Supplementary Data

Supplementary Materials and Methods

Vectors and constructs

GFP-, MYC- and FLAG-tagged Smurf2(WT), mutant MYC-Smurf2(CG) and HA-tagged ubiquitin were previously described (6). pRK5-HA-Ubiquitin-K48 mutant was a gift from Ted Dawson (Addgene plasmid #17605). GFP- and FLAG-tagged mutant Smurf2(C716G) were subcloned into pEGFP-C2 or pBabe-puro vector, respectively, by PCR amplification from pRK-MYC-Smurf2(CG) vector. The following primers were used: 5'-caccgaattcatgtctaaccccggaggccggag-3' (forward primer containing EcoRI site) and 5'-atatgtcgactcattccacagcaaatccac-3' (reverse primer containing SalI site). The PCR products were digested with EcoRI and SalI restriction enzymes (NEB). N-terminal FLAG-tagged human Topo IIα (pRK-FLAG-Topo IIα) was constructed by PCR, using the following primers: 5'-caccatcgatgaagtgtcaccattgcagc-3' (forward primer containing ClaI site) and 5'-atatgtcgacttaaaacagatcatcttcatctg-3' (reverse primer containing SalI site) and a template plasmid encoding the full-length cDNA of human Topo IIα (Origene). The PCR product was digested with ClaI and SalI and inserted into pRK2-FLAG vector. N-terminal mCherry-tagged human Topo IIα was constructed by similar procedure, using another pair of primers: 5'-caccctcgagacatggaagtgtcaccattgc-3' (forward primer containing XhoI site) and 5'-atatcccgggttaaaacagatcatcttca-3' (reverse primer containing XmaI site). For this construct, the PCR product was digested with XhoI and XmaI and inserted into pmCherry-C1 vector (Clontech). All constructs were sequence verified.

siRNAs

To knock down the expression of Top1, Topo IIα, Topo IIβ, and Smurf2, we used Dicer-substrate siRNA (DsiRNA) duplexes obtained from Integrated DNA Technologies (IDT, Israel). DsiRNA sequences are shown below.

Top1-#1: 5'-GAAUAUUAUCACCAACCUAAGCAAA-3' and

3'-UUCUUAUAAUAGUGGUUGGAUUCGUUU-5'

Top1-#2: 5'-GAAGUCCGGCAUGAUAACAAGGUTA-3' and

3'-UUCUUCAGGCCGUACUAUUGUUCCAAU-5'

Top1-#3: 5'-UCACAGGUCAAUAAACUUAGAGGAA-3' and

3'-AAAGUGUCCAGUUAUUUGAAUCUCCUU-5'

Topo IIα: 5'-GCACAUCAAAGGAAGCUAAAGAATA-3' and

3'-GUCGUGUAGUUUCCUUCGAUUUCUUAU-5'

Topo IIβ: 5'-GCAUUCCAAAGAAGACUACAACACC-3' and

3'-ACCGUAAGGUUUCUUCUGAUGUUGUGG-5'

Smurf2, 3'UTR: 5'-GCAGAGUUUCAAAGAAUAUGCUGAA-3' and

3'-GUCGUCUCAAAGUUUCUUAUACGACUU-5'

Smurf2, CDS: 5'-AGUUAAUCCGGAACAUUUAUCCUAT-3' and

3'-CGUCAAUUAGGCCUUGUAAAUAGGAUA-5'

NS (non-specific): 5'-CGUUAAUCGCGUAUAAUACGCGUAT-3' and

3'-CAGCAAUUAGCGCAUAUUAUGCGCAUA-5'

Cell transfections and generation of stable cell lines

Plasmids transfections were performed using either branched polyethylelimine (PEI, Sigma) or FUGENE 6 (Promega) according to the manufacturer's instructions. For siRNAs transfections, Oligofectamine (Invitrogen) was used, and the efficiency of knockdown was assessed 72 hrs after transfection.

For generating Smurf2 stable knock-down, cells were infected with lentiviruses containing pLKO.1-Smurf2-puro vector (Sigma), and selected with puromycin. To generate GFP-Smurf2 and mCherry-Topo IIα stably expressing cells, U2OS were transfected using FUGENE 6. Following the transfection, cells were propagated in the presence of G-418 (550 μg/ml) for at least 2 weeks. Population of positive fluorescent cells was then enriched using MoFlo Astrios cell sorter (Beckman Coulter). For generation of Smurf2-reconstituted MEFs, pBabe-FLAG-Smurf2-puro based retroviral system was used (6).

Generation of Smurf2^{CRISPR} U2OS cells

We generated the U2OS Smurf2KO cell line using the CRISPR/Cas9 genome editing system (Origene). Briefly, cells were grown in 10 cm plate to obtain 50%-60% confluence. Then, cells were co-transfected with 5μg of Smurf2 gRNA vector and 5μg of donor plasmid using FUGENE 6. Forty-eight hours after transfection, cells were split into 1:10 ratio and grown for an additional three days. In order to dilute cells containing the donor plasmid as episomal form, cells were split seven times before puromycin selection (2.5 μg/ml). The efficiency of gene knockout was analyzed using western blotting.

Protein extraction and Western Blot

For the preparation of whole cell lysates (WCL), cells were resuspended in RIPA buffer (50 mM Tris-HCl [pH 7.8], 1% Nonidet P40 Substitute (Sigma), 150 mM NaCl, 0.1% SDS, 0.5% p/v

sodium deoxycholate) supplemented with protease (Roche) and phosphatase inhibitors (Sigma). Samples were maintained on ice for 30 minutes and then sonicated for 1 min at 30% amplitude. Fractionated samples were prepared as described (32). For protein extraction from mouse tissues, the collected organs were homogenized in RIPA buffer using TissueRuptor (Qiagen) and, following sonication, cleared using centrifugation. Protein concentrations were assessed using Pierce BCA protein assay kit (ThermoScientific). Samples were analyzed by western blot analysis using the following antibodies: anti-Topo IIα (ab52934, 1:3000, Abcam), anti-Topo IIβ (ab72334, 1:1000, Abcam), anti-Top1 (#3552-1, 1:5000, Epitomics), anti-β-actin (#600401886, 1:3000, Rockland), anti-α-tubulin (T9026, 1:4000, Sigma), anti-H2B (ab1790, 1:1000, Abcam), anti-Smurf2 (12024, 1:2000, Cell Signaling), anti-MYC (2278S, 1:1000, Cell Signaling), anti-FLAG (TA50011, 1:3000, Origene), anti-HA (715500, 1:250, Invitrogen), anti-poly-Ub-Lys48 (8081S, 1:1000, Cell Signaling), and anti-MAP1LC3A/B (AHP2167, 1:500, BIO-RAD). Horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at the dilution of 1:10,000.

Immunoprecipitation

For co-immunoprecipitation (co-IP) experiments, cells were lysed using a freeze-thawing (FT) protocol. In brief, a FT lysis buffer (600 mM KCl, 20 mM Tris-HCl [pH 7.8], 20% glycerol, protease and phosphatase inhibitors) was added to cellular pellets and samples were immediately subjected to three cycles of freeze and thawing using liquid nitrogen and a water bath at 37 ˚C. Then, three volumes of resuspension buffer (45 mM Tris-HCl [pH 7.8], 2.25 mM EDTA, 0.1% Nonidet P40 Substitute) were added and samples were centrifuged. Lysates were incubated overnight at 4 ˚C with either anti-Smurf2 (sc-25511, Santa Cruz) or anti-MYC (sc-40, Santa Cruz) antibodies. Protein G-Sepharose beads (4 Fast Flow, GE Healthcare) were then added and the

samples were incubated for additional 2 hrs at 4 ˚C under rotation. Subsequently, beads were washed four times with an ice-cold lysis buffer and boiled for 10 minutes in 5X SDS sample buffer (50 mM Tris-HCl [pH 8], 5 mM EDTA, 5% SDS, 50% glycerol, 50 mM DTT, 0.05% p/v bromophenol blue, 6% 2-mercaptoethanol). To immunoprecipitate FLAG-tagged proteins, agarose beads conjugated with FLAG antibody (FLAG-M2 affinity gel, Sigma) were added and samples were kept for 2 hrs at 4˚C under rotation. Then, beads were washed and IPs recovered as described above.

Immunofluorescence and confocal analysis

Cells were cultured overnight on poly-D-lysine covered glass slides and fixed either in 2% formaldehyde at room temperature for 20 minutes, or in methanol/acetone solution (1:1) at -20˚C for 5 minutes. Then, cells were permeabilized with 0.5% Triton X-100 in PBS, blocked in 3% BSA and stained for 1 hour at room temperature with antibody against Topo IIα (sc-365918, Santa Cruz; 1:100). Subsequently, cells were washed and incubated for 1 hour with goat anti-mouse secondary antibody conjugated with Rhodamine Red™-X (code 115-296-071, Jackson ImmunoResearch Laboratories). DNA was stained with Hoechst 33258 (B2883, Sigma), and cells were analyzed using a LSM780 Inverted Confocal Microscope (Zeiss) through a Plan-Apochromat 63x/1.40 Oil DIC M27 objective. All comparative images were obtained under identical microscope and camera settings.

For anaphase bridges counting assay, cells were arrested in M-phase using 0.1 μg/ml colcemid for 14 hrs and then released. After 4 hrs from release, cells were washed once with 1X PBS and fixed in 2% formaldehyde. Cells were then permeabilized with 0.5% Triton X-100 in PBS for 10 minutes and DNA was stained with Hoechst.

For PLA assay, the Duolink In Situ Red Starter Kit Mouse/Rabbit (DUO92101, Sigma) was used according to the manufacturer's instructions. In this assay, we used antibodies against FLAG tag (Sigma F7425, 1:1000) or Topo IIα (sc-365918, 1:100). All comparative PLA images were obtained under identical microscope and camera settings, and quantified using ImageJ (NIH). A minimum five different fields with 15-25 cells per field were quantified.

Topoisomerase I activity assay

The activity of DNA topoisomerase I (Top1) was measured on the nuclear extract prepared from U2OS cells using a DNA relaxation assay (TG1015, TopoGen), according to the manufacturer's instructions.

qRT-PCR

Total RNA was extracted from Smurf2 knock-down and control U2OS and HCT116 cells using RNeasy mini kit (Qiagen), according to the manufacturer's instructions. Total RNA was then reverse-transcribed with random primers using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Topo IIα cDNA levels were determined using Fast SYBR Green Master mix and ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific). The experiments were performed three times with three technical replicates for each experiment. Topo IIα gene expression was calculated using $2^{-\Delta\Delta Ct}$ method and normalized to GAPDH gene. The following primers were used for Topo IIα expression analysis: Forward: 5'-accattgcagcctgtaaatga-3' and Reverse: 5'-gggcggagcaaaatatgttcc-3'. Primers used for GAPDH: Forward 5'-ggagcgagatccctccaaaat-3' and Reverse 5'-ggctgttgtcatacttctcatgg-3'.

Cell viability assay

The viability of cells following etoposide treatment was assessed using the XTT assay. In brief, cells were seeded at equal density ($3x10^3$cells/well) in 96-well plates in hexaplicates. Etoposide

(Calbiochem) was administered to cells 24 hrs later at concentrations ranging from 0.25 μM to 20 μM. Seventy-two hours later, XTT reagent (Biological Industries) was added to each well and, after 5 hrs of incubation, plates were read using Eon Microplate Spectrophotometer (BioTek). The absorbance was detected at a test wavelength of 475 nm and a reference wavelength of 660 nm.

Bi-parametric Flow Cytometry

Cells were analyzed by two-dimensional flow cytometry as we previously described (6). Briefly, cells were fixed in 80% ethanol and permeabilized with 0.25% Triton X-100 in PBS. Subsequently, cells were incubated for 1 hour at RT with anti-pH3-Ser10 antibodies: with Alexa Flour®488 conjugate (#3465, Cell Signaling) to stain siRNA-transfected cells, or pH3-Ser10 Alexa Flour®647 conjugate (#9716, Cell Signaling) to detect mitotic population in Smurf2^{CRISPR} and control cells. Both antibodies were diluted 1:50 in 1% BSA. After primary antibody incubation, cells were treated with 20 μg/ml RNAse (R6513, Sigma) at 37°C for 30 min, and stained with propidium iodide (PI) for DNA quantitation. Data were collected using Flow Analyzer - Gallios (Beckman Coulter), counting 20,000 events/sample; and FlowJo software was used for analysis.

Statistical analyses

Analysis of the expression of Smurf2 and Topo IIα protein levels in human normal and cancer tissues were performed using Mann-Whitney and Wilcoxon Signed Ranks tests. Spearman's rank correlation test was used to measure the statistical dependence between Smurf2 and Topo IIα protein expression levels in tissues. χ^2-test was used to determine statistical significance between percent of ana/telophase cells *vs.* control samples. Two-tailed student t-test was applied for statistical analysis of data obtained in the XTT viability assay, for analysis of Topo IIα mRNA expression in Smurf2 knockdown human cells, and for PLA data analysis. Data with P-values of less than 0.05 were considered statistically significant.

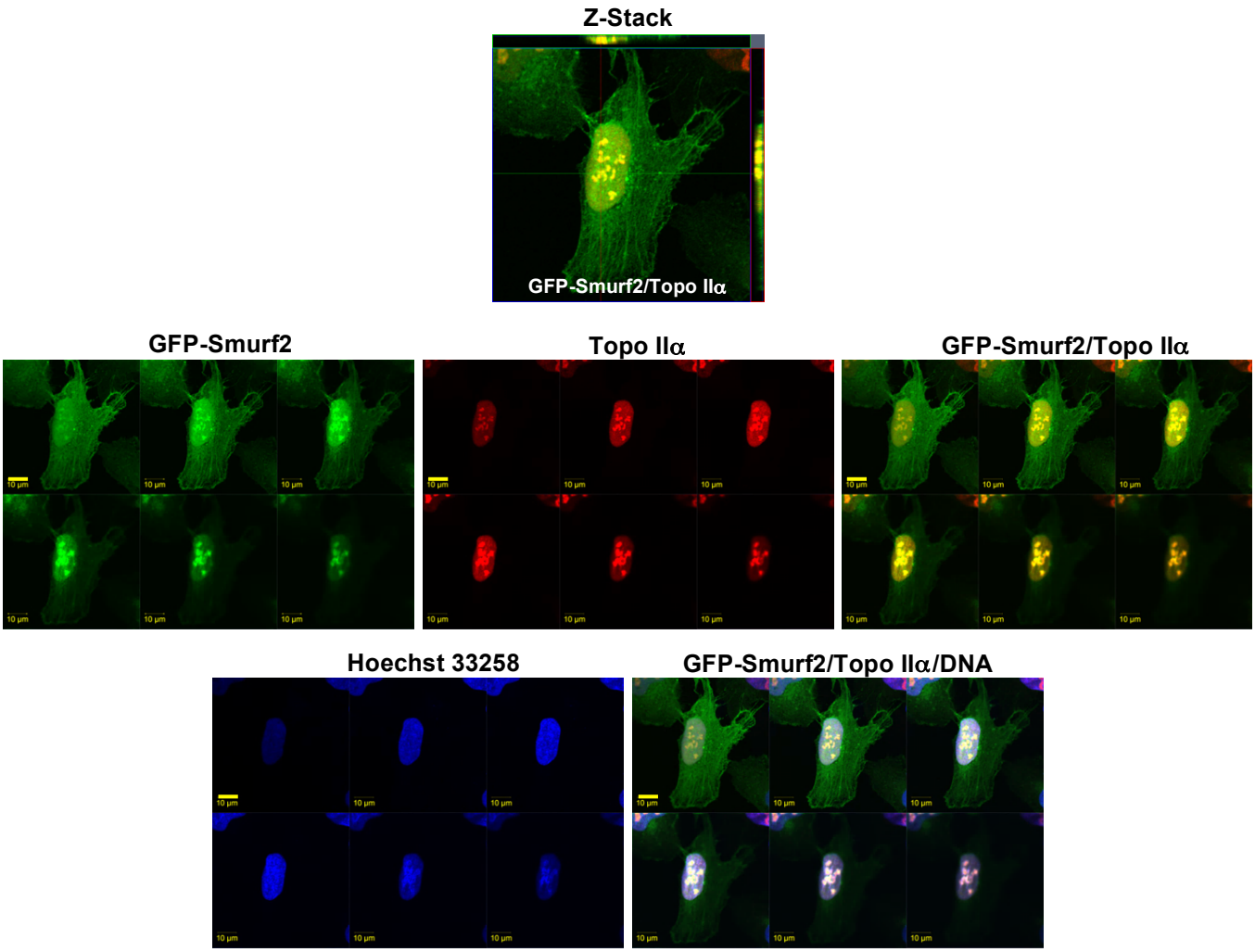

Figure S1. Smurf2 co-localizes with Topo IIα through the nuclear volume. Z-stack analysis of Smurf2/Topo IIα- stained U2OS cells. DNA was counterstained with Hoechst. Bars, 10 µm.

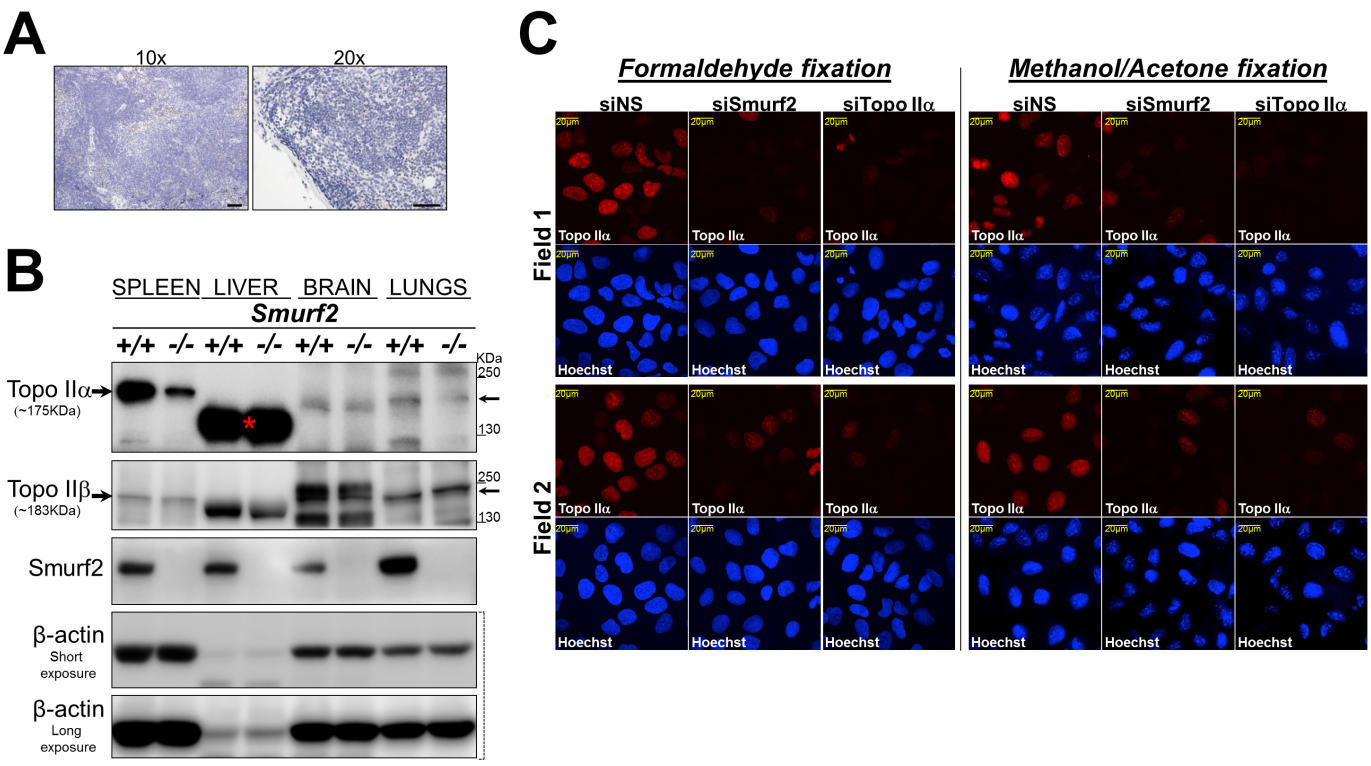

Figure S2. Smurf2 depletion reduces cellular levels of Topo IIα in cells and tissues. **A**, validation of the specificity of the DAB-staining used in IHC of mouse spleen tissues derived from Smurf2KO and WT mice. Samples were processed for the DAB staining, but omitting Topo IIα-staining step. The nuclei were counterstained with hematoxylin (blue). Two representative images are shown. Bars, 50 µm. **B**, western blot analysis of Topo IIα expression levels in different mouse tissues derived from Smurf2KO and littermate control mice. Note, the decreased Topo IIα protein levels in Smurf2KO spleen and lungs tissues. *-non-specific band. **C**, confocal images showing reduced levels of Topo IIα in Smurf2 knockdown U2OS cells, independent on cell fixation procedure. Two different fields with approximately 15-20 cells per field cells are shown for each fixation. siRNA for Topo IIα was used to demonstrate the specificity of anti-Topo IIα antibody. DNA was counterstained with Hoechst. Bars, 20 µm.

A

% cells in	1st experiment		2nd experiment		3rd experiment		Mean±S.D.	
	control	Smurf2^{CRISPR}	control	Smurf2^{CRISPR}	control	Smurf2^{CRISPR}	control	Smurf2^{CRISPR}
G1	51.7	51.2	47.9	43.8	45.1	48.4	48.2 ± 3.3	47.8 ± 3.7
S	22.3	25.2	22.5	26.7	24.4	24.8	23.1 ± 1.2	25.6 ± 1
G2	24.4	22.5	28.1	27.7	28.2	24.6	26.9 ± 2.2	24.9 ± 2.6
M	1.4	1.1	1.2	1.3	2.2	2.1	1.6 ± 0.6	1.5 ± 0.5

B

% cells in	1st experiment			2nd experiment			3rd experiment			Mean±S.D.		
	siNS	siSmurf 2	siTopo IIα	siNS	siSmurf 2	siTopo IIα	siNS	siSmurf 2	siTopo IIα	siNS	siSmurf2	siTopo IIα
G1	58.7	57.1	61.0	60.3	55.5	58.0	46.0	55.5	55.6	55 ± 7.8	56 ± 0.9	58.2 ± 2.7
S	18.9	20.2	20.1	17.3	17.6	16.0	18.9	17.4	17.1	18.4 ± 0.9	18.4 ± 1.6	17.7 ± 2.1
G2	20.9	21.4	17.6	21.0	25.8	24.5	32.3	25.0	24.8	24.7 ± 6.6	24.1 ± 2.3	22.3 ± 4.1
M	1.4	1.1	1.2	1.4	1.1	1.6	2.8	2.1	2.4	1.9 ± 0.8	1.4 ± 0.6	1.7 ± 0.6

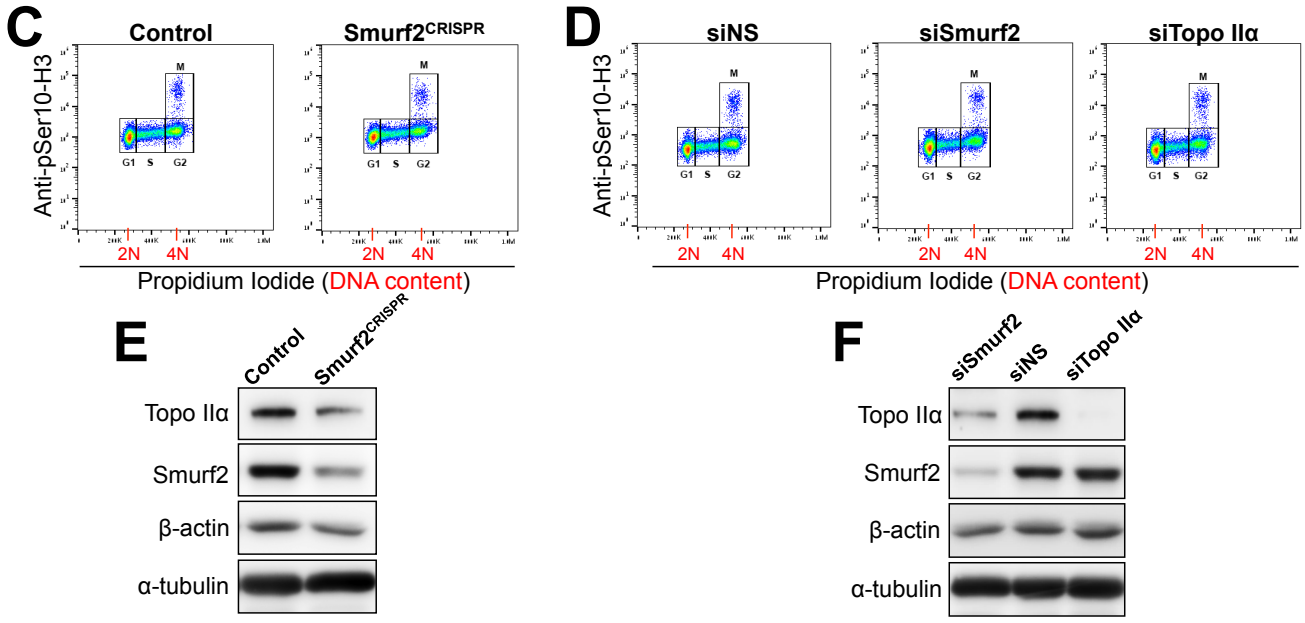

Figure S3. Effects of Smurf2 depletion on the cell cycle of U2OS cells. **A**, bi-parametric flow cytometry analysis of cell cycle distribution of Smurf2^{CRISPR} vs. control cells. Three independent experiments, as well as a summary (Mean±S.D.) of all three experiments, are presented in the figure. **B**, distribution of U2OS cells in cell cycle following Smurf2 or Topo IIα knockdown. Cells were collected for bi-parametric cell cycle analysis 72 hrs after siRNA transfection. Three independent experiments are shown. **C,** representative flow cytometry data described in (A). **D,** representative flow cytometry data described in (B). **E&F,** western blot analyses validating efficient Smurf2 inactivation in the analyzed samples. Whole cell extracts were prepared concomitantly to FACS analysis shown in (C and D).

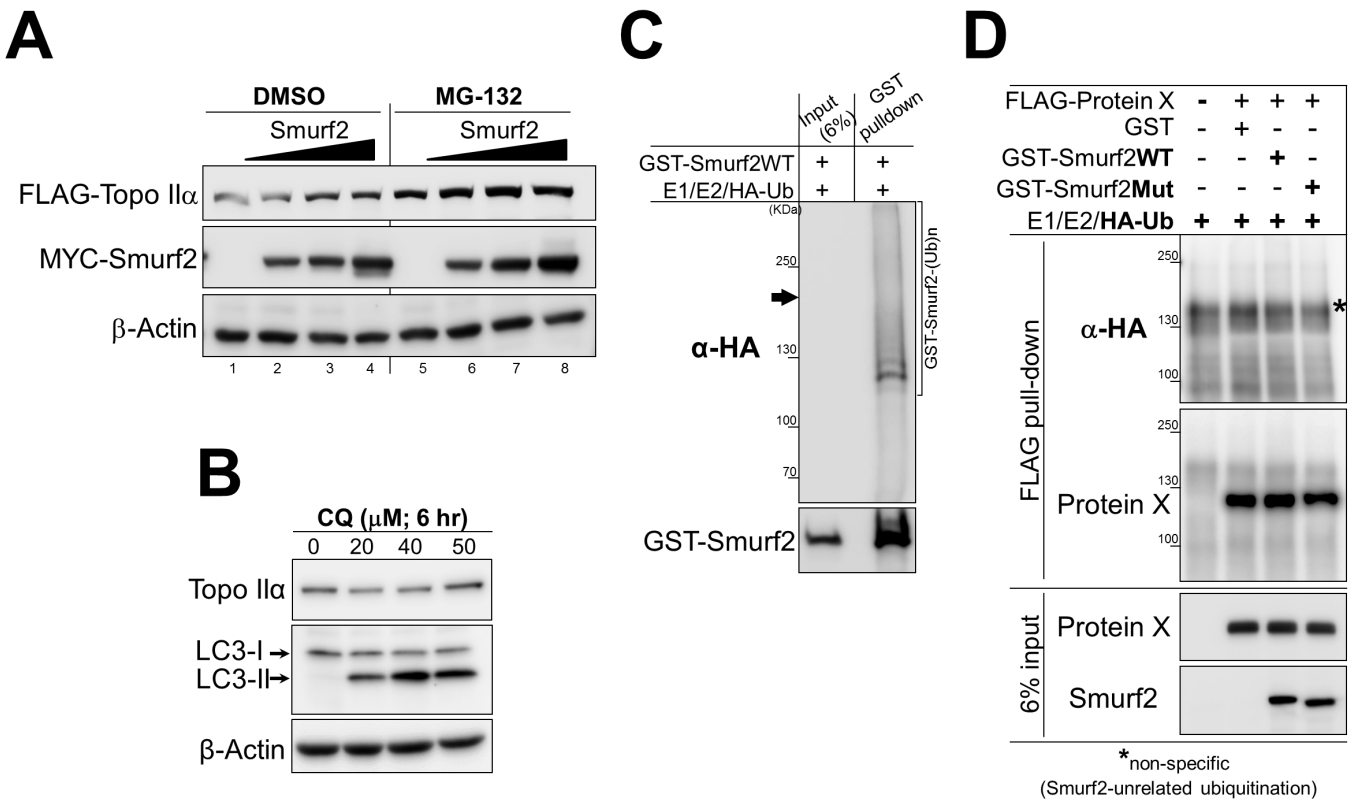

Figure S4. Effects of Smurf2 on stability of Topo IIα. **A**, MYC-Smurf2 protects FLAG-Topo IIα from proteasome-specific degradation in Smurf2 dose-dependent manner in HEK-293T cells. **B**, western blot analysis of Topo IIα cellular levels following inhibition of the lysosomal protein turnover with chloroquine (CQ). Inhibition of the lysosomal degradation pathway is demonstrated by membrane probing with anti-MAP1LC3A/B antibody that recognizes both the LC3-I and LC3-II forms of MAP1LC3A and MAP1LC3B. **C**, western blot analysis of Smurf2 ubiquitination pattern (auto-ubiquitination). GST-Smurf2 was pulled down from *in vitro* ubiquitination assay using GST-beads. Arrow shows the position of monoubiquitinated-Topo IIα detected in Topo IIα ubiquitination assay conducted in the tube (described in Fig. 4G). Note, the absence of this band in Smurf2 auto-ubiquitination assay. **D**, *in vitro* ubiquitination assay conducted on another, Topo IIα-unrelated, nuclear protein (protein-X) showing the absence of Topo IIα-specific monoubiquitination produced by Smurf2WT.

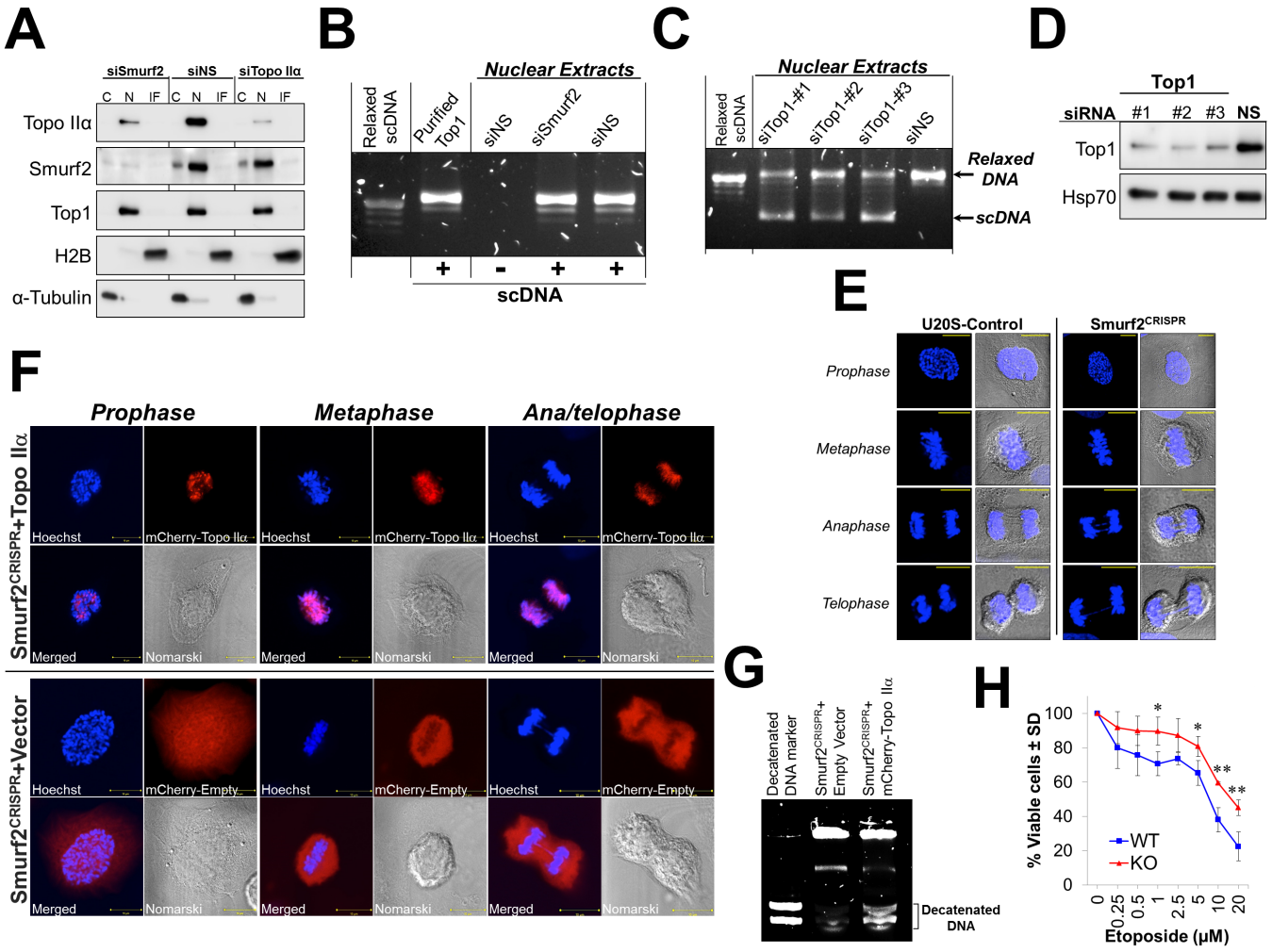

Figure S5. Effects of Smurf2 on Topo IIα and Top1 activities, DNA bridge formation, and cell sensitivity to Topo II poison etoposide. **A**, western blot analyses of cellular fractions prepared from Smurf2- and Topo IIα-knockdown U2OS cells. Nuclear fractions were used in DNA decatenation and Top1 activity assays. **B**, Top1 activity assay. scDNA, supercoiled DNA. **C**, validations of Top1 assay sensitivity using three different siRNAs for Top1. **D**, western blot analysis showing the efficiency of Top1 knockdown used in panel C. **E**, confocal images of Hoechst-stained U2OS cells sequestered at different stages of mitosis showing chromatin bridge formation in ana/telophase Smurf2^{CRISPR} cells. The particular stage of mitosis in these cells was determined based on the distinct cell morphology and DNA distribution pattern. Bars, 10 μm. **F**, confocal images of Smurf2^{CRISPR} cells expressing either mCherry-Topo IIα or an empty mCherry vector recorded at different stages of mitosis. Note the exclusion of a fluorescence signal generated by an empty vector from chromatin templates. Bars, 10 μm. **G**, DNA decatenation assay in Topo IIα-reconstituted cells. **H**, sensitivity of Smurf2KO MEFs cells to etoposide. Data are represented as the average values of three independent experiments performed in hexaplicates±SD. *P < 0.05, **P < 0.01.

Supplementary Table S1. The number/percentage of TMA samples in which a direct correlation between Smurf2 and Topo IIα was observed.

Samples	Samples with a direct Smurf2-Topo IIα correlation in:	
	Intensity	**%Positive cells**
Prostate Normal Tissues	19/31 (61.3%)	9/31 (29%)
Prostate Cancer Tissues	46/149 (30.9%)	61/149 (40.9%)
Breast Cancer Tissues	45/98 (45.9%)	71/98 (72.45%)