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The Ubiquitin specific protease USP34 regulates Axin stability and Wnt/β-catenin signaling.

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Abstract

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. The analysis of purified AXIN-containing protein complexes by LC-MS/MS 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 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.

Keywords: Axin/ubiquitin/ubiquitin specific protease/Wnt signaling

40 **Introduction**

41

42 During embryonic development and tissue homeostasis in adults, the Wnt family of
43 secreted glycoproteins modulates several cell behaviors including differentiation, proliferation,
44 cell movement and polarity (32, 37). Malfunctioning Wnt-activated signaling pathways are
45 associated with multiple human diseases including cancer (10, 38). The etiology of colon
46 carcinoma is a particularly striking example that reflects the critical importance of the integrity
47 of this signaling cascade during intestinal epithelium homeostasis (45). Approximately 80% of
48 all colon cancers are molecularly rooted in mutations of Wnt pathway components. These
49 primarily consist of inactivating mutations in the gene coding for the tumor suppressor
50 Adenomatous Polyposis Coli (APC) (44, 47, 51) but also of activating mutations in the
51 transcription factor β-catenin (39) and loss of function mutations in the scaffolding protein Axin
52 (22).

53 APC and Axin are the core components of a cellular machinery dubbed the "destruction
54 complex" that promotes the phosphorylation of the cytoplasmic pool of β-catenin (24). Axin,
55 through binding to the destruction complex kinases Casein Kinase 1 alpha (CK1α) and Glycogen
56 Synthase Kinase 3 (GSK3), orchestrates β-catenin phosphorylation (31). Phospho-β-catenin is in
57 turns recognized by the $SCF^{\beta\text{-}TrCP}$ (Skp1-Cullin1-FBOX) E3 ubiquitin ligase that
58 polyubiquitinates β-catenin and promotes its proteolysis by the proteasome (26, 59). The
59 destruction complex thereby maintains low levels of cytosolic β-catenin in the absence of Wnt
60 stimulation. The recognition of Wnt ligands by the cell surface receptor complex Frizzled-
61 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 co-operate 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 post-translational 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 degradation 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 (USP) (also termed de-ubiquitinating enzymes or 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 re-utilization 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 re-localization 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 co-receptor (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 either by the modulation of β-catenin nuclear export (18), the

sequestration of β-catenin away from an active transcription complex (41) or via 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 re-utilized 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 co-receptor (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 nucleus of 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, 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 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. Human point mutant β-CATENIN (pt.mutant-hβCATENIN-CBP-HA-SBP) (34) and human DISHEVELLED-2

(pGLUE-hDSH2) (3) were described previously. USP34core (residues 1892-2241) was expressed and purified as a HIS-tagged protein from *E. coli*. USP2core 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's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) 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 (2μg/ml). Transient cDNA transfections were performed following the manufacturer's recommendations using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

For siRNA experiments, cells were transfected with 20nM of siRNA with recommended amounts of Lipofectamine RNAiMax (Invitrogen). Previously validated siRNAs against *β-CATENIN*, *AXIN1*, *AXIN2* (34), and Control-non targeting (Dharmacon, Lafayette, CO) were used, while a set of 4 siRNAs targeting *USP34* was obtained from Dharmacon (cat# LQ-006082-00-0002) and tested using western blotting. Within this set, USP34 siRNA "A" was the most effective and its target sequence was: 5'-GCAGGGAAGUUCUGACGAA-3'. The target sequences of the other USP34 siRNAs were: "B": 5'-CAACAGAUCAGUAGUAAUU-3'; "C": 5'-GCAGCUAUCCAGUAUAUUA-3'; "D": 5'-CCAUGUGACUGGAGAUUUA-3'.

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

Wnt3A conditioned media

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

Western Blotting/Antibodies

Protein lysates were resolved with SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were stained with antibodies indicated in the Figure legends, then incubated with horseradish peroxidase-conjugated secondary antibody and detected by chemiluminescence. Antibodies: α-β-CATENIN (#9587, Cell Signaling Technologies); rabbit monoclonal α-AXIN1 (#2074, Cell Signaling Technologies); polyclonal α-AXIN1 (obtained from J. Woodgett, Mt. Sinai Toronto); p44/42 MAP Kinase (ERK) (#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); peroxidase-conjugated secondary anti-goat/rabbit/mouse (705-035-147, 711-035-152, 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, cells were lysed with tandem affinity purification lysis buffer (10% glycerol, 50mM Hepes-KOH pH 8.0, 100mM KCl, 2mM EDTA, 0.1% NP40, 2mM DTT, 10mM NaF, 0.25mM $NaOVO_3$, protease inhibitors (Sigma)), lysates were cleared by centrifugation at 16,000g for 10min then incubated at 4°C with 100µl packed streptavidin resin (GE Healthcare). 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 using 100µl of calmodulin resin (GE Healthcare). Following washes, the protein complexes were eluted with two 100µl elutions with Calmodulin Elution Buffer (50mM Ammonium Bicarbonate pH 8.0, 10mM EGTA) and directly digested with sequencing-grade trypsin (Promega). The resulting peptide mixture was then analyzed by 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 using a normalized implementation of SEQUEST. The resulting peptide identifications returned by SEQUEST were filtered and assembled into protein identifications using the transproteomic pipeline softwares running on a Sorcerer platform (SageNResearch).

TopFlash reporter assays

Lentivirus containing the superTopFlash 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, media was replaced with a 1:1 mix of fresh DMEM:Wnt3A or DMEM:Control conditioned media. Cells were then assayed 24h after stimulation, performed in accordance with the Dual Luciferase assay protocol (Promega) using the Envision Multilabel Plate Reader (PerkinElmer).

Co-affinity purification

For co-affinity purification of endogenous proteins, HEK293T cells ($5x10^6$) stably expressing pGLUE-HA-hAXIN1 or pGLUE-HA-RADIL were lysed in tandem affinity purification lysis buffer (10% glycerol, 50mM Hepes-KOH pH 8.0, 100mM KCl, 2mM EDTA, 0.1% NP40, 2mM DTT, 10mM NaF, 0.25mM $NaOVO_3$, protease inhibitors (Sigma)). Lysates were cleared by centrifugation at 16,000g for 10min and affinity purification was performed using streptavidin resin. Purified protein complexes were then analyzed by western blotting, using antibodies noted in Figure legends.

K48 Ubiquitin chain cleavage

1µg of purified K48 chains from Boston Biochem (UC-230) were incubated in USP Assay Buffer (20mM Tris pH 8.0, 2mM $CaCl_2$, and 2mM β-mercaptoethanol) with 20nM USP2

core, 100nM USP34 core, 1µg of affinity-purified AXIN Complex, or 1µg of AXIN Complex (USP34 shRNA). The samples were incubated at 37ºC for 30min and the reaction was stopped by addition of SDS sample buffer. The appearance of mono-ubiquitin was monitored by western blot using α-UBIQUITIN antibody (Sigma U5379).

UBL-PLA$_2$Assay

20nM USP2 core, 20nM USP34 core or 1µg of total protein from purified AXIN complexes was mixed with 30nM Ub-PLA$_2$ and 20µM NBD C6HPC (PLA$_2$ substrate, Invitrogen) in a total volume of 100µL/well in a black 96-well-plate (Greiner Bio-One). Data were collected 45min after addition of Ub-PLA$_2$ and NBD C6HPC on a Perkin-Elmer Envision fluorescence plate reader with excitation and emission filters of 475nm and 555nm respectively. Net RFU was then used to calculate signal (isopeptidase or complexes + reporter) to background (reporter) ratio. *UBL-selectivity assays:* Relative isopeptidase activity against various UBL-PLA$_2$ fusions was determined by adding the USP34 core to a final concentration of 20nM in combination with 20µM NBD C6-HPC and 30nM of the individual UBL-PLA$_2$ reporter constructs and expressed as a percentage of 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. (www.lifesensors.com) 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 2X 100mm petri dishes of HEK293T were

transfected with pIRES-puro plasmids expressing STREP-HA-USP34 core domain (amino acids 1696-2400), or a catalytically inactive STREP-HA-USP34 core domain (C1903S). 16 hours before lysis, STREP-HA-AXIN1 cells were treated with 1μM MG132. Cells were lysed separately in TAP lysis buffer supplemented with protease inhibitors in the absence (USP34) or presence (AXIN) of 5mM NEM. Proteins were affinity purified using streptavidin beads. After extensive washes in TAP lysis buffer and two washes in DUB buffer (Tris 50mM pH8, NaCl 150mM, EDTA 2mM, MgCl2 2mM, DTT 2mM), an equivalent amount of AXIN1 was incubated for 1 hour at 37°C with the USP34 core domains as indicated in the figure. Proteins were resolved by SDS-PAGE and blotted with FLAG-antibodies to detect ubiquitin conjugates and HA to monitor AXIN and USP34 core domains expression.

Immunofluorescence

Cells were seeded on poly-D-lysine treated coverslips and, when indicated, reverse transfected with siRNA. 48h after transfection cells were fixed with 4% paraformaldehyde/PBS for 20min, then permeabilized and blocked with 0.2% Triton X-100 and 10% normal donkey serum/PBS for 20min. Where indicated, cells were treated with 5ng/ml Leptomycin B (LC Laboratories, MA) for 3 hours. Cells were then stained for indirect immunofluorescence using polyclonal α-AXIN1 antibodies (provided by Dr. Woodget, Mount Sinai, Toronto) and Alexa488 conjugated anti-rabbit antibodies. Cells were mounted with Vectorshield (Vector) and examined by laser scanning confocal microscope (Zeiss LSM 510).

Cycloheximide chase

HEK293T cells expressing scramble or *USP34* shRNA were seeded on 6 wells plate. Cells were treated with 3μM XAV939 for 16 hours, were then washed twice with PBS and subjected to 10μg/mL cycloheximide for the indicated times. Cells were lysed using TAP lysis buffer supplemented with protease inhibitors. An equivalent amount of proteins were resolved using SDS-PAGE followed by western blotting using AXIN1 or TUBULIN antibodies.

β-CATENIN stabilization assay

RKO cells were reverse transfected with siRNA. 48 hours following transfection, cells were stimulated with control or Wnt3A conditioned media (CM) treatment for different times (0.5h – 6h). Cells were washed and lysed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 0.1% SDS) for 15min then cleared by centrifugation at 16,000g for 10min before being resuspended in SDS sample buffer and resolved by SDS-PAGE. β-catenin accumulation was monitored by western blot.

AXIN ubiquitination assay

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

Real Time qPCR

Total RNA from SW480 cells treated with control or USP34 siRNAs was purified using Tri-Reagent (Sigma). After DNaseI (Invitrogen) treatment, RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Primer sequences used are: *CYCLOPHILIN* 5'-GGAGATGGCACAGGAGGAA-3', 5'-GCCCGTAGTGCTTCAGTTT-3'; *NKD1* 5'-TGAGAAGAAGATGGAGAGAGTGAGCGA-3', 5'-GGTGACCTTGCCGTTGTTGTCAAA-3'; *TNFRSF19* 5'-GGAGTTGTCTAAGGAATGTGG-3', 5'- GCTGAACAATTTGCCTTCTG-3'. Primer pair efficiencies were validated as previously described (6). Quantitative RT-PCR analysis was carried out in triplicate using an Applied Biosystems Prism 7900HT instrument. Each reaction contained 12.5ng of cDNA, 150nM of each primer, and Power SYBR green PCR Master Mix (Applied Biosystems). Gene expression analysis was performed using the comparative CT method, normalized to *CYCLOPHILIN* expression, and fold changes were calculated relative to control siRNA treated cells.

Results

Targeted proteomic analysis identifies USP34 as an Axin associated protein.

To further understand the regulation of Axin and its mechanism of action, we isolated human AXIN1 and AXIN2 protein complexes and analyzed their compositions 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 using the 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 previously uncharacterized protein UBIQUITIN-SPECIFIC PROTEASE 34 (USP34) (Fig. 1A, blue circle and table 1), which contains 3546 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 co-immunoprecipitation 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 blot 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 co-precipitate with USP34 (Fig. 1B, lane 4). Probably reflecting the transient nature of the interaction, attempts to perform endogenous co-immunoprecipitation 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 ubiquitin-specific proteases (USP) 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 mono-ubiquitin as detected by western blotting (Fig. 2A, lane 3).

As an alternative approach to monitor USP activity, we employed the newly developed UB-PLA_2 assay (42) to quantify ubiquitin isopeptidase activity present in purified AXIN complexes. Briefly, this assay consists of a fusion protein containing UBIQUITIN fused to the N-terminus of PHOSPHOLIPASE A2 (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 the methods section. Robust isopeptidase activity could be detected in AXIN complexes when compared to control complexes that exhibited background activity (Fig. 2B, compare lanes 3&5). We then tested whether the isopeptidase activity present in the AXIN complexes was attributable to USP34, in both 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&B compare lanes 1&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 UB-AXIN conjugates were detected by western blot using 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 sulphydryl alkylating agent N-ethylmaleimide (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 poly-ubiquitination 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 UBIQUITIN-AXIN (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 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 ubiquitin-proteasome system (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-AXIN1 levels (Fig. 3A, compare lanes 1&2 on western blots). This could be efficiently rescued when the cells were treated with 5μM MG132 for 10h 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 studying the stability of endogenous AXIN1 (Fig. 3A, western blot on bottom right). Importantly, two independent USP34 siRNAs (A & D) had the same effect on AXIN stability (data not 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 pre-requisite 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 GFP-AXIN1 expressing cells with control or USP34 siRNAs for 48 hours and added XAV939 for the last 12 hours. We showed that XAV939 reversed the degradation of AXIN resulting from USP34 depletion (Fig. 3B, compare lanes 2&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 hours. 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 when 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 hours and endogenous USP34 protein levels were subsequently measured using western blot with anti-USP34 antibodies. All four USP34 siRNAs could block protein expression with varying 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 (10638bp) 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 activation of the reporter, respectively (Fig. 4B, lanes 4&10) when compared with cells treated with control conditioned media. *USP34*-depleted cells showed a reduction of the 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&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 the β-catenin transactivation properties (Fig. 4C, lanes 6-8). 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 knock-down 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-media (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 kinetic and 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 as 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. As 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&4). The constitutive reporter activity was β-CATENIN dependent since β-CATENIN knockdown virtually eliminated the TopFlash signal (Fig. 4E, lanes 3&6). *USP34* knockdown also inhibited β-CATENIN signaling indicating that it is also required in this context (Fig. 4E, lanes 2&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). Knock-down 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&5 vs lanes 1&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 nucleus of colon cancer cells (1), to undergo nucleo-cytoplasmic shuttling (11, 57) and to translocate to the nucleus following 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 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 results by depleting *AXIN1* and *AXIN2* transcripts in SW480 and HCT116 by siRNA. As suggested by the Major et al. study (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 co-transfected together, *AXIN1/2* 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&5). This is in sharp contrast to what is usually observed in cells presumed to have intact Wnt pathway components such as human HEK293 kidney cells or RKO colon carcinoma cells. At 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-media (Fig. 5B, lanes 2&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&6 to lanes 2&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 above results 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 nucleus of these cells (Fig. 6A, left panels). Pre-treatment 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 nucleus 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 nucleo-cytoplasmic shuttling as it accumulates in the nucleus when cells are treated with the CRM1-dependent nuclear export inhibitor Leptomycin B (LMB) (11, 57). We thus asked whether the stabilization of AXIN by USP34 is required to observe the accumulation of AXIN in the nucleus following 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 nucleus 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 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, this study highlighted 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 reveal 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 others 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 identifed 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 if 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 co-receptor 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

583 functions. The use of two colon cancer cell lines where the destruction complex machinery is
584 defective and where AXIN accumulates in the nucleus, has allowed us to uncover this novel
585 function. Further work is now needed to address how AXIN exert this positive role in the
586 nucleus. Two possibilities are that AXIN serves as escort protein for β-CATENIN and
587 influences its residency in the nucleus or that AXIN is an integral part of the β-CATENIN
588 transcriptional machinery participating in the recognition of target genes as recently found for
589 Dsh (15). In any case, Axin is likely not absolutely required for β-catenin signaling but rather
590 could determine the duration and the strength of signaling by regulating the availability of
591 activated nuclear β-catenin. What emerges from our study however is that the tight regulation of
592 the balance of AXIN ubiquitation/de-ubiquitination is likely an important control point during
593 Wnt signaling.

594 **Compounds interfering with Axin stability as drug targets**

595 Since the nuclear localization of AXIN and β-CATENIN are associated with Wnt
596 pathway activation and are constitutively found in the nucleus in several human cancers, the
597 control of their residency in the nucleus by modulating USP34 activity could represent a novel
598 therapeutic approach for not only the treatment of cancers but of the numerous human diseases
599 exhibiting spurious Wnt/β-CATENIN pathway activity. Current strategies for small molecule
600 inhibitors of the Wnt pathway have relied, with limited success, on compounds inhibiting
601 protein-protein interactions (14, 28). The protease activity of USPs, however, makes them
602 highly amenable to inhibition by small molecules (13). That promoting AXIN stability or
603 degradation both lead to inhibition of Wnt signaling suggests that the precise control of AXIN
604 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.

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812

813 **Figure legends**

814

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817 (A) Human AXIN1 & AXIN2 protein interactions network. Lines represent interactions found in
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823

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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. 48h post-transfection the cells were fixed or lysed and the levels of Venus-AXIN1 were evaluated by fluorescence microscopy (left panels) or western blotting using anti-GFP antibodies (top right panels). Where indicated, MG132 was added for the last 10h 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 (A) was repeated but instead of MG132 the TANKYRASE inhibitor XAV939 was added for the last 12h. AXIN1 levels were monitored by fluorescence microscopy (left panels) and western blotting using 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 3A and B. Regions of interest were drawn randomly around the periphery of cells taking the phase contrast images as template, and the pixel intensity of the fluorescence images were quantified using ImageJ (N=20-25 cells). Error bars represent S.E.M; Single star represents statistical significance compared to Ctl siRNA condition, whereas double star represents statistical significance compared to USP34 siRNA condition. D) USP34 regulates the turnover of AXIN. HEK293 cells expressing control or USP34 shRNA were treated with 3μM XAV939 for 12h. Cells were washed twice and were switched to media containing 10μg/ml cycloheximide for the indicated times. Lysates were collected and proteins resolved on a 8% SDS PAGE gel. AXIN levels were determined by western blot using anti-AXIN1 antibodies (left), quantified by densitometry and normalized to TUBULIN levels for each time points (right).

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 3-6) or *β-CATENIN* siRNA (Lane 7) were resolved by SDS-PAGE and probed using 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 1,4,7,10) *β-CATENIN* (lanes 2,5,8,11) or

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FIG. 5. Positive role of AXIN during Wnt signaling in colon cancer cells.

TopFlash assays in (A) HCT116 and SW480 colon cancer cells or (B) HEK293T and RKO cells treated with control, *β-CATENIN* or *AXIN* siRNAs. In (A) *AXIN1+2* siRNAs inhibit the constitutive Wnt pathway activation due to mutations in APC (SW480) or β-CATENIN (HCT116) while in (B) AXIN1+2 knock-downs potentiate the Wnt3A-stimulated activity of the reporter. (C) The activation of the Wnt pathway in HEK293T cells using a degradation resistant β-CATENIN mutant (pt.β-CATENIN) is inhibited by *AXIN1+2* siRNAs. Figures are representative of at least three independent experiments performed in duplicates where the error

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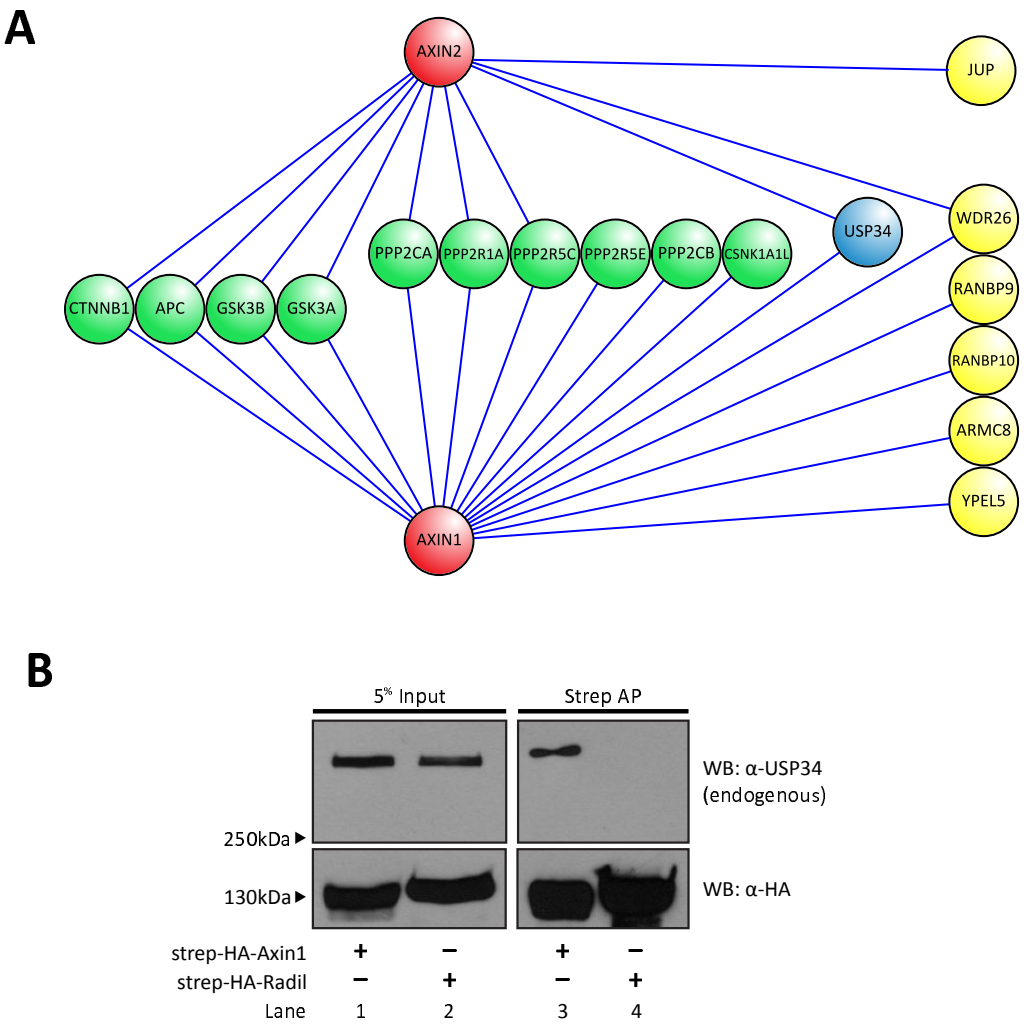

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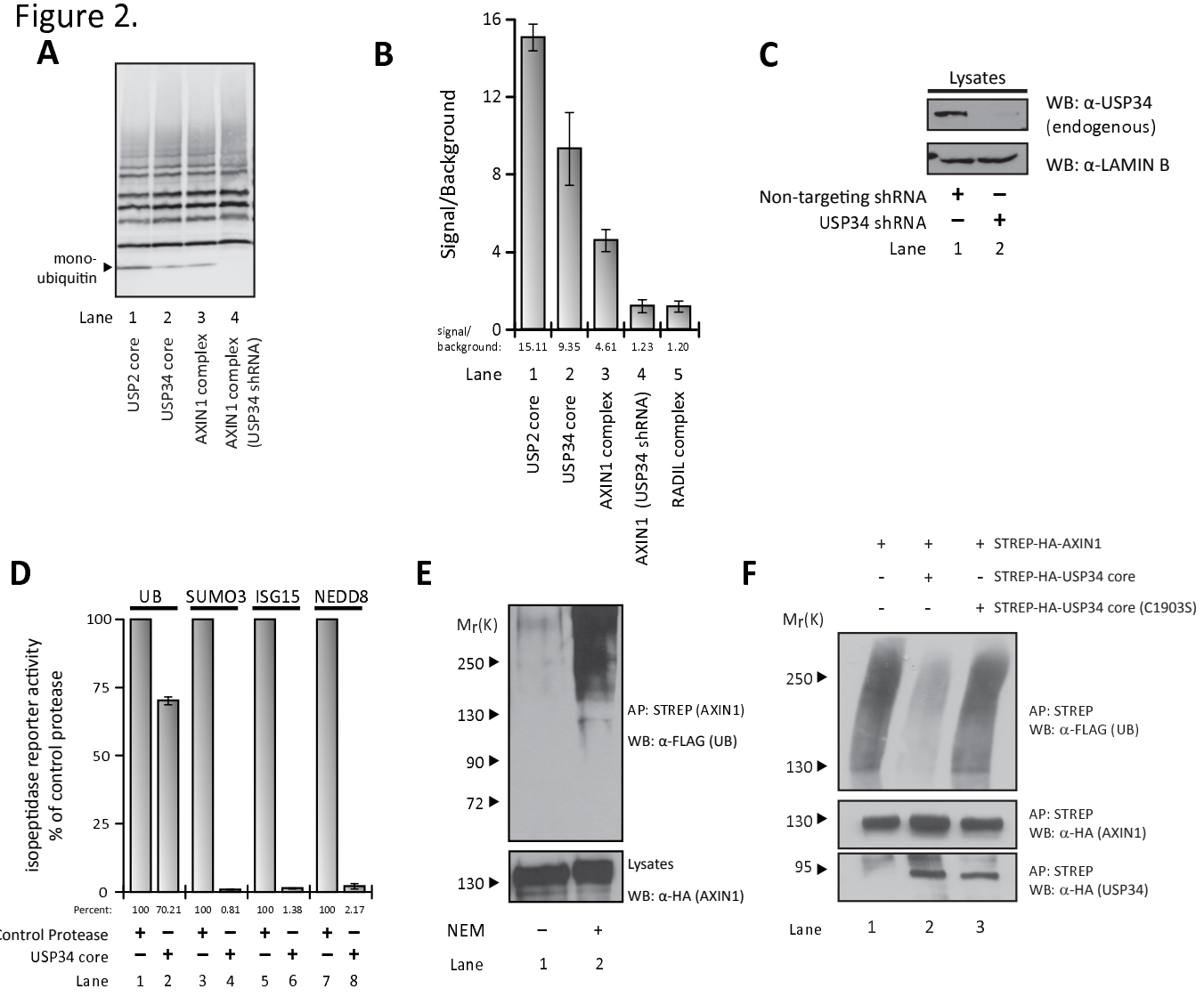

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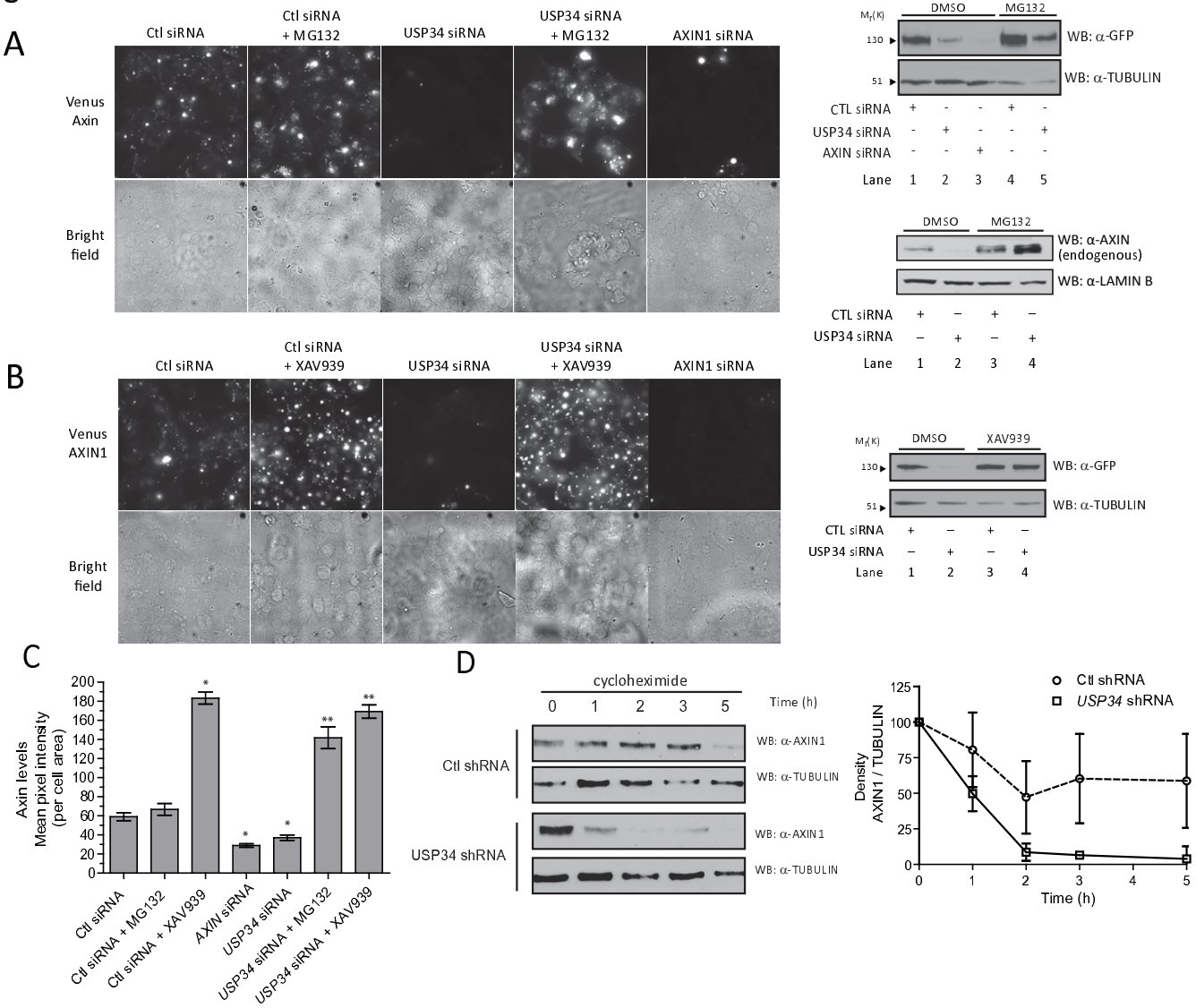

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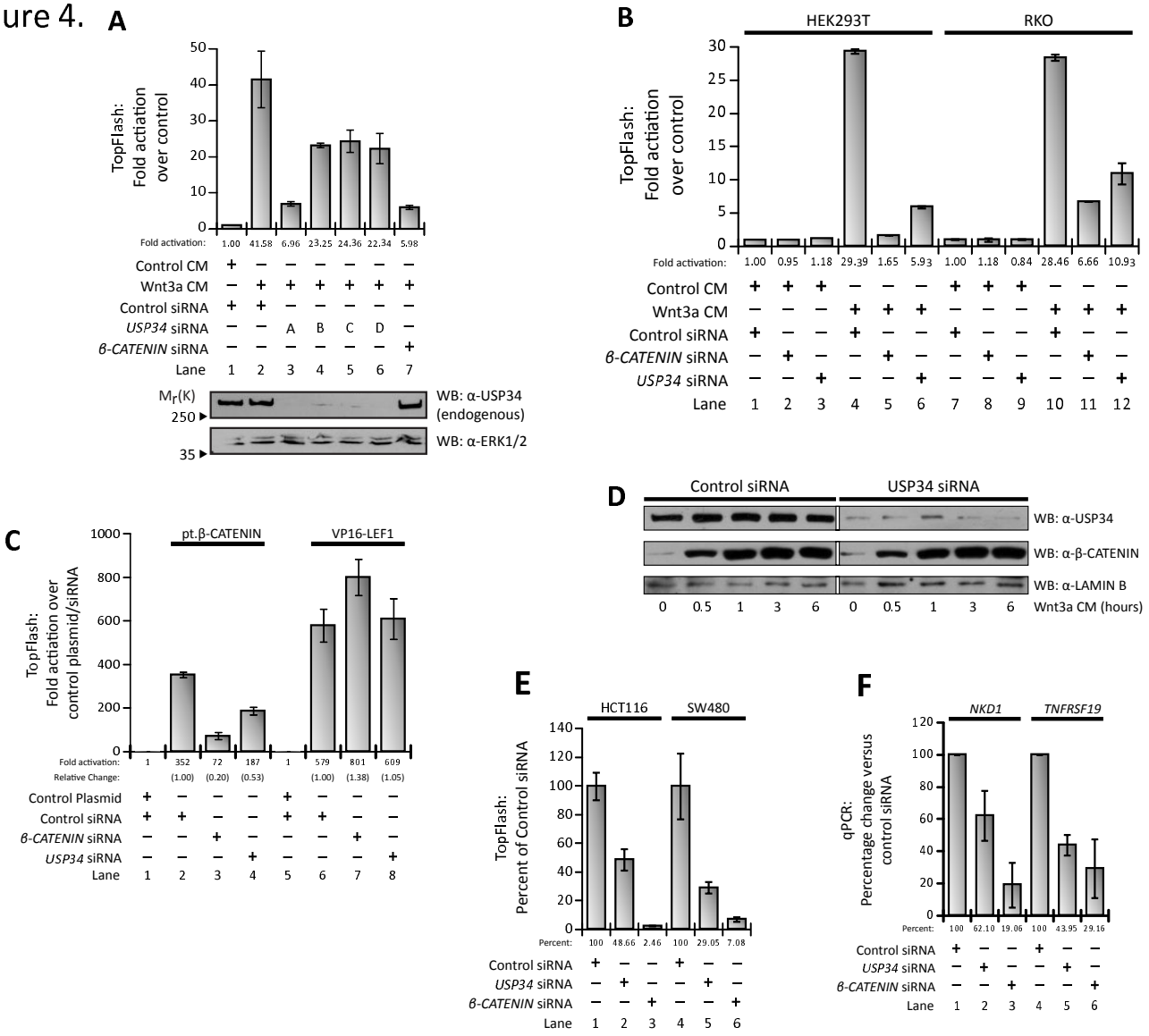

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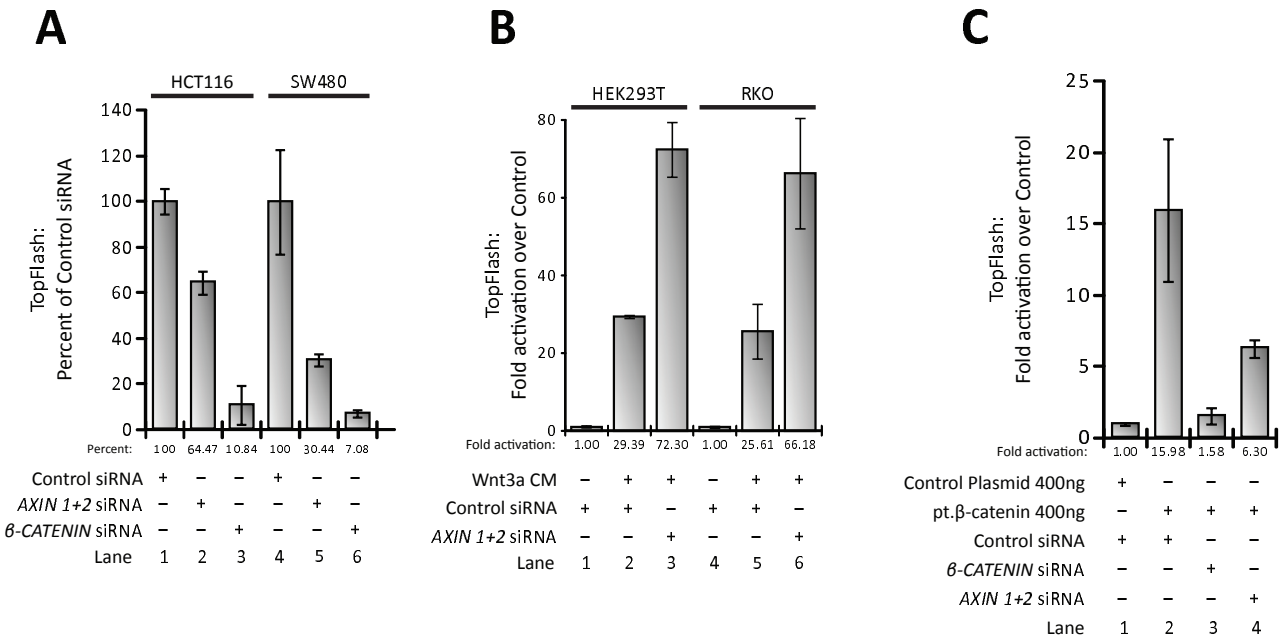

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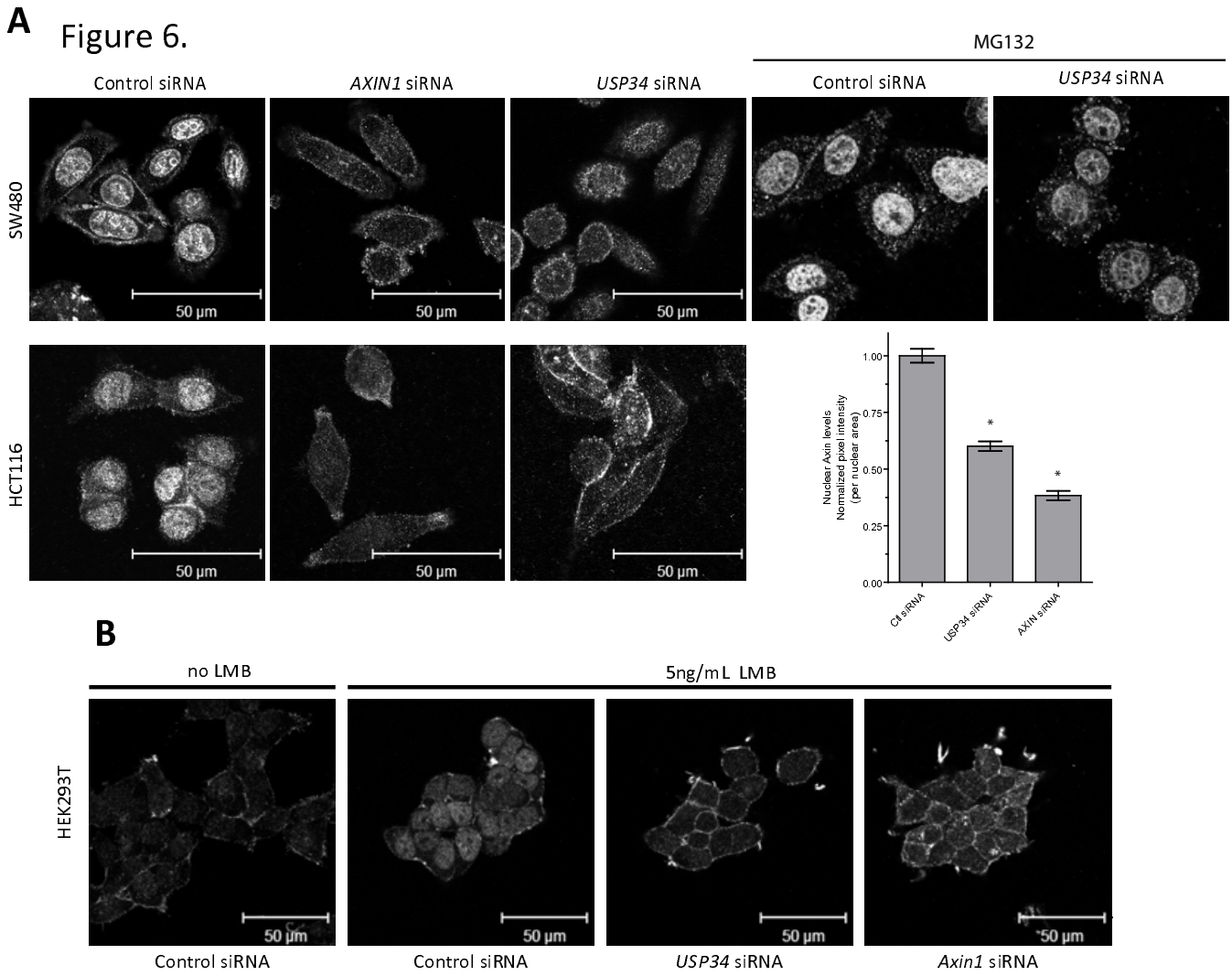

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Table 1.

A. Axin 1

Protein	Total Peptides	Unique Peptides	Protein Accession	#n pulldowns
Axin 1	197	47	NP_003493.1	4
ubiquitin C	46	42	NP_055524.2	1
GSK3-α	36	23	NP_002084.2	4
USP34	35	28	NP_055524.3	3
GSK3-β	33	22	NP_063937.2	4
ubiquitin B precursor; polyubiquitin B	18	1	NP_066289.1	1
PP2A Regulatory subunit B56-ε	11	7	NP_006237.1	3
WD repeat domain 26	11	9	NP_079436.3	3
PP2 Regulatory subunit A-α	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

B. Axin 2

Protein	Total Peptides	Unique Peptides	Protein Accession	#n pulldowns
Axin 2	155	19	NP_004646.2	4
Nucleolin	70	10	NP_005372.2	1
GSK3-β	42	14	NP_002084.2	4
GSK3-α	34	12	NP_063937.2	4
β-Catenin	28	9	NP_001895.1	2
APC	27	16	NP_000029.2	4
Ubiquitin Specific Protease 34 (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 A-α	3	3	NP_055040.2	1
Wiskott-Aldrich syndrome protein family member 2	3	2	NP_008921.1	1
PP2A Regulatory subunit B56-γ	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

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