protein complexes the pre-

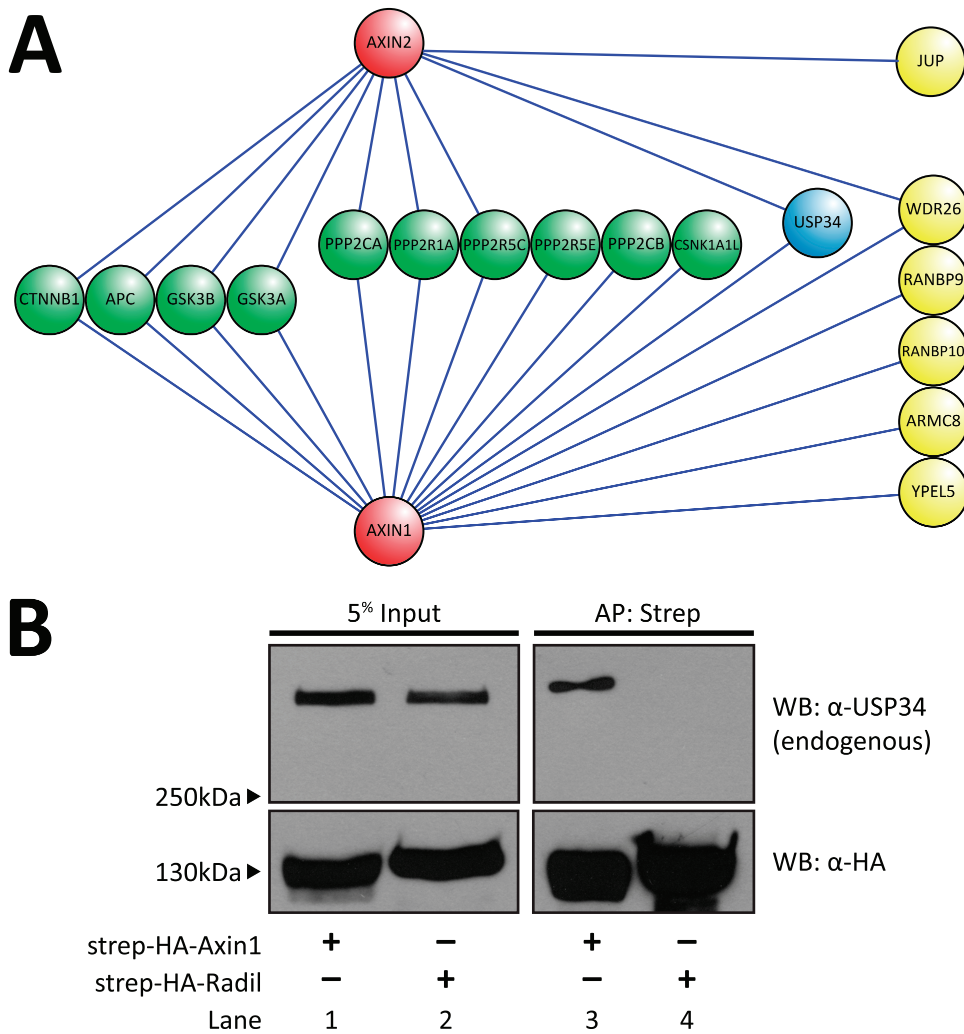

FIG. 1. Identification of ubiquitin-specific protease 34 (USP34) as an axin-interacting protein. (A) Human AXIN1 and AXIN2 protein interaction network. Lines represent interactions found in AXIN1 and AXIN2 (red circles) pulldown experiments by using LC-MS/MS. Green circles are previously described associated proteins, yellow circles are new associations, and the blue circle represents USP34. (B) Confirmation of the axin-USP34 interaction using co-affinity purification. In HEK293T cells, endogenous USP34 associates with AXIN1 (lane 3) but not with the unrelated protein RADIL (lane 4).

viously uncharacterized protein USP34 (Fig. 1A, blue circle, and Table 1), which contains 3,546 amino acids and possesses a central ubiquitin hydrolase domain characteristic of DUBs (33).

The presence of endogenous USP34 in AXIN1 complexes was then confirmed in coimmunoprecipitation studies (Fig. 1B). Cell lysates from HEK293T cells stably expressing SBP-HA-CBP-AXIN1 were subjected to affinity purification using streptavidin affinity chromatography to isolate axin protein complexes and probed for endogenous USP34 using Western blotting with anti-USP34 polyclonal antibodies (Fig. 1B). Importantly, a cell line stably expressing a control protein (RADIL) identically tagged and expressed at similar levels did not coprecipitate with USP34 (Fig. 1B, lane 4). Probably reflecting the transient nature of the interaction, attempts to perform endogenous coimmunoprecipitation of AXIN1 and USP34 were challenging. By stabilizing ubiquitinated axin with MG132, we were, however, able to reproducibly detect small amounts of USP34 in AXIN1 immunoprecipitates (data not shown). We therefore conclude that axin and USP34 are present in the same protein complex.

USP34 confers ubiquitin specific protease activity to the axin complex. Since USP34 belongs to the family of USPs, we next tested the prediction that USP34 confers ubiquitin-protease activity to the axin complex. To test this possibility, we performed ubiquitin-protease assays using purified axin protein complexes. Axin complexes were isolated from SBP-HA-CBP-AXIN1 expressing cells using a single streptavidin affinity chromatography step and were incubated with recombinant K48-linked polyubiquitin chains. The presence of USP activity in the axin complexes was revealed by the production of a band corresponding to cleaved monoubiquitin as detected by Western blotting (Fig. 2A, lane 3).

As an alternative approach to monitor USP activity, we used the newly developed UB-PLA_2 assay (42) to quantify ubiquitin isopeptidase activity present in purified axin complexes.

TABLE 1. Results of a representative LC-MS/MS analysis of AXIN1 and AXIN2 affinity-purified protein complexes[a]

Protein	No. of peptides		Protein accession no.	No. of pulldowns
	Total	Unique		
AXIN1-type proteins				
AXIN1	197	47	NP_003493.1	4
Ubiquitin C	46	42	NP_055524.2	1
GSK3α	36	23	NP_002084.2	4
USP34	35	28	NP_055524.3	3
GSK3β	33	22	NP_063937.2	4
Ubiquitin B precursor; polyubiquitin B	18	1	NP_066289.1	1
PP2A regulatory subunit B56ε	11	7	NP_006237.1	3
WD repeat domain 26	11	9	NP_079436.3	3
PP2 regulatory subunit Aα	9	6	NP_055040.2	3
β-Catenin	7	7	NP_001895.1	1
Armadillo repeat containing 8	6	3	NP_056211.2	2
PP2 catalytic subunit α	5	3	NP_002706.1	3
PP2 catalytic subunit β	5	3	NP_004147.1	3
RAN binding protein 9	5	4	NP_005484.2	3
Yippee-like 5	5	4	NP_057145.1	3
8-Oxoguanine DNA glycosylase isoform 2d	3	2	NP_002533.1	1
APG4 autophagy 4 homolog B	3	2	XP_001126088.1	1
RAN binding protein 10	3	3	NP_065901.1	3
APC	2	2	NP_000029.2	4
CK1α	2	2	NP_660204.1	4
DEP domain containing 2 isoform a	2	2	NP_079146.2	1
PP2A regulatory subunit B56-γ	2	2	NP_848701.1	3
AXIN2-type proteins				
AXIN2	155	19	NP_004646.2	4
Nucleolin	70	10	NP_005372.2	1
GSK3β	42	14	NP_002084.2	4
GSK3α	34	12	NP_063937.2	4
β-Catenin	28	9	NP_001895.1	2
APC	27	16	NP_000029.2	4
USP34	21	13	NP_055524.3	3
Junction plakoglobin	9	6	NP_068831.1	3
PP2 catalytic subunit α/β	5	4	NP_002706.1	2
abl-interactor 1	5	3	NP_005461.2	1
PP2 regulatory subunit Aα	3	3	NP_055040.2	1
Wiskott-Aldrich syndrome protein family member 2	3	2	NP_008921.1	1
PP2A regulatory subunit B56γ	2	2	NP_848701.1	1
α-Catenin	2	1	NP_001894.2	1
Nucleoporin	2	2	NP_055484.2	1
Wiskott-Aldrich syndrome protein family member 1	2	2	NP_003922.1	1

[a] The total peptides column indicates the total number of peptides successfully matched to protein identity, whereas the unique peptides column gives the number of distinct peptide fragments identified. Four independent purifications of AXIN1 and AXIN2 complexes were analyzed by LC-MS/MS, and the results of a representative pulldown experiment are shown in this table. The frequency with which a protein was identified in the four pulldowns is noted in the "No. of pulldowns" column.

Briefly, this assay consists of a fusion protein containing UBIQUITIN fused to the N terminus of PLA_2 used as a reporter enzyme. Since PLA_2 requires a free N terminus to be catalytically active, the UB-PLA_2 fusion is inactive, and its enzymatic activity is only restored upon cleavage of the α-peptide-linked ubiquitin moiety. Since most USPs to date can cleave the α- or ε-linkage with equal efficiency (25, 30), the UB-PLA_2 assay can act as a sensitive and quantitative reporter of ubiquitin isopeptidase activity. Affinity-purified axin or RADIL (control) protein complexes were assayed using the UB-PLA_2 assay as described in Materials and Methods. Robust isopeptidase activity could be detected in axin complexes compared to control complexes that exhibited background activity (Fig. 2B, compare lanes 3 and 5). We then tested whether the isopeptidase activity present in the axin complexes was attributable to USP34 in both of these assays by depleting USP34 levels in SBP-HA-CBP-AXIN1 cells with the stable expression of a *USP34* shRNA that reduced its protein levels by 90% (Fig. 2C). We found that the affinity-purified fraction isolated from equivalent number of cells expressing the *USP34* shRNA was largely devoid of ubiquitin isopeptidase activity (Fig. 2A, lane 4, and Fig. 2B, lane 4). We also expressed and purified the USP34 core region as a recombinant protein in *E. coli* and found that it exhibited robust ubiquitin isopeptidase activity similar to the USP2 core region, which is included as a positive control for the assay (Fig. 2A and B, compare lanes 1 and 2). Variants of this assay where the ubiquitin protein fused to the N terminus of PLA_2 is replaced with other ubiquitin-like proteins such as SUMO, NEDD8, or ISG15 allow the determination of the cleavage specificity of the isopeptidase. Using these different reporters, we showed that the recombinant core USP34 enzyme exhibits specificity for ubiquitin cleavage (Fig. 2D).

These results led us to investigate whether ubiquitin proteases could control the ubiquitination of axin. We used SBP-HA-CBP-AXIN1 stable cells in which we transfected a FLAG-ubiquitin expression plasmid. Axin was then affinity purified by streptavidin affinity chromatography, and the ubiquitin-axin (UB-axin) conjugates were detected by Western blotting with FLAG antibodies. Under our normal protein isolation conditions (first incubated with 1% SDS to disrupt protein-protein interactions), only small amounts of ubiquitinated axin could be detected (Fig. 2E, lane 1). However, since USP34 is present in axin complexes, it may cleave axin-linked ubiquitin chains. We thus inhibited USP activity by incorporating the sulfhydryl alkylating agent NEM in the lysis buffer. NEM is known to react with the catalytic cysteine residue within USP core domains to irreversibly inhibit their protease activity. Under conditions where NEM is present, we detected robust polyubiquitination of axin (Fig. 2E, lane 2). To directly show that USP34 can cleave ubiquitin chains conjugated to axin, we performed an *in vitro* deubiquitination reaction. We purified UB-axin conjugates and the catalytic core domain of USP34 by affinity purification from HEK293 transfected cells. We then incubated equivalent amount of UB-axin with (Fig. 2F, lane 2) or without (Fig. 2F, lane 1) USP34 core proteins and showed that the core domains could efficiently cleave the ubiquitin chains associated with axin. As a control, we generated a catalytically inactive USP34 core domain (C1903S), performed the same experiment, and showed that it had no effect on UB-axin conjugates (Fig. 2F, lane 3).

Our results suggest that axin protein complexes exhibit ubiquitin-protease activity and that ubiquitin proteases regulate the steady-state ubiquitination of axin. We also conclude that USP34 can directly deubiquitinate axin.

USP34 regulates the stability of axin. Since axin's stability has been found to be controlled by the UPS (20, 23), we tested whether USP34 was involved in this process. We treated cells stably expressing Venus-AXIN1 with control or USP34 siRNAs and determined the impact on the steady-state levels of axin proteins by fluorescence microscopy and Western blotting. Consistent with USP34 regulating the ubiquitination status of axin, its knockdown led to a robust decrease in Venus-

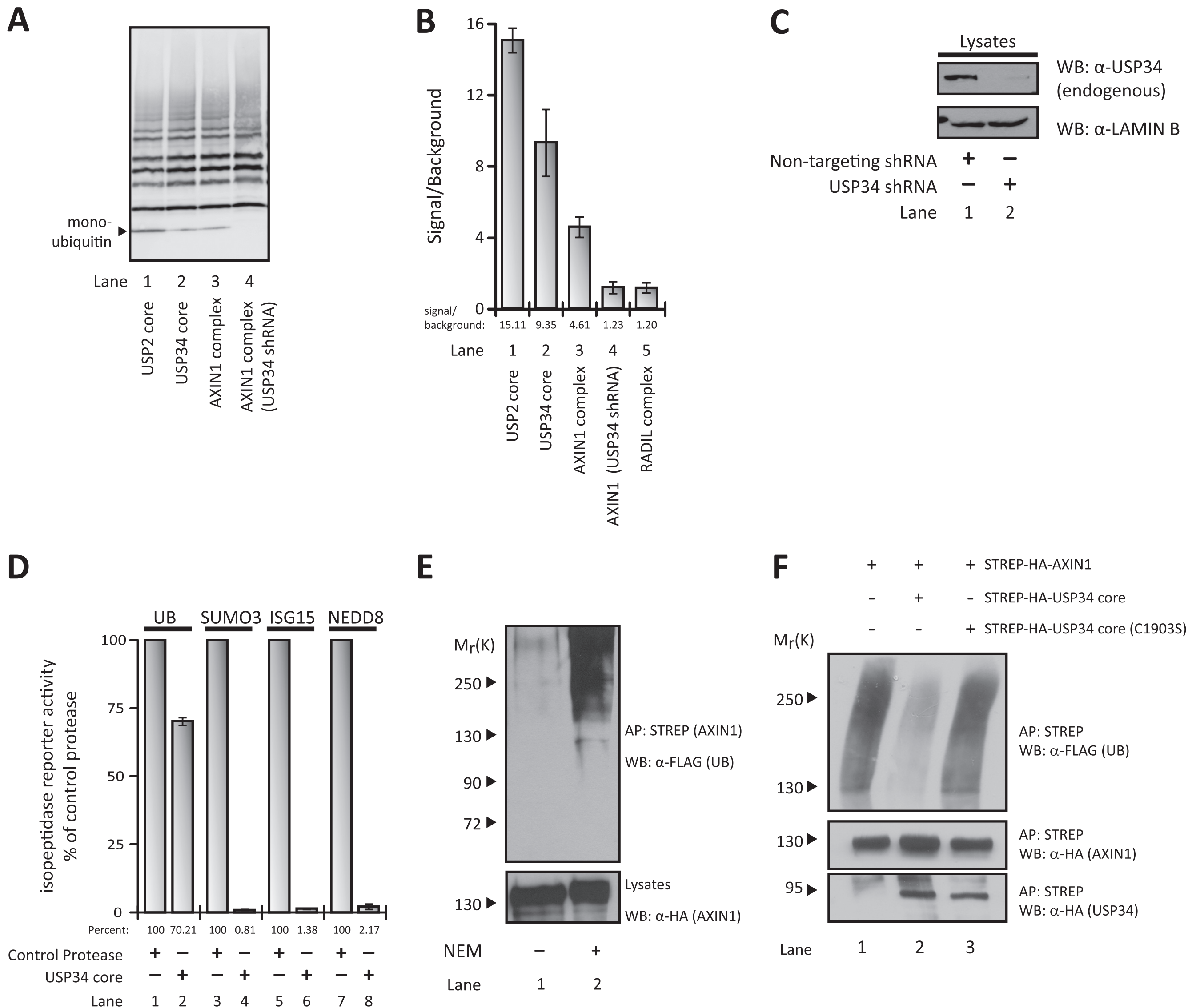

FIG. 2. USP34 confers ubiquitin protease activity to the axin protein complex. (A) Cleavage of K48-linked ubiquitin by recombinant USP2 and USP34 core domains and by purified AXIN1 protein complexes but not by AXIN1 protein complexes isolated from cells where USP34 expression was knocked down using shRNA. Cleavage efficiency is monitored with the appearance of monoubiquitin from the polyubiquitin chains. (B) Quantification of ubiquitin protease activity using the ubiquitin-PLA2 assay. Purified AXIN1 complexes from SBP-HA-CBP-AXIN1 cells but not from cells expressing a *USP34* shRNA exhibited USP activity. A similar amount of the unrelated RADIL protein complex showed no activity. Recombinant USP2 and USP34 were used as positive controls in this assay. (C) Western blot verification of endogenous *USP34* knockdown in HEK293T SBP-HA-CBP-AXIN1 cells stably expressing *USP34* shRNA. (D) Cleavage specificity of the USP34 core domains. UB-, SUMO3-, ISG15-, and NEDD8-PLA$_2$ assays were used to demonstrate that the USP34 core domain preferentially cleaves ubiquitin. (E) Ubiquitinated axin is sensitive to USP activity. Cells stably expressing SBP-HA-CBP-AXIN1 were transfected with FLAG-ubiquitin. Input lysates prepared for streptavidin affinity purification were left untreated (lane 1) or treated with the nonspecific cysteine protease inhibitor NEM (lane 2). Ubiquitin-linked axin conjugates were resolved by SDS-PAGE and detected by using anti-FLAG antibodies (top panel). Equivalent pulldown of axin was monitored using anti-HA antibodies (bottom panel). The inhibition of USP activity robustly increased the amount of ubiquitinated axin (compare lane 2 to lane 1). (F) The USP34 core domain deubiquitinates axin *in vitro*. FLAG-ubiquitin-axin conjugates, wild-type (WT), and catalytically inactive (C1903S) core domains of USP34 were separately purified from transfected cells using affinity purification. FLAG-UB-axin proteins were then incubated alone (lane 1) or with WT (lane 2) or catalytically inactive USP34 core domain (lane 3) proteins for 1 h. The reaction was stopped by addition of 2× sample buffer, and samples were run on an 8% SDS-PAGE. UB-axin conjugates were detected by using FLAG antibodies and AXIN1 and the core domains of USP34 with HA antibodies.

AXIN1 levels (Fig. 3A, compare lanes 1 and 2 on the Western blots). This could be efficiently rescued when the cells were treated with 5 μM MG132 for 10 h prior to assay (Fig. 3A, compare lanes 2 and 5). These results are consistent with the possibility that reducing USP34 function leads to an increase in axin ubiquitination, thereby targeting it for proteolysis by the 26S proteasome. Similar results were obtained when we studied the stability of endogenous AXIN1 (Fig. 3A, Western blot at bottom right). Importantly, two independent USP34 siRNAs (A and D) had the same effect on axin stability (data not

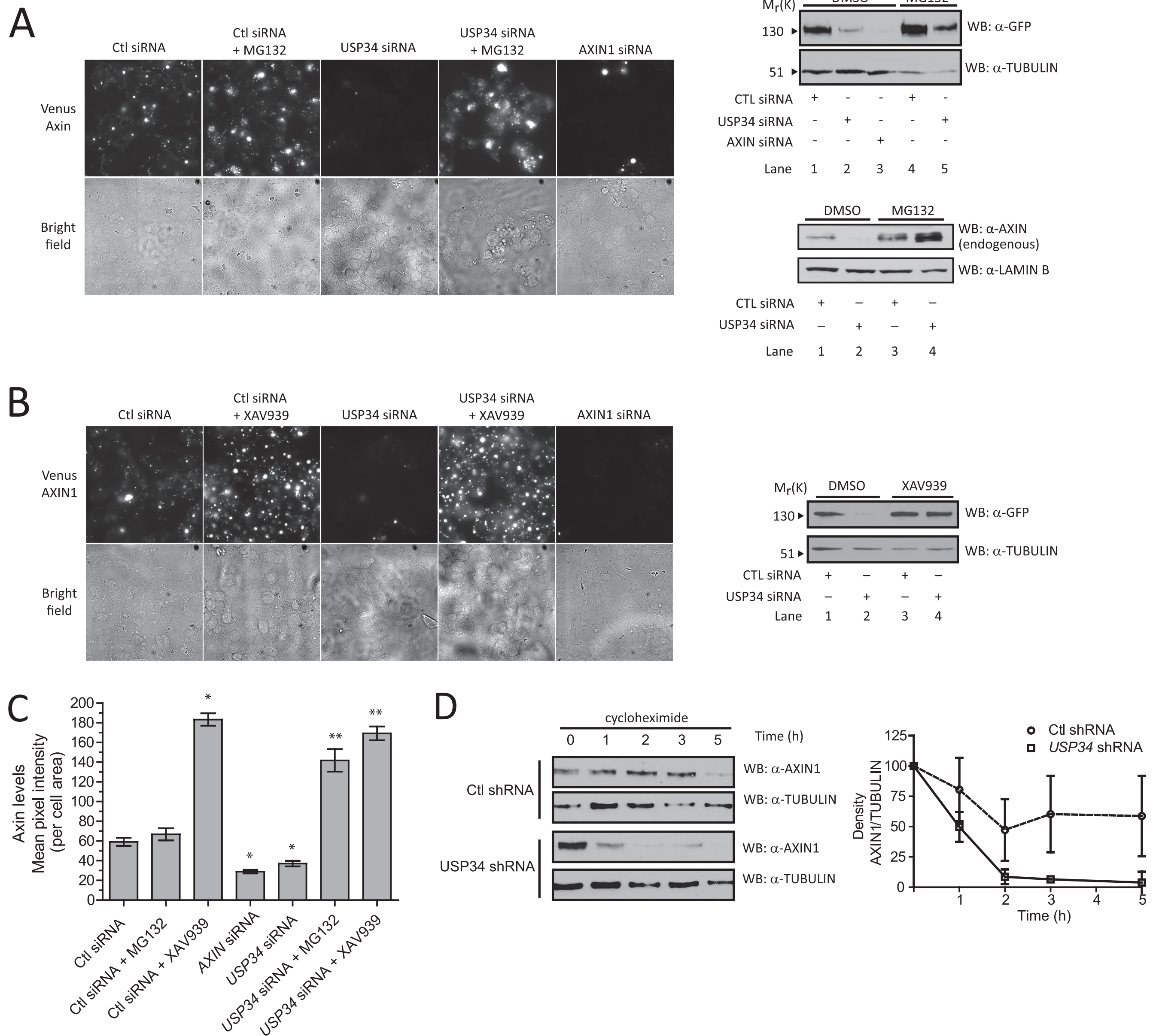

FIG. 3. USP34 regulates the stability of axin. (A) A HEK293 cell line stably expressing Venus-AXIN1 was derived. These cells were transfected with control, AXIN1, or USP34 siRNAs. At 48 h posttransfection the cells were fixed or lysed, and the levels of Venus-AXIN1 were evaluated by fluorescence microscopy (left panels) or Western blotting with anti-GFP antibodies (top right panels). Where indicated, MG132 was added for the last 10 h to inhibit the proteasome. A similar experiment using WT-HEK293 cells and probing for endogenous AXIN1 with a monoclonal rabbit antibody was also performed (bottom right panels). (B) The same experiment performed in panel A was repeated but instead of MG132 the tankyrase inhibitor XAV939 was added for the last 12 h. AXIN1 levels were monitored by fluorescence microscopy (left panels) and Western blotting with anti-GFP antibodies (right panels). Both MG132 and XAV939 were able to rescue the degradation of AXIN1 resulting from the depletion of USP34. (C) Quantification of panels A and B. Regions of interest were drawn randomly around the periphery of cells taking the phase-contrast images as a template, and the pixel intensities of the fluorescence images were quantified by using ImageJ ($n = 20$ to 25 cells). Error bars represent the standard error of the mean (SEM); a single asterisk represents statistical significance compared to the control (Ctl) siRNA condition, whereas double asterisks represent statistical significance compared to the USP34 siRNA condition. (D) USP34 regulates the turnover of axin. HEK293 cells expressing control or USP34 shRNA were treated with 3 μM XAV939 for 12 h. Cells were washed twice and were switched to medium containing 10 μg of cycloheximide/ml for the indicated times. Lysates were collected and proteins resolved on by SDS–8% PAGE. Axin levels were determined by Western blotting with anti-AXIN1 antibodies (left), quantified by densitometry, and normalized to tubulin levels for each time point (right).

shown). To quantify axin levels, we have also measured the pixel intensities in images taken for several independent cells in the different conditions (Fig. 3C).

Recently, the tankyrase-dependent parsylation of axin was demonstrated to be a prerequisite for its ubiquitination, and the small molecule tankyrase inhibitor XAV939 was shown to stabilize axin and to inhibit Wnt signaling (20). If the degradation of axin induced by the depletion of *USP34* is mediated

through the regulation of its ubiquitination, treating *USP34*-depleted cells with XAV939 should rescue the degradation of axin. To test this prediction, we incubated green fluorescent protein (GFP)-AXIN1 expressing cells with control or USP34 siRNAs for 48 h and added XAV939 for the last 12 h. We showed that XAV939 reversed the degradation of axin resulting from USP34 depletion (Fig. 3B, compare lanes 2 and 4 on Western blot). To determine whether USP34 controls the turnover of axin, we performed a cycloheximide chase analysis. To perform this experiment, we used HEK293 cells expressing control or USP34 shRNA and first stabilized the endogenous pool of axin by treating the cells with XAV939 for 16 h. We then washed the XAV939 to restore the ubiquitination of axin and incubated the cells for different times in the presence of the protein synthesis inhibitor cycloheximide. We observed that the knockdown of USP34 leads to a precocious turnover of axin proteins compared to control shRNA-expressing cells (Fig. 3D). We conclude that USP34 controls the levels of axin by opposing its tankyrase-dependent ubiquitination.

USP34 positively regulates β-catenin-dependent transcription downstream of the destruction complex. We next assessed the functional importance of USP34 for Wnt signal transduction using RNAi. The effectiveness of four independent siRNAs designed to target the USP34 mRNA was first established by immunoblotting. RKO cells were transfected with control or USP34 siRNAs for 48 h and endogenous USP34 protein levels were subsequently measured by using Western blot with anti-USP34 antibodies. All four USP34 siRNAs could block protein expression with various efficiencies (Fig. 4A, bottom panels), and similar results were obtained with these siRNAs in downstream experiments. This is especially relevant since we were unable to clone and express a full-length USP34 cDNA (10,638 bp) to perform rescue experiments. We also attempted to rescue the USP34 siRNA effect with a cDNA expressing only the core domain but observed no effect (data not shown). Given that the core domain was sufficient to deubiquitinate AXIN1 *in vitro* (Fig. 2F), this suggests that other domains of USP34 are required *in vivo*, possibly to control the subcellular localization of USP34 or to regulate its activity. Since it consistently yielded the best knockdown we therefore carried all subsequent experiments with siRNA A. HEK293T and RKO cell lines stably expressing a β-catenin luciferase reporter and a *Renilla* luciferase control protein were then transfected with control, β-*CATENIN*, or *USP34* siRNAs. In the control-transfected HEK293T and RKO cells, addition of Wnt3A led to 29- and 28-fold activations of the reporter, respectively (Fig. 4B, lanes 4 and 10) compared to cells treated with control conditioned medium. *USP34*-depleted cells showed a reduction in Wnt3A-mediated activation to 5.9- and 10.9-fold in the HEK293T and RKO cells, respectively (Fig. 4B, lanes 6 and 12). The impact of *USP34* depletion was comparable, albeit less dramatic, to the depletion of β-*CATENIN* (Fig. 4B, lanes 5 and 11). We therefore conclude that USP34 acts as a positive regulator of Wnt signaling.

To functionally position USP34 within the Wnt pathway we next performed epistasis experiments where we tested the ability of the *USP34* siRNA to block pathway activation at different levels. Strikingly, *USP34* depletion inhibited the β-catenin reporter activity driven by the ectopic expression of a degradation-resistant form of β-catenin (Fig. 4C, compare lanes 4 and 2), as well as by Dishevelled (data not shown), but not by the constitutively activated chimeric VP16-LEF1 protein, a fusion protein between the activation domain of VP16 and LEF1 (19) known to be insensitive to β-catenin transactivation properties (Fig. 4C, lanes 6 to 8). These results position the function of USP34 downstream of the β-catenin stabilization step and argue that USP34 activity is important for the full activation of target genes. If USP34 functions downstream of the destruction complex, its knockdown should not influence the stabilization of β-catenin in response to Wnt pathway activation. To test this prediction, we used RKO cells, which lack β-catenin at adherent junctions. Under resting conditions these cells have minimal amount of cytosolic β-catenin whose levels can be strongly induced by Wnt3A conditioned medium (Fig. 4D). We performed a time course experiment of Wnt3A-induced β-catenin stabilization in control or *USP34* siRNA-treated cells and observed that both the kinetics and the magnitude of β-catenin stabilization were unchanged (Fig. 4D, middle panels). Importantly, we show that USP34 was efficiently knocked down in *USP34* siRNA-treated cells (Fig. 4D, upper panels).

To further support the site of USP34 action downstream of the destruction complex, we tested the effect of USP34 depletion on the constitutive Wnt signaling observed in SW480 and HCT116 colon cancer cells. These two cell lines were chosen since they harbor inactivating APC and activating β-catenin mutations, respectively. To monitor β-catenin-dependent transcription, the cell lines were transduced with lentivirus coding for the β-catenin responsive luciferase reporter TopFlash and for *Renilla* luciferase under the control of the constitutive EF1α promoter as a normalization probe. Since the Wnt pathway is strongly and constitutively activated in these cells (39, 55), a high ratio of firefly/*Renilla* luciferase activity was predictably observed (Fig. 4E, lanes 1 and 4). The constitutive reporter activity was β-catenin dependent since β-catenin knockdown virtually eliminated the TopFlash signal (Fig. 4E, lanes 3 and 6). *USP34* knockdown also inhibited β-catenin signaling, indicating that it is also required in this context (Fig. 4E, lanes 2 and 5). To confirm whether the results obtained using the synthetic TopFlash reporter could be applicable to *bona fide* Wnt target genes, we examined the impact of *USP34* depletion on the transcript levels of *NAKED1* and *TNFRSF19*, two genes strongly regulated by β-catenin in colon cancer cells (35). Knockdown of USP34 in SW480 cells reduced the steady-state levels of the *NAKED1* and *TNFRSF19* transcripts by 38 and 56%, respectively (Fig. 4F, compare lanes 2 and 5 versus lanes 1 and 4). We conclude that USP34 is required at a step subsequent to β-catenin stabilization.

Role of axin downstream of the β-catenin destruction complex during Wnt signaling. Axin has previously been shown to localize to the nuclei of colon cancer cells (1), to undergo nucleocytoplasmic shuttling (11, 57), and to translocate to the nucleus after Wnt stimulation in normal cells (61). Furthermore, we noted in a recent whole-genome siRNA screen of the Wnt pathway that depletion of the two *AXIN* genes in colon cancer cells with stabilized β-catenin consistently led to the inhibition of β-catenin-mediated transcription (35). These results argue that axin, in addition to its well-described function as a negative regulator of the pathway, may be required downstream of the destruction complex to fulfill positive regulatory roles during pathway activation. We first confirmed these re-

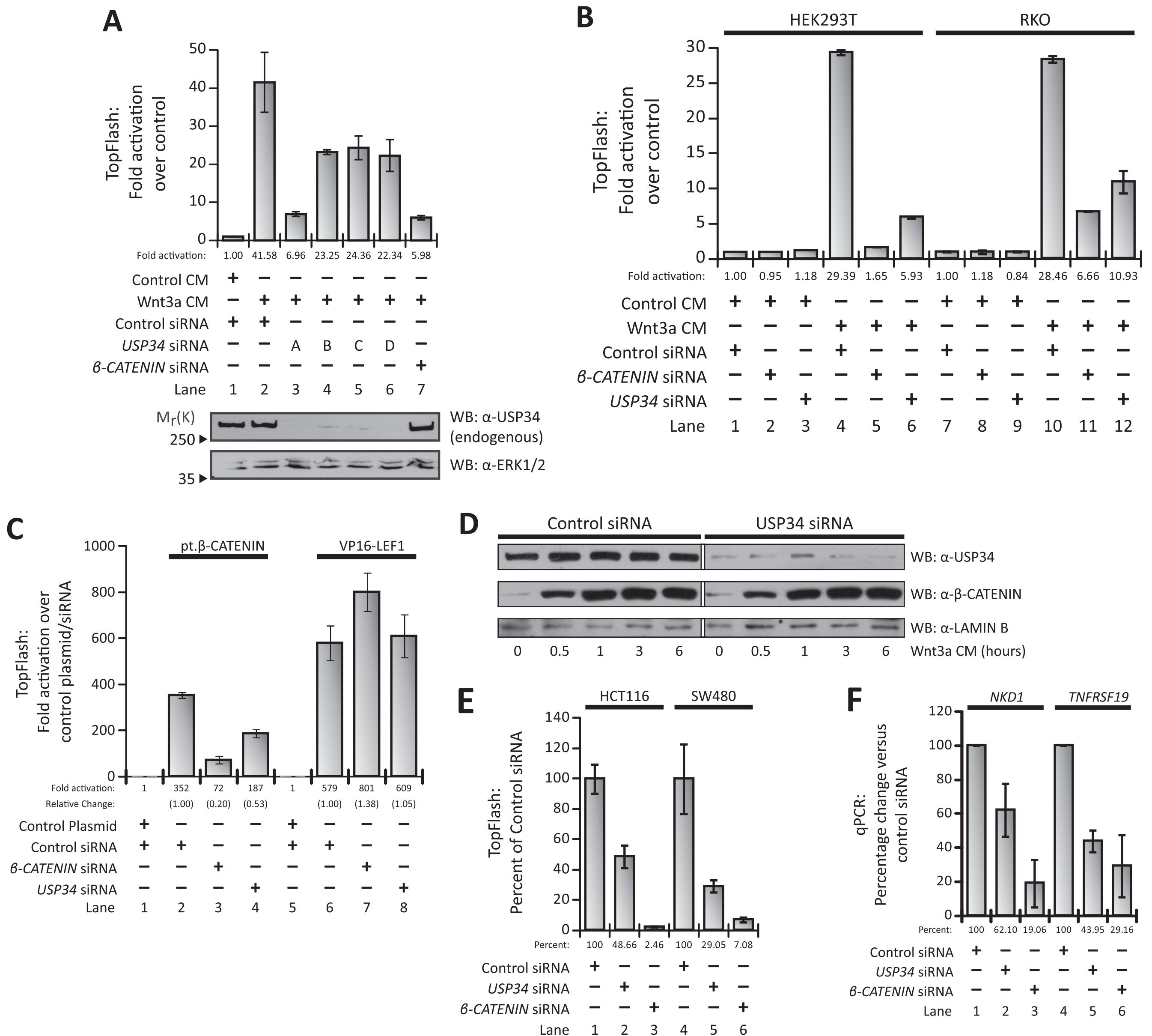

FIG. 4. USP34 has a positive regulatory function in Wnt signaling. (A) Validation of USP34 siRNAs. Lysates from RKO cells treated with control siRNA (lanes 1&2), four different *USP34* siRNAs (lanes 3 to 6) or β-*CATENIN* siRNA (lane 7) were resolved by SDS-PAGE and probed with anti-USP34 antibodies to monitor USP34 knockdown and anti-ERK antibodies as loading controls (bottom panels). Each *USP34* siRNA was able to reduce the Wnt3A stimulated activation of the TopFlash reporter (top panel). (B) TopFlash assays were performed in HEK293T and RKO cells treated with control (lanes 1, 4, 7, and 10), β-*CATENIN* (lanes 2, 5, 8, and 11), or *USP34* (lanes 3, 6, 9, and 12) siRNA and stimulated with control conditioned medium (lanes 1, 2, 3, 7, 8, and 9) or Wnt3A conditioned medium (lanes 4, 5, 6, 10, 11, and 12). USP34 knockdown inhibited Wnt3A-mediated activation of the reporter in both cell lines (compare lanes 4 and 6 to lanes 10 and 12). (C) Epistasis analysis of USP34 function. TopFlash assays in HEK293T cells showed that *USP34* and β-*CATENIN* siRNAs antagonized the activation of the pathway by overexpression of the stabilized form of β-catenin (compare lanes 2, 3, and 4) but not by the chimeric VP16-LEF1 protein (compare lanes 6, 7, and 8). (D) USP34 knockdown does not influence Wnt3A-induced stabilization of β-catenin. RKO cells treated with control or *USP34* siRNA were stimulated with Wnt3A conditioned medium for the indicated durations, and lysates were probed for β-catenin levels by using Western blotting. (E) TopFlash assays in HCT116 and SW480 cells treated with *USP34* siRNA showed that the constitutive activation of the β-catenin reporter in these cells requires USP34 function (compare lanes 1 and 2 to lanes 4 and 5). (F) Quantitative RT-PCR analysis of SW480 cells treated with *USP34* siRNA shows reduced expression of Wnt target genes *NKD1* and *TNFRSF19*. The levels are expressed as a percentage of the control siRNA.

sults by depleting *AXIN1* and *AXIN2* transcripts in SW480 and HCT116 by siRNA. As suggested by Major et al. (35), we found that independent knockdown of *AXIN1* and *AXIN2* using established siRNAs (34) inhibited Wnt reporter activity in both cell lines (data not shown). When transfected together, *AXIN1* and *AXIN2* siRNAs reduced the constitutive activity of the reporter to 64 and 30% of control siRNA-treated HCT116 and SW480 cells, respectively (Fig. 5A, lanes 2 and 5). This is in sharp contrast to what is usually observed in cells presumed to have intact Wnt pathway components, such as human

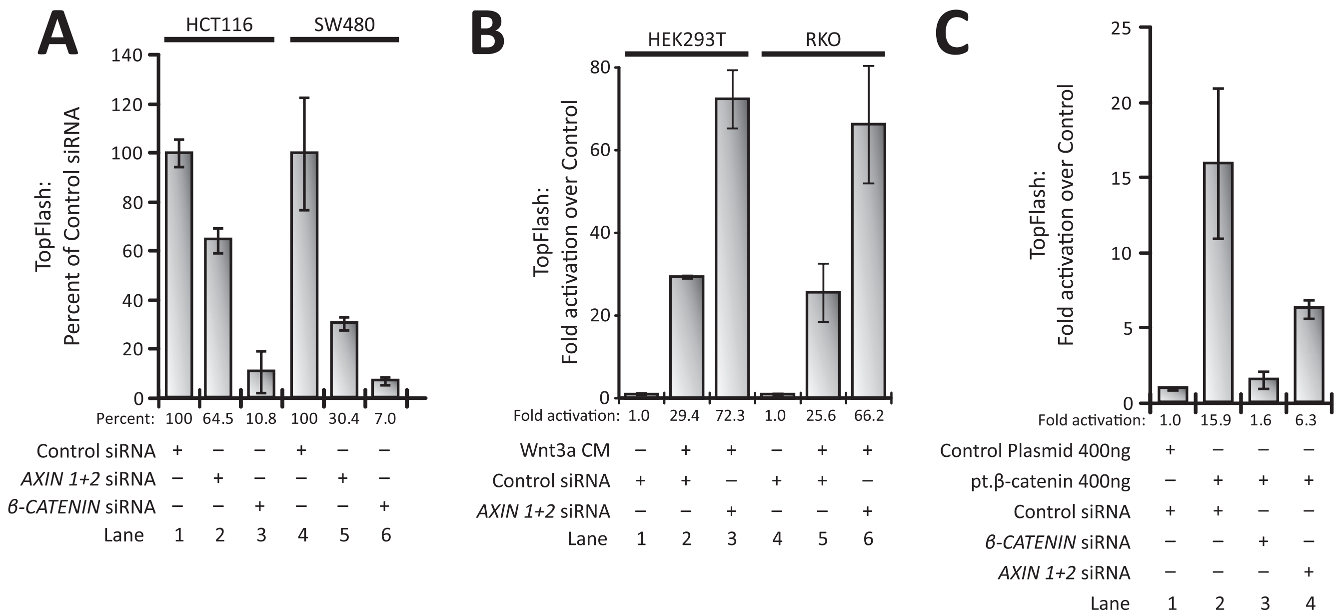

FIG. 5. Positive role of axin during Wnt signaling in colon cancer cells. TopFlash assays in HCT116 and SW480 colon cancer cells (A) or HEK293T and RKO cells treated with control, β-catenin, or AXIN siRNAs (B) were performed. In panel A, *AXIN1* and *AXIN2* siRNAs inhibit the constitutive Wnt pathway activation due to mutations in APC (SW480) or β-catenin (HCT116), while in panel B AXIN1 plus AXIN2 knockdowns potentiate the Wnt3A-stimulated activity of the reporter. (C) Activation of the Wnt pathway in HEK293T cells using a degradation resistant β-catenin mutant (pt.β-catenin) is inhibited by *AXIN1* plus *AXIN2* siRNAs. Figures are representative of at least three independent experiments performed in duplicates, where the error bars represent the standard errors. In panel A, TopFlash levels are expressed as the percent activation compared to the basal constitutive levels observed with control siRNA (100%).

HEK293 kidney cells or RKO colon carcinoma cells. In a resting state, these cells exhibit a low level of spontaneous activity of the β-catenin reporter that can be strongly induced by treatment with Wnt3A conditioned medium (Fig. 5B, lanes 2 and 5). In this context, however, and as expected from the known negative role of axin proteins in Wnt signal transduction, the reduction of function for AXIN1 and AXIN2 potentiated the Wnt3A-mediated activation (Fig. 5B, compare lanes 3 and 6 to lanes 2 and 5). However, when a degradation-resistant mutant of β-catenin is introduced in HEK293T cells to constitutively activate the pathway, a condition mimicking the mutated state of the signaling cascade in HCT116 colon cancer cells, the activity of the TopFlash reporter was antagonized by *AXIN* knockdown (Fig. 5C, compare lanes 4 and 2).

Since activation of the Wnt pathway in SW480 and HCT116 cells results from β-catenin escaping its normal regulation by the destruction complex, the results presented above suggest that axin performs a positive regulatory function downstream of β-catenin stabilization when the destruction complex is disassembled. This prompted us to examine the subcellular localization of axin in these cells with an anti-AXIN1 peptide antibody by indirect immunofluorescence. Consistent with previous reports (1), axin is localized mostly to the nuclei of these cells (Fig. 6A, left panels). Pretreatment with *AXIN1* siRNA largely eliminated the observed immunoreactivity, confirming the specificity of the AXIN1 antibody (Fig. 6A, middle panels). Treatment of SW480 and HCT116 cells with *USP34* siRNA established that the accumulation of axin in the nuclei of colon cancer cells depends on USP34 (Fig. 6A). Treatment of the cells with MG132 could rescue the nuclear localization of axin in *USP34* siRNA-treated cells. Together with the results described above, this supports a role for USP34 in controlling axin stability and exclude that the reduction of nuclear axin could be due to an effect on nuclear import and/or export.

At steady state, in cells with normal Wnt signaling, axin is mostly found in the cytoplasm but undergoes nucleocytoplasmic shuttling as it accumulates in the nucleus when cells are treated with the CRM1-dependent nuclear export inhibitor LMB (11, 57). We thus sought to determine whether the stabilization of axin by USP34 is required to observe the accumulation of axin in the nucleus after LMB treatment. As shown in Fig. 6B, whereas axin is predominantly nuclear in control-transfected HEK293T cells incubated with LMB, axin does not accumulate in the nuclei of *USP34*-depleted cells. These results indicate that the activity of USP34 is important for the nuclear accumulation of axin.

We conclude from these observations that in transformed colon cancer cells, where Wnt signaling is constitutively activated, axin plays a positive regulatory role. In two genetically different colon cancer cells exhibiting defective Wnt signaling and in LMB-treated HEK293T cells where the Wnt pathway is normal, axin exhibits a USP34-dependent nuclear accumulation. The simplest explanation for these results is that the inhibition of Wnt signaling resulting from USP34 depletion (Fig. 4) is a consequence of axin destabilization and impaired nuclear accumulation.

DISCUSSION

The ubiquitin-proteasome system controls multiple steps in Wnt signaling through the regulation of protein stability or

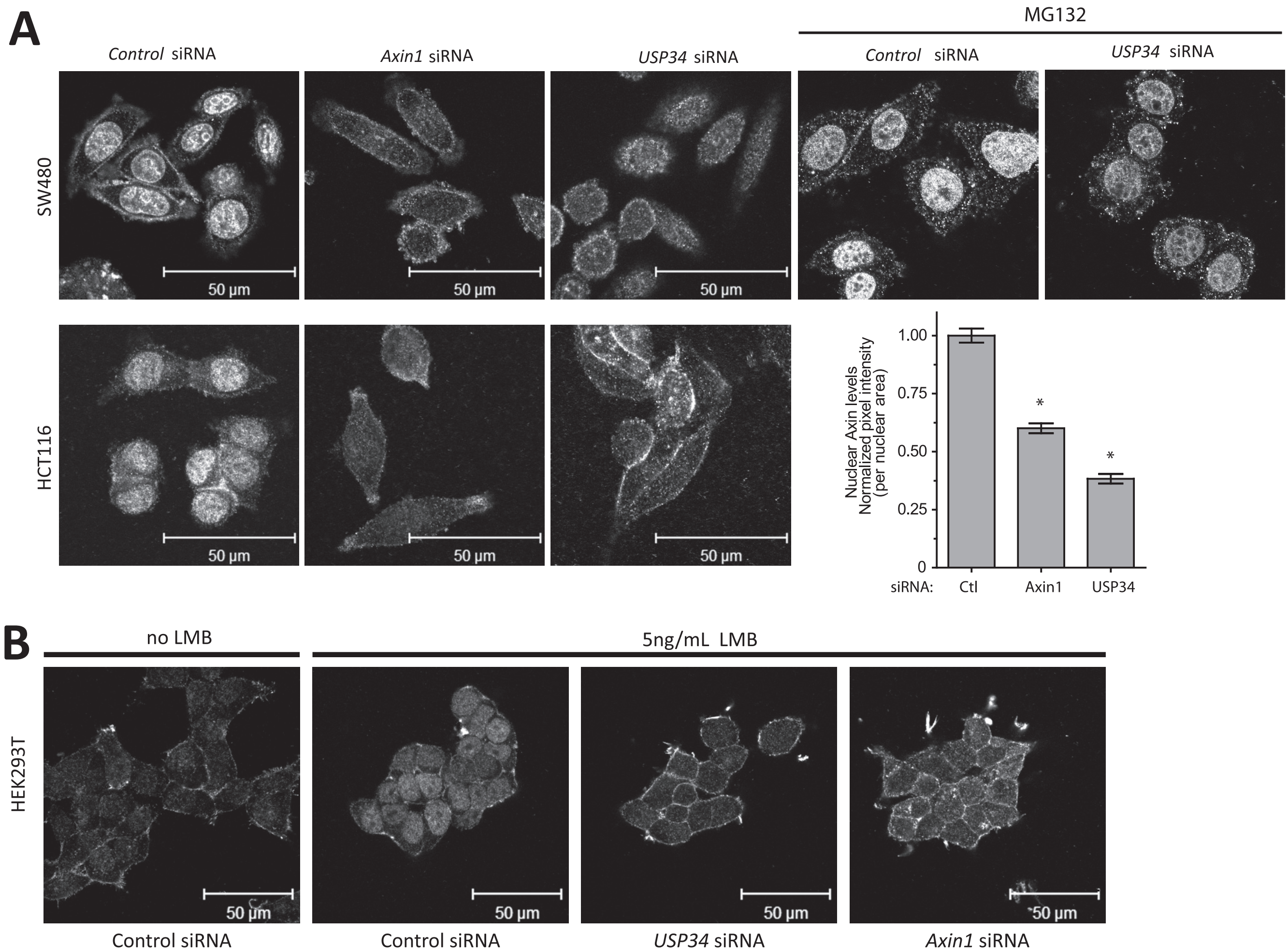

FIG. 6. USP34 controls the nuclear accumulation of axin. (A) AXIN1, detected by using polyclonal anti-AXIN1 antibodies, localizes to the nuclei of SW480 and HCT116 colon cancer cells (left panels). The specificity of the antibody was controlled using AXIN1 siRNA (middle panels). USP34 siRNA (right panels) inhibits the strong nuclear localization of axin seen in cells treated with control siRNA (left panels). Where indicated, 1 μM MG132 was added for the last 12 h. For quantification, regions of interest were drawn around the nuclear region, as indicated by counterstaining and the pixel intensity quantified using ImageJ ($n > 80$ cells). Error bars represent the SEM. An asterisk indicates statistical significance compared to the Ctl siRNA condition. (B) USP34 depletion also inhibits the nuclear accumulation of axin in HEK293T cells observed when the CRM1-dependent nuclear export is blocked with LMB.

function. Recently, axin has been shown to be parsylated by tankyrase, a step required for its ubiquitin-dependent degradation (20). Although the precise mechanisms controlling axin levels are still unknown, we highlighted here the importance of axin as a key regulatory node in Wnt signaling. Our findings add to the mechanism controlling axin levels by identifying USP34 as the ubiquitin-specific protease opposing the tankyrase-dependent ubiquitination of axin and show that this regulation is important for the nuclear accumulation of axin during Wnt signaling to positively influence β-catenin-mediated transcription.

USP34 regulates axin stability. Our data agree with those of other researchers to show that axin levels are dynamically regulated by the ubiquitin-proteasome system (20, 23). Recently, the tankyrase-mediated parsylation of axin was showed to be required for its ubiquitination and degradation. The E3 ubiquitin ligase catalyzing axin ubiquitination remains to be identified, but our findings suggest that USP34 counteracts this reaction. Recently, the SUMOylation of axin on a C-terminal domain and its phosphorylation by GSK3 were shown to protect axin from ubiquitination (23). Although the precise functional interplay between these processes and USP34 needs to be studied, one possibility is that these signals recruit USP34 to axin to promote its deubiquitination. Another important question to be addressed is the understanding of the cellular signals that control USP34 and ultimately axin ubiquitination and stability. For example more work is now needed to determine whether the catalytic activity of USP34 is regulated during Wnt signaling and/or whether axin is recruited to USP34 following the disassembly of the destruction complex.

Positive role for nuclear axin during Wnt/β-catenin signaling. Although the precise mechanistic details remain unclear, our study suggests that axin plays a positive role in the nucleus for the transmission of Wnt/β-catenin signaling. In cells with normal Wnt pathways, axin undergoes nuclear-cytoplasmic shuttling (11, 57), a process suggested to be important for the

nuclear export of β-catenin in the absence of Wnt signaling. However, the precise function of the nuclear pool of axin and the regulatory mechanisms influencing this localization are still poorly defined. Our study contributes to the understanding of this process by unraveling a positive regulatory role of axin in the nucleus during signaling and identifying USP34 as a protein influencing its stability. The positive signaling role for nuclear axin has eluded the numerous screens and studies performed on the Wnt pathway. It is likely that, under normal circumstances, this positive function is masked by the strong negative regulatory task of axin within the destruction complex. Supporting this, the positive roles of GSK3 and axin at the plasma membrane when they are recruited to the Wnt coreceptor LRP6 have also escaped these screens. Similarly, the appreciation that APC has dual positive and negative regulatory functions for Wg signaling has only been uncovered recently using a repressor screen in *Drosophila* (52). Alternatively, it is possible that in lower organisms where the majority of the pioneer screens have been performed, axin does not perform nuclear functions. The use of two colon cancer cell lines where the destruction complex machinery is defective and where axin accumulates in the nucleus has allowed us to uncover this novel function. Further work is now needed to address how axin exert this positive role in the nucleus. Two possibilities are that axin serves as escort protein for β-catenin and influences its residency in the nucleus or that axin is an integral part of the β-catenin transcriptional machinery participating in the recognition of target genes as recently found for Dsh (15). In any case, axin is likely not absolutely required for β-catenin signaling but rather could determine the duration and the strength of signaling by regulating the availability of activated nuclear β-catenin. What emerges from our study, however, is that the tight regulation of the balance of axin ubiquitination/deubiquitination is likely an important control point during Wnt signaling.

Compounds interfering with axin stability as drug targets. Since the nuclear localization of axin and β-catenin are associated with Wnt pathway activation and are constitutively found in the nucleus in several human cancers, the control of their residency in the nucleus by modulating USP34 activity could represent a novel therapeutic approach for not only the treatment of cancers but of the numerous human diseases exhibiting spurious Wnt/β-catenin pathway activity. Current strategies for small molecule inhibitors of the Wnt pathway have relied, with limited success, on compounds inhibiting protein-protein interactions (14, 28). The protease activity of USPs, however, makes them highly amenable to inhibition by small molecules (13). That promoting axin stability or degradation both lead to inhibition of Wnt signaling suggests that the precise control of axin levels dictates the outcome of signaling but also indicates that the ubiquitin-proteasome system may be adjusting the availability of different pools of axin underlying its negative function within the destruction complex and its positive role in the nucleus.

ACKNOWLEDGMENTS

We are grateful to Daniel Durocher, Ben Nicholson, Michael Mattern, and the members of the Angers lab for providing comments on the manuscript. We also thank Anne-Claude Gingras for her expertise and David Sterner and Xufan Tian for cloning, expressing, and purifying USP34.

S.A. holds the Canada Research Chair in Functional Architecture of Signal Transduction. This study was supported by a grant from the Canadian Institute for Health Research (CIHR 171619) to S.A. Work at Progenra, Inc., is supported by NIH grants HL083527, CA115205, DK071391, and CA132246.

REFERENCES

1. **Anderson, C. B., K. L. Neufeld, and R. L. White.** 2002. Subcellular distribution of Wnt pathway proteins in normal and neoplastic colon. Proc. Natl. Acad. Sci. U. S. A. **99:**8683–8688.
2. **Angers, S., et al.** 2006. Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. Nature **443:**590–593.
3. **Angers, S., et al.** 2006. The KLHL12-Cullin-3 ubiquitin ligase negatively regulates the Wnt-β-catenin pathway by targeting Dishevelled for degradation. Nat. Cell Biol. **8:**348–357.
4. **Axelrod, J. D., J. R. Miller, J. M. Shulman, R. T. Moon, and N. Perrimon.** 1998. Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Genes Dev. **12:** 2610–2622.
5. **Bilic, J., et al.** 2007. Wnt induces LRP6 signalosomes and promotes dishevelled-dependent LRP6 phosphorylation. Science **316:**1619–1622.
6. **Bookout, A. L., C. L. Cummins, D. J. Mangelsdorf, J. M. Pesola, and M. F. Kramer.** 2006. High-throughput real-time quantitative reverse transcription PCR. Curr. Protoc. Mol. Biol. Chapter **15:**Unit 15 8.
7. **Capelluto, D. G., et al.** 2002. The DIX domain targets dishevelled to actin stress fibres and vesicular membranes. Nature **419:**726–729.
8. **Caspi, M., A. Zilberberg, H. Eldar-Finkelman, and R. Rosin-Arbesfeld.** 2008. Nuclear GSK-3beta inhibits the canonical Wnt signalling pathway in a beta-catenin phosphorylation-independent manner. Oncogene **27:**3546–3555.
9. **Choi, J., S. Y. Park, F. Costantini, E. H. Jho, and C. K. Joo.** 2004. Adenomatous polyposis coli is downregulated by the ubiquitin-proteasome pathway in a process facilitated by axin. J. Biol. Chem. **279:**49188–49198.
10. **Clevers, H.** 2006. Wnt/β-catenin signaling in development and disease. Cell **127:**469–480.
11. **Cong, F., and H. Varmus.** 2004. Nuclear-cytoplasmic shuttling of axin regulates subcellular localization of beta-catenin. Proc. Natl. Acad. Sci. U. S. A. **101:**2882–2887.
12. **Davidson, G., et al.** 2005. Casein kinase 1γ couples Wnt receptor activation to cytoplasmic signal transduction. Nature **438:**867–872.
13. **Daviet, L., and F. Colland.** 2008. Targeting ubiquitin specific proteases for drug discovery. Biochimie **90:**270–283.
14. **Emami, K. H., et al.** 2004. A small molecule inhibitor of β-catenin/CREB-binding protein transcription. Proc. Natl. Acad. Sci. U. S. A. **101:**12682–12687.
15. **Gan, X. Q., et al.** 2008. Nuclear Dvl, c-Jun, β-catenin, and TCF form a complex leading to stabilization of β-catenin-TCF interaction. J. Cell Biol. **180:**1087–1100.
16. **Hamada, F., and M. Bienz.** 2004. The APC tumor suppressor binds to C-terminal binding protein to divert nuclear beta-catenin from TCF. Dev. Cell **7:**677–685.
17. **Hart, M., et al.** 1999. The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. Curr. Biol. **9:**207–210.
18. **Henderson, B. R.** 2000. Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. Nat. Cell Biol. **2:**653–660.
19. **Hsu, S. C., J. Galceran, and R. Grosschedl.** 1998. Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with beta-catenin. Mol. Cell. Biol. **18:**4807–4818.
20. **Huang, S. M., et al.** 2009. Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling. Nature **461:**614–620.
21. **Itoh, K., B. K. Brott, G. U. Bae, M. J. Ratcliffe, and S. Y. Sokol.** 2005. Nuclear localization is required for Dishevelled function in Wnt/β-catenin signaling. J. Biol. **4:**3.
22. **Jin, L. H., et al.** 2003. Detection of point mutations of the Axin1 gene in colorectal cancers. Int. J. Cancer **107:**696–699.
23. **Kim, M. J., I. V. Chia, and F. Costantini.** 2008. SUMOylation target sites at the C terminus protect axin from ubiquitination and confer protein stability. FASEB J. **22:**3785–3794.
24. **Kimelman, D., and W. Xu.** 2006. beta-catenin destruction complex: insights and questions from a structural perspective. Oncogene **25:**7482–7491.
25. **Larsen, C. N., B. A. Krantz, and K. D. Wilkinson.** 1998. Substrate specificity of deubiquitinating enzymes: ubiquitin C-terminal hydrolases. Biochemistry **37:**3358–3368.
26. **Latres, E., D. S. Chiaur, and M. Pagano.** 1999. The human F box protein beta-Trcp associates with the Cul1/Skp1 complex and regulates the stability of beta-catenin. Oncogene **18:**849–854.
27. **Lee, E., A. Salic, R. Kruger, R. Heinrich, and M. W. Kirschner.** 2003. The

roles of APC and axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS Biol. **1:**E10.
28. **Lepourcelet, M., et al.** 2004. Small-molecule antagonists of the oncogenic Tcf/β-catenin protein complex. Cancer Cell **5:**91–102.
29. **Li, Q., et al.** 2009. Axin determines cell fate by controlling the p53 activation threshold after DNA damage. Nat. Cell Biol. **11:**1128–1134.
30. **Lin, H., L. Yin, J. Reid, K. D. Wilkinson, and S. S. Wing.** 2001. Divergent N-terminal sequences of a deubiquitinating enzyme modulate substrate specificity. J. Biol. Chem. **276:**20357–20363.
31. **Liu, C., et al.** 2002. Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell **108:**837–847.
32. **Logan, C. Y., and R. Nusse.** 2004. The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. **20:**781–810.
33. **Love, K. R., A. Catic, C. Schlieker, and H. L. Ploegh.** 2007. Mechanisms, biology and inhibitors of deubiquitinating enzymes. Nat. Chem. Biol. **3:**697–705.
34. **Major, M. B., et al.** 2007. Wilms tumor suppressor WTX negatively regulates WNT/β-catenin signaling. Science **316:**1043–1046.
35. **Major, M. B., et al.** 2008. New regulators of Wnt/beta-catenin signaling revealed by integrative molecular screening. Sci. Signal **1:**ra12.
36. **Mao, J., et al.** 2001. Low-density lipoprotein receptor-related protein-5 binds to axin and regulates the canonical Wnt signaling pathway. Mol. Cell **7:**801–809.
37. **Moon, R. T., J. D. Brown, and M. Torres.** 1997. WNTs modulate cell fate and behavior during vertebrate development. Trends Genet. **13:**157–162.
38. **Moon, R. T., A. D. Kohn, G. V. De Ferrari, and A. Kaykas.** 2004. WNT and beta-catenin signaling: diseases and therapies. Nat. Rev. Genet. **5:**691–701.
39. **Morin, P. J., et al.** 1997. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science **275:**1787–1790.
40. **Neufeld, K. L., et al.** 2000. Adenomatous polyposis coli protein contains two nuclear export signals and shuttles between the nucleus and cytoplasm. Proc. Natl. Acad. Sci. U. S. A. **97:**12085–12090.
41. **Neufeld, K. L., F. Zhang, B. R. Cullen, and R. L. White.** 2000. APC-mediated downregulation of beta-catenin activity involves nuclear sequestration and nuclear export. EMBO Rep. **1:**519–523.
42. **Nicholson, B., et al.** 2008. Characterization of ubiquitin and ubiquitin-like-protein isopeptidase activities. Protein Sci. **17:**1035–1043.
43. **Nijman, S. M., et al.** 2005. A genomic and functional inventory of deubiquitinating enzymes. Cell **123:**773–786.
44. **Powell, S. M., et al.** 1992. APC mutations occur early during colorectal tumorigenesis. Nature **359:**235–237.
45. **Radtke, F., H. Clevers, and O. Riccio.** 2006. From gut homeostasis to cancer. Curr. Mol. Med. **6:**275–289.
46. **Rosin-Arbesfeld, R., F. Townsley, and M. Bienz.** 2000. The APC tumour suppressor has a nuclear export function. Nature **406:**1009–1012.
47. **Rubinfeld, B., et al.** 1993. Association of the APC gene product with beta-catenin. Science **262:**1731–1734.
48. **Salahshor, S., and J. R. Woodgett.** 2005. The links between axin and carcinogenesis. J. Clin. Pathol. **58:**225–236.
49. **Schwarz-Romond, T., C. Merrifield, B. J. Nichols, and M. Bienz.** 2005. The Wnt signalling effector Dishevelled forms dynamic protein assemblies rather than stable associations with cytoplasmic vesicles. J. Cell Sci. **118:**5269–5277.
50. **Stadeli, R., R. Hoffmans, and K. Basler.** 2006. Transcription under the control of nuclear Arm/beta-catenin. Curr. Biol. **16:**R378–R385.
51. **Su, L. K., B. Vogelstein, and K. W. Kinzler.** 1993. Association of the APC tumor suppressor protein with catenins. Science **262:**1734–1737.
52. **Takacs, C. M., et al.** 2008. Dual positive and negative regulation of wingless signaling by adenomatous polyposis coli. Science **319:**333–336.
53. **Tamai, K., et al.** 2004. A mechanism for Wnt coreceptor activation. Mol. Cell **13:**149–156.
54. **Tauriello, D. V., et al.** 2010. Loss of the tumor suppressor CYLD enhances Wnt/beta-catenin signaling through K63-linked ubiquitination of Dvl. Mol. Cell **37:**607–619.
55. **Thompson, B., F. Townsley, R. Rosin-Arbesfeld, H. Musisi, and M. Bienz.** 2002. A new nuclear component of the Wnt signalling pathway. Nat. Cell Biol. **4:**367–373.
56. **Tran, H., F. Hamada, T. Schwarz-Romond, and M. Bienz.** 2008. Trabid, a new positive regulator of Wnt-induced transcription with preference for binding and cleaving K63-linked ubiquitin chains. Genes Dev. **22:**528–542.
57. **Wiechens, N., K. Heinle, L. Englmeier, A. Schohl, and F. Fagotto.** 2004. Nucleo-cytoplasmic shuttling of axin, a negative regulator of the Wnt/β-catenin pathway. J. Biol. Chem. **279:**5263–5267.
58. **Willert, K., S. Shibamoto, and R. Nusse.** 1999. Wnt-induced dephosphorylation of axin releases beta-catenin from the axin complex. Genes Dev. **13:**1768–1773.
59. **Winston, J. T., et al.** 1999. The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IκBα and β-catenin and stimulates IκBα ubiquitination in vitro. Genes Dev. **13:**270–283.
60. **Xu, H. T., et al.** 2005. Expressions of axin and β-catenin in non-small cell lung cancer. Zhonghua Bing Li Xue Za Zhi **34:**519–523. (In Chinese.)
61. **Yokoyama, N., D. Yin, and C. C. Malbon.** 2007. Abundance, complexation, and trafficking of Wnt/beta-catenin signaling elements in response to Wnt3a. J. Mol. Signal. **2:**11.
62. **Zeng, X., et al.** 2008. Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled, and axin functions. Development **135:**367–375.
63. **Zeng, X., et al.** 2005. A dual-kinase mechanism for Wnt coreceptor phosphorylation and activation. Nature **438:**873–877.
64. **Zhao, B., C. Schlesiger, M. G. Masucci, and K. Lindsten.** 2009. The ubiquitin specific protease 4 (USP4) is a new player in the Wnt signalling pathway. J. Cell Mol. Med. **13:**1886–1895.