Cell Reports, Volume *33*

Supplemental Information

DNA and RNA Cleavage Complexes and Repair Pathway for TOP3B RNA- and DNA-Protein Crosslinks

Sourav Saha, Yilun Sun, Shar-yin Naomi Huang, Simone Andrea Baechler, Lorinc Sandor Pongor, Keli Agama, Ukhyun Jo, Hongliang Zhang, Yuk-Ching Tse-Dinh, and Yves Pommier

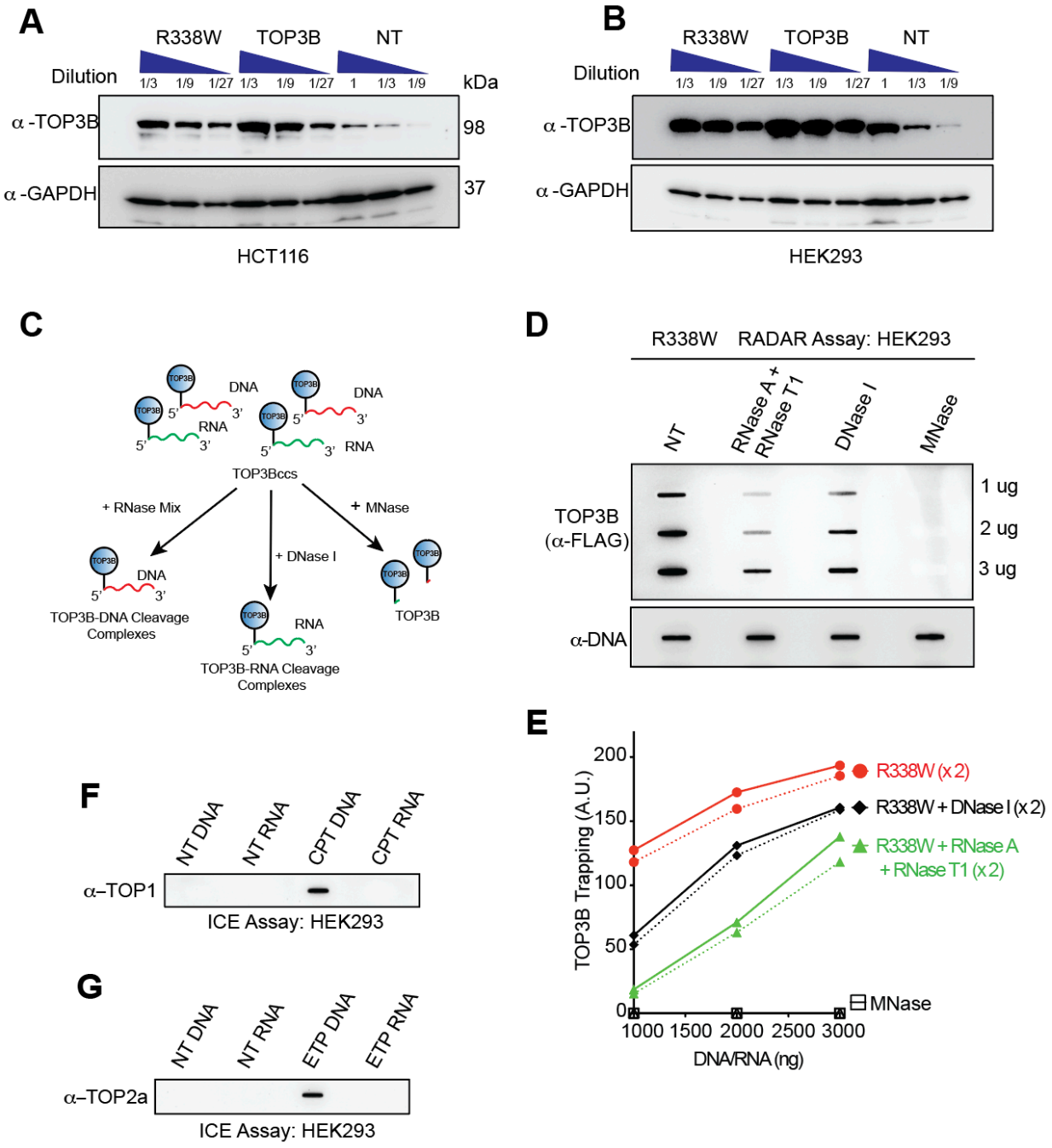

Figure S1

Figure S1. TOP3Bccs form both in DNA and RNA. Related to Figure 1.

(A & B) Western blots showing over-expression of WT-TOP3B and R338W-TOP3B compared to endogenous TOP3B both in HCT116 (A) and HEK293 cells (B). HEK293 and HCT116 cells were transfected with the indicated FLAG-tagged TOP3B constructs for 72 h and subjected to Western blotting with anti-TOP3B antibody.

(C & D) TOP3Bccs in DNA and RNA (red and green) in HEK293 cells transfected with FLAG-tagged R338W-TOP3B plasmid construct. Protein-nucleic acid adducts were isolated by RADAR assay and samples were digested either with excess RNase A (200 µg/mL) and RNase T1 (200

units/ml), or with DNase I (10 units) or micrococcal nuclease (MNase, 300 units). Samples were ethanol-precipitated, resuspended, slot-blotted and TOP3Bccs were detected with anti-FLAG antibody. Equal loading was tested by slot-blotting and probing with anti-dsDNA antibody. The figure is representative of two independent experiments.

(E) Quantitation of TOP3Bccs in 2 independent (x2) RADAR assays as shown in (D). TOP3Bccs were measured by densitometric analyses of slot-blot signals and plotted as a function of nucleic acid (DNA and RNA) concentration.

(F & G) Detection of TOP1cc and TOP2ccs by ICE assay in HEK293 cells after treatment with camptothecin (1 μM, 1 h) or etoposide (50 μM, 1 h). Equal numbers of cells were lysed in 1% sarkosyl and ICE (In vivo complex of enzymes) bioassays using cesium chloride gradient ultracentrifugation were performed to separate DNA (middle of the gradient) and RNA (bottom of the gradient) from free proteins (top of the gradient). The figure is representative of three independent experiments.

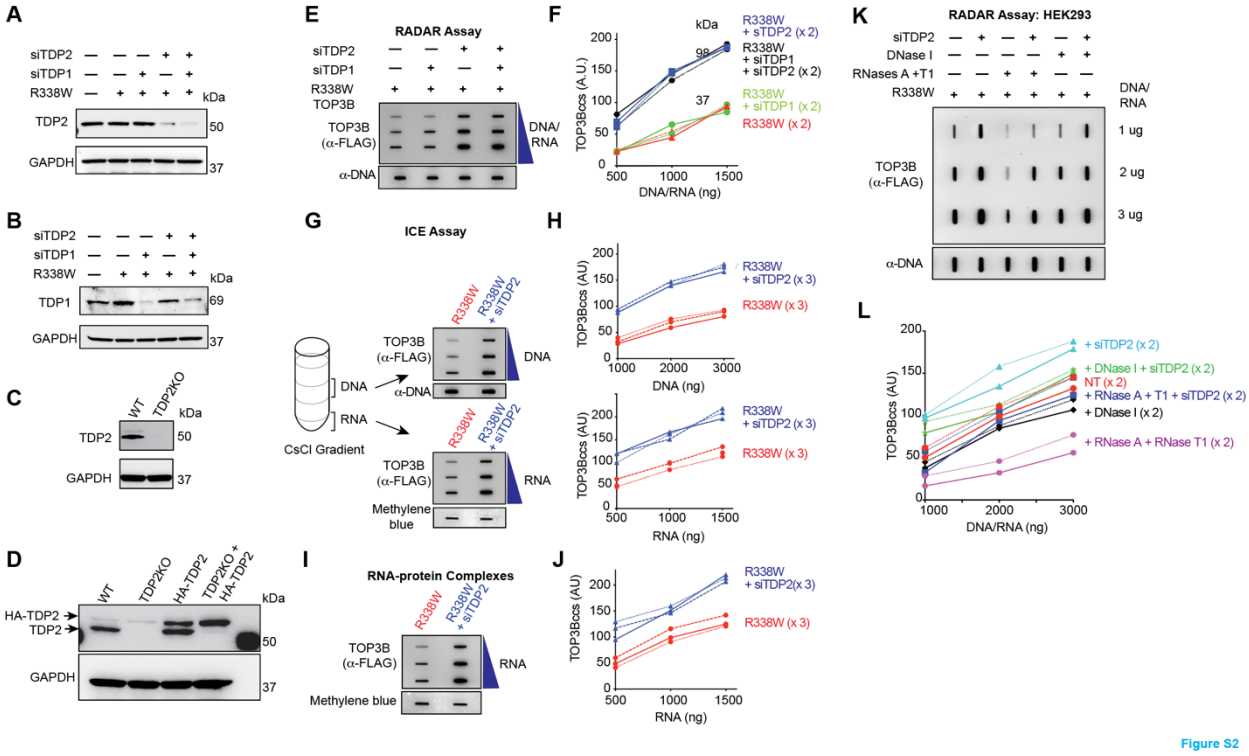

Figure S2. TDP2 Processes both RNA and DNA TOP3Bccs. Related to Figure 2.

(A-B) Western blots showing the efficiency of TDP1 and TDP2 knock-down (GAPDH as loading control). HEK293 cells were co-transfected with R338W-TOP3B and siRNA constructs against TDP1, TDP2 or both TDP1 and TDP2, as indicated. Cell lysates were subjected to Western blotting with anti-TDP1 and anti-TDP2 antibody.

(C) Western blot showing lack of TDP2 expression in TDP2KO HCT116 cells.

(D) Western blots showing expression levels of endogenous TDP2 and ectopically expressed HA-TDP2. Wild-type (WT) and TDP2KO HCT116 cells were transfected with FLAG-tagged R338W-TOP3B alone or with HA-tagged TDP2 and incubated for 72 h. Cell lysates were subjected to Western blotting and immunoblotted with anti-TDP2 antibody (GAPDH as loading control).

(E) DNA and RNA TOP3Bccs detected by RADAR assays. HEK293 cells were transfected with the indicated siRNAs and co-transfected with R338W-TOP3B. After 72 h, protein-nucleic acid adducts were isolated by RADAR assay, slot-blotted and TOP3Bccs were detected with anti-FLAG antibody. Equal loading was tested by slot-blotting and probing with anti-dsDNA antibody. The figure is representative of two independent experiments.

(F) Quantitation of two independent experiments as shown in (E). TOP3Bccs were measured by densitometric analyses of slot blot signals and plotted as a function of total nucleic acid (DNA and RNA) concentration.

(G) DNA and RNA TOP3Bccs detected by ICE assays. HEK293 cells were transfected with R338W-TOP3B-FLAG alone or co-transfected with siTDP2. After 72 h, ICE bioassay was performed to separate DNA and RNA fractions. TOP3Bccs were detected using anti-FLAG antibody. Equal loading was tested by slot-blotting and probing with anti-dsDNA antibody or methylene blue staining (RNA). The figure is representative of three independent experiments.

(H) Quantitation of 3 independent experiments as shown in (G). TOP3Bccs were measured by densitometric analyses of slot blot signals and plotted as a function of DNA and RNA concentration.
(I) Detection of RNA TOP3Bccs. HEK293 cells were transfected with R338W-TOP3B alone or co-transfected with siTDP2. After 72 h, covalent protein-RNA adducts were isolated using TRIzol®, slot-blotted and TOP3Bccs were detected using anti-FLAG Antibody. Equal loading was tested by slot-blotting and methylene blue staining. The figure is representative of three independent experiments.
(J) Quantitation of 3 independent experiments as shown in (I). TOP3Bccs were measured by densitometric analyses of slot blot signals and plotted as a function of RNA concentration.
(K) HEK293 cells were transfected with R338W-TOP3B alone or with siTDP2 construct as indicated. After 72 h nucleic acid-containing protein adducts were isolated by RADAR assay. Samples were digested either with excess RNase A (200 μg/mL) and RNase T1 (200 units/ml), or with DNase I (10 units/). Samples were ethanol precipitated, resuspended, slot-blotted and TOP3Bccs were detected with anti-FLAG antibody. Equal loading was tested by slot-blotting and probing with anti-dsDNA antibody. The figure is representative of two independent experiments.
(L) Quantitation of 2 independent experiments (x2) as shown in panel K. TOP3Bccs were measured by densitometric analyses of slot blot signals and plotted as a function of total nucleic acid (DNA and RNA) concentration.

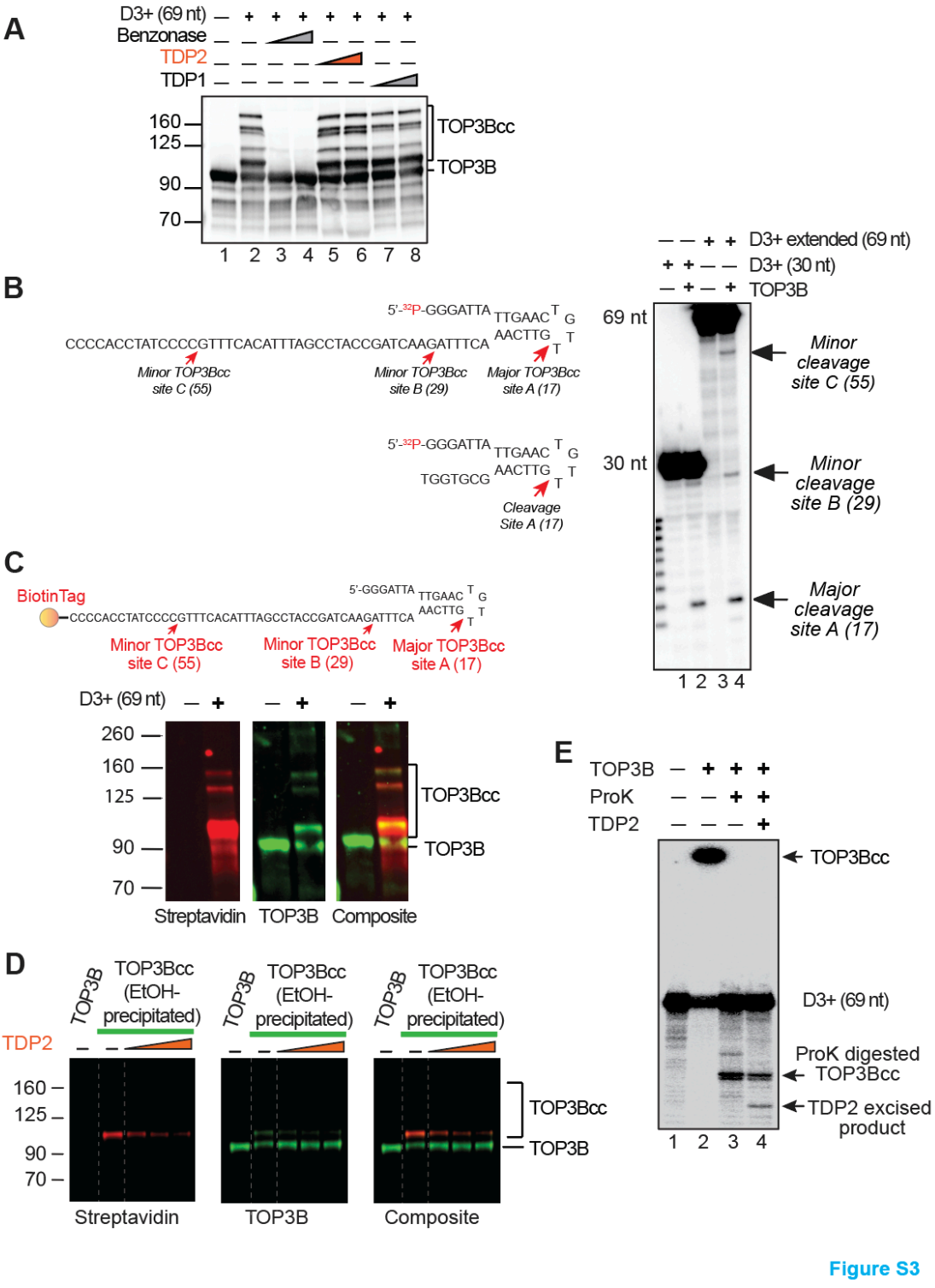

Figure S3

Figure S3. Processing Conditions of TOP3Bcc by Recombinant Human TDP2. Related to Figure 3.

(A) Full gel image of the experiment shown in Figure 3B showing the presence of several TOP3Bccs after D3+ extended oligonucleotide reacted with recombinant TOP3B (see Figure 3A).

(B) Representative gel showing *in vitro* cleavage assay of the D3+ substrate and its D3+ extended derivative to map the cleavage sites generated by TOP3B. The D3+ substrate (10 nM) and modified long D3+ substrate (10 nM) were labeled at the 5'-end with [γ-^{32}P] ATP and reacted with recombinant human TOP3B (22 nM). Reactions were performed at 37°C for 30 minutes and stopped by adding 0.2% SDS. Samples were separated in 20% denaturing polyacrylamide gels. TOP3B cleavage sites are indicated.

(**C**) Representative gel showing TOP3B cleavage complex (TOP3Bccs) formation after incubating purified recombinant human TOP3B (4 μM) with 3'-end biotin-labeled D3+ extended oligonucleotide (300 nM) to detect TOP3Bcc as upper-shifted bands. Samples were resolved by 6% SDS-PAGE and probed both with streptavidin (red channel) and anti-TOP3B (green channel) antibody. The composite channel shows the TOP3Bcc bands detected both by the anti-TOP3B antibody and the biotinylated DNA substrate covalently linked to TOP3B.

(**D**) Recombinant human TDP2 can excise denatured TOP3B from TOP3Bccs. The 3'-end biotin-labeled modified D3+ extended oligonucleotide substrate (300 nM) was reacted with purified recombinant human TOP3B (4 uM) at 30°C for 30 minutes. Reaction were stopped by adding 0.2% SDS and TOP3Bccs were denatured using ethanol. Following ethanol precipitation, samples were incubated with increasing concentrations of TDP2 (2.25, 4.5, 9 μM) at 25°C overnight. Samples were resolved by 6% SDS-PAGE and probed both with streptavidin and anti-TOP3B antibodies.

(**E**) TDP2 can process proteinase K digested TOP3Bccs. 3'-end radiolabeled (using 3′-[α-32P] cordycepin 5′-triphosphate) modified D3+ extended oligonucleotide (80 nM) was reacted with recombinant human TOP3B (4 μM) at 30°C for 1 hour. The samples were digested with proteinase K (overnight) followed by heat-inactivation (95°C for 5 minutes). The resulting samples were incubated with recombinant TDP2 (1 μM) at 25°C for 30 minutes. TOP3Bcc formation results in in the retention of the radiolabeled oligonucleotide in the well of the gel (lane 2). As TOP3B forms cleavage complexes at three different sites on the D3+ extended substrates, digestion of TOP3Bcc with proteinase K results in three different species of TOP3Bccs (lane 3). TDP2-mediated processing of the proteolyzed TOP3Bccs releases the oligonucleotide substrate (lane 4; TDP2 excised product).

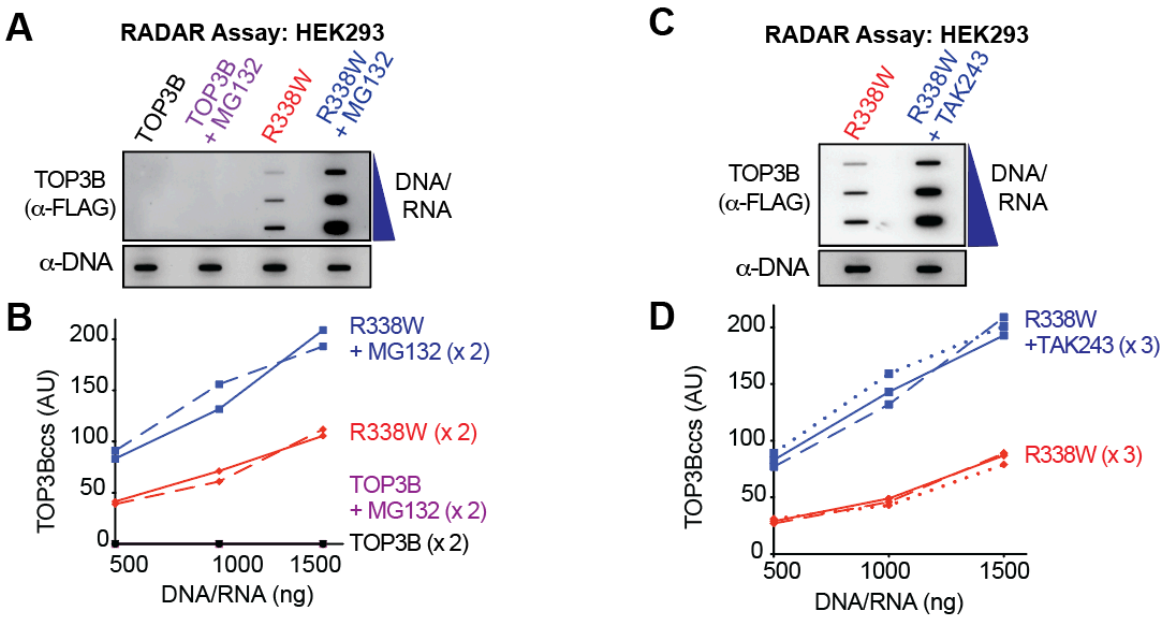

Figure S4

Figure S4. Cellular TOP3Bccs are Ubiquitinated and Degraded by the Proteasomal Pathway. Related to Figure 4.

(A) Proteasome inhibition enhances cellular TOP3Bccs. HEK293 cells were transfected with FLAG-tagged wild-type TOP3B and R338W-TOP3B for 72 h. Before harvest, cells were treated with MG132 (10 μM, 2 h). TOP3Bccs were detected by RADAR assays using anti-FLAG antibody. Equal loading was tested by slot-blotting and probing with anti-dsDNA antibody. The figure is representative of two independent experiments.

(**B**) Quantitation of 3 independent experiments as shown in (A). TOP3Bccs were measured by densitometric analyses of slot blot signals and plotted as a function of DNA/RNA concentration.

(C) Ubiquitination inhibition enhances cellular TOP3Bccs. HEK293 cells were transfected with FLAG-tagged R338W-TOP3B for 72 h. Before harvesting, the cells were treated with the UAE inhibitor TAK243 (10 μM, 2 h). TOP3Bccs were detected by RADAR assays using anti-FLAG antibody. Equal loading was tested by slot-blotting and probing with anti-dsDNA antibody. The figure is representative of three independent experiments.

(**D**) Quantitation of 3 independent experiments as shown in (C). TOP3Bccs were measured by densitometric analyses of slot blot signals and plotted as a function of DNA/RNA concentration.

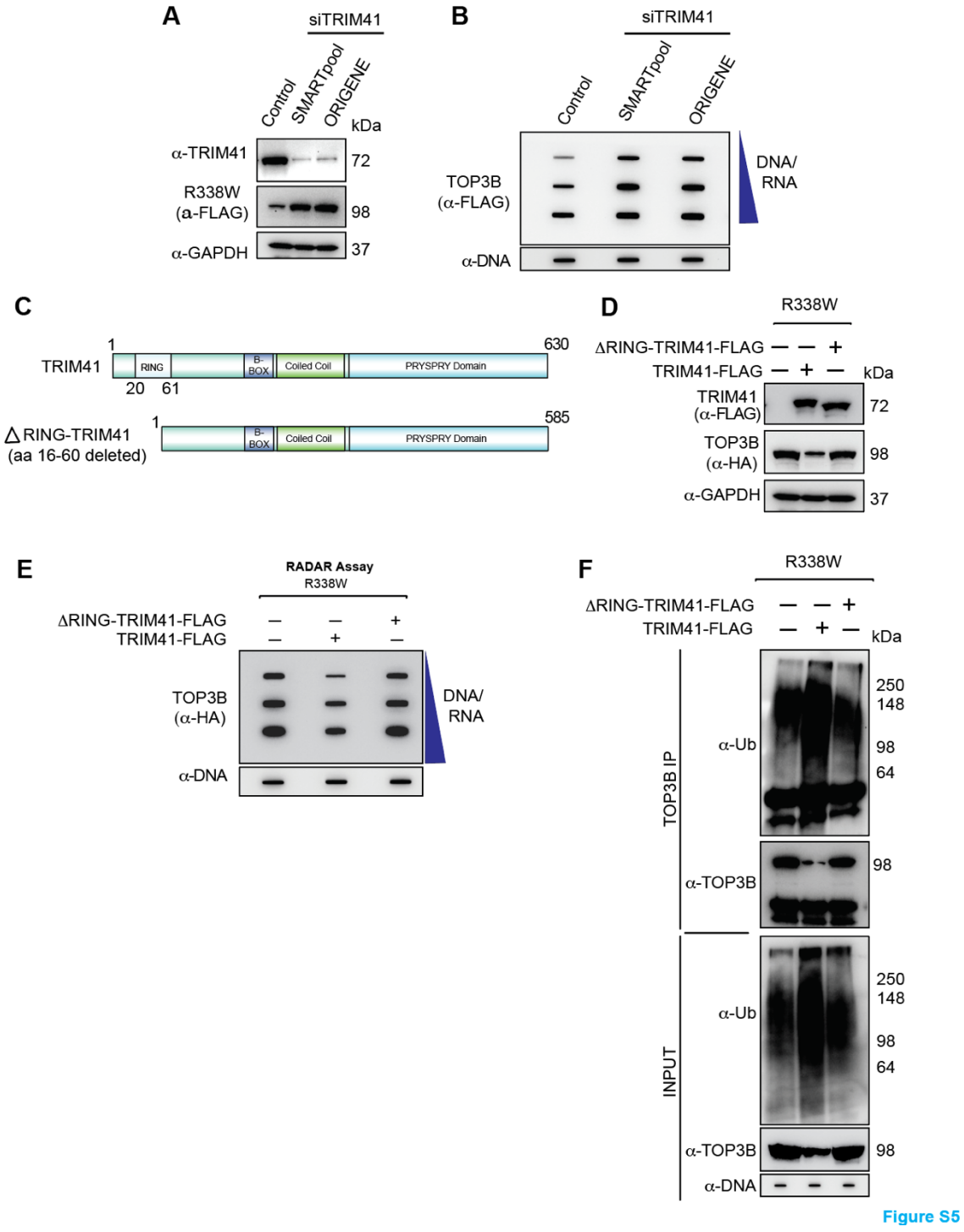

Figure S5

Figure S5. E3 ligase activity of TRIM41 is required for ubiquitination and repair of TOP3Bcc. Related to Figure 5.

(A) Immunoblots showing TRIM41 and R338W-TOP3B expression level after transfecting HCT116 cells with two different sets of siRNAs against TRIM41 (Human TRIM41 SMART pool, Dharmacon and three pairs of siRNA duplexes for TRIM4, ORIGENE). HCT116 cells were either

transfected with FLAG-tagged R338W-TOP3B plasmid construct alone or co-transfected with siTRIM41construct for 72 h. GAPDH was included as loading control.
(B) HCT116 cells were transfected with FLAG-tagged R338W-TOP3Bs alone or co-transfected with siTRIM41constructs for 72 h. Cells were harvested and nucleic acids and protein-nucleic acid adducts isolated by RADAR assay. TOP3Bcc were detected with anti-FLAG antibody.
(C) Schematic representation of the domain organization of human TRIM41 (1-630 aa) and RING domain deletion mutant of TRIM41 (deletion of amino acids 16-60; ΔRING-TRIM41).
(D) Immunoblots showing WT TRIM4, ΔRING-TRIM41 and R338W-TOP3B expression after transfection of HCT116 cells with HA-tagged R338W-TOP3B plasmid construct alone or co-transfected with either FLAG-tagged TRIM41 or FLAG-tagged ΔRING-TRIM41. GAPDH was included as loading control.
(E) HCT116 cells were either transfected with HA-tagged R338W-TOP3B plasmid construct alone or co-transfected with either FLAG-tagged TRIM41 or FLAG-tagged ΔRING-TRIM41. After 48 h cells were harvested and nucleic acids and protein-nucleic acid adducts were isolated by RADAR assay. TOP3Bccs were detected using anti-HA antibody.
(F) HCT116 cells were either transfected with HA-tagged R338W-TOP3B plasmid construct alone or co-transfected with either FLAG-tagged TRIM41 or FLAG-tagged ΔRING-TRIM41. After 48 h, RADAR assay samples were prepared, and equal amounts of RADAR assay samples were immunoprecipitated with anti-TOP3B antibody. IP samples and the INPUT RADAR assay samples were digested with micrococcal nuclease (MNase), resolved on SDS-PAGE and immunoblotted with anti-Ub and anti-TOP3B antibody. Equal loading of INPUT RADAR samples was tested by slot-blotting and probing with anti-dsDNA antibody.

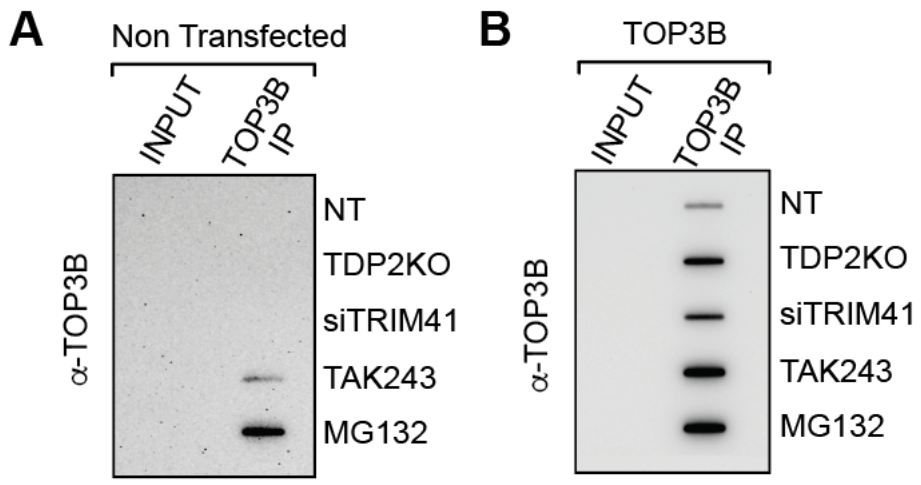

Figure S6

Figure S6. Endogenous and WT-TOP3Bcc could be detected following IP enrichment of TOP3Bccs. Related to Figure 1 and 6.

(A) Endogenous TOP3Bccs can be detected by blocking the ubiquitin-proteasome pathway. RADAR assay samples were prepared from wild-type, TDP2 knockout, TRIM41 knocked-down and MG132- or TAK243-treated HCT116 cells. RADAR assay samples were immunoprecipitated with anti-TOP3B antibody, eluted from the beads, ethanol precipitated and resuspended. IP samples and the INPUT RADAR assay samples were slot blotted and TOP3Bccs were detected with anti-TOP3B antibody.

(B) TOP3Bccs after transfection of WT-TOP3B. RADAR assay samples were prepared from wild-type, TDP2 knockout, TRIM41 knocked-down and MG132- or TAK243-treated HCT116 cells. RADAR assay samples were immunoprecipitated with anti-TOP3B antibody, eluted from the beads, ethanol precipitated and resuspended. IP samples and the INPUT RADAR assay samples were slot blotted and TOP3Bccs were detected with anti-TOP3B antibody.

Table S1. All molecular cloning and mutagenesis primers used in this study. Related to STAR Methods.

Primers	Sequence	SOURCE
TDP2 forward primer for cloning into pcDNA3-HA	5'- TATAGGATCCGAGTTGGGGAGTTGCCTG-3'	IDT oligo
TDP2 reverse primer for cloning into pcDNA3-HA	3'- GCGCGAATTCTTACAATATTATATCTAA-5'	IDT oligo
TOP3B forward primer for cloning into pcDNA3-HA	5'- GCTTGGATCCAAGACTGTGCTCATGGTT-3'	IDT oligo
TOP3B reverse primer for cloning into pcDNA3-HA	3'-CCAAGAATTCTCATACAAAGTAGGCGGC-5'	IDT oligo
TOP3B forward primer for cloning into Gateway entry vector pENTR3C	5'-CGGGGTACCATGAAGACTGTGCTCATGG-3'	IDT oligo
TOP3B reverse primer for cloning into Gateway entry vector pENTR3C	5'-AGGCTACATCAGCTACGT ACGGACAGAGACCACC-3'	IDT oligo
P337V TOP3B-Myc-FLAG mutagenesis primers	5'-GGTGGTCTCTGTCCGTA CGTAGCTGATGTAGCCT-3' 5'-AGGCTACATCAGCTACG TACGGACAGAGACCACC-3'	IDT oligo
R338W-TOP3B-Myc-FLAG mutagenesis primers:	5'-GGTCTCTGTCCATGGG TAGCTGATGTAGCCT-3' 5'-AGGCTACATCAGCTAC CCATGGACAGAGACC-3'	IDT oligo
TRIM41 RING Domain Deletion Primers	5'-CGGGAGGAGGAGGAGGAG-3' 5'-CTGAAGGGTCTGCACAGG-3'	IDT oligo
Ubiquitin K6R mutagenesis primer	5'-ATCTTCGTGAGGACCCTGACTGG-3'	IDT oligo
Ubiquitin K11R mutagenesis primer	5'-CTGACTGGTAGGACCATCACTC-3'	IDT oligo
Ubiquitin K27R mutagenesis primer	5'-GAGAATGTCAGGGCAAAGATCC-3'	IDT oligo
Ubiquitin K29R mutagenesis primer	5' GTCAAGGCAAGGATCCAAGAC-3'	IDT oligo
Ubiquitin K33R mutagenesis primer	5'-ATCCAAGACAGGGAAGGCATC-3'	IDT oligo
Ubiquitin K48R mutagenesis primer	5'-TTTGCTGGGAGACAGCTGGAA-3'	IDT oligo
Ubiquitin K63R mutagenesis primer	5'-AACATCCAGAGAGAGTCCACCC-3'	IDT oligo