Mitochondrial tyrosyl-DNA phosphodiesterase 2 and its TDP2^{S} short isoform

Appendix

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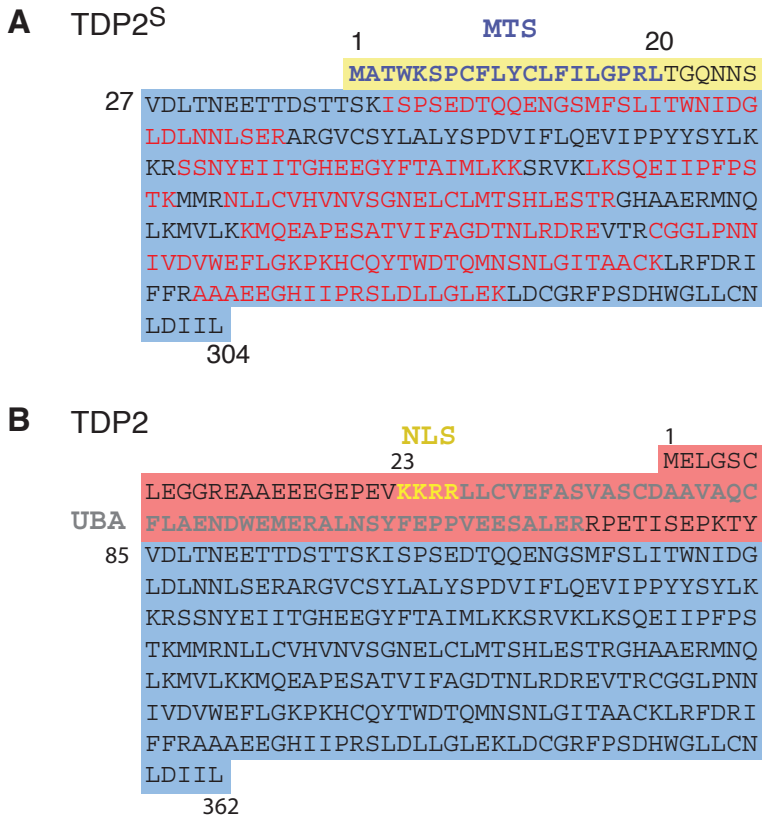
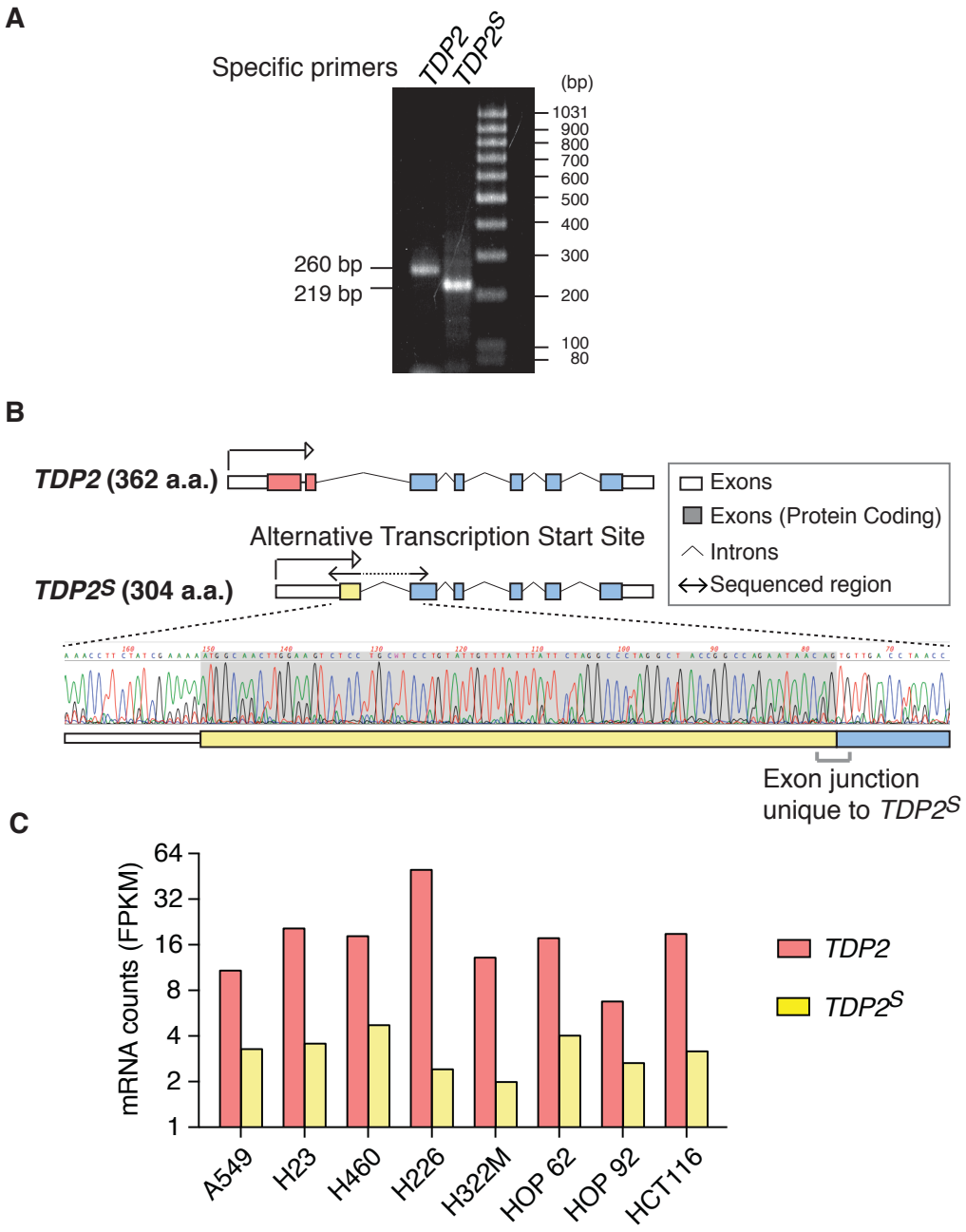


Appendix Fig S1. Comparison of the protein sequence for the two TDP2 isoforms.

A Protein sequence of TDP2^{S} short isoform (a.a. 1-304). The identical catalytic C-terminal domain between the two TDP2 isoforms is shaded in blue. The N-terminal sequence unique to TDP2^{S} is shaded in yellow, with the mitochondrial targeting sequence (MTS: a.a. 1-20) in bold blue letters. The peptides identified by mass spectrometry in the pull-down are annotated in red letters (12 unique peptides, 58% coverage).

B Protein sequence of TDP2 canonical full-length isoform (a.a. 1-362). The identical catalytic C-terminal domain between the two TDP2 isoforms is shaded in blue. The N-terminal leading sequence unique to TDP2, encoded by the first two exons, is shaded in red. The nuclear localization sequence (NLS: aa 23-26) is marked in bold yellow letters while the ubiquitin-associated (UBA) domain is annotated in bold green letters.



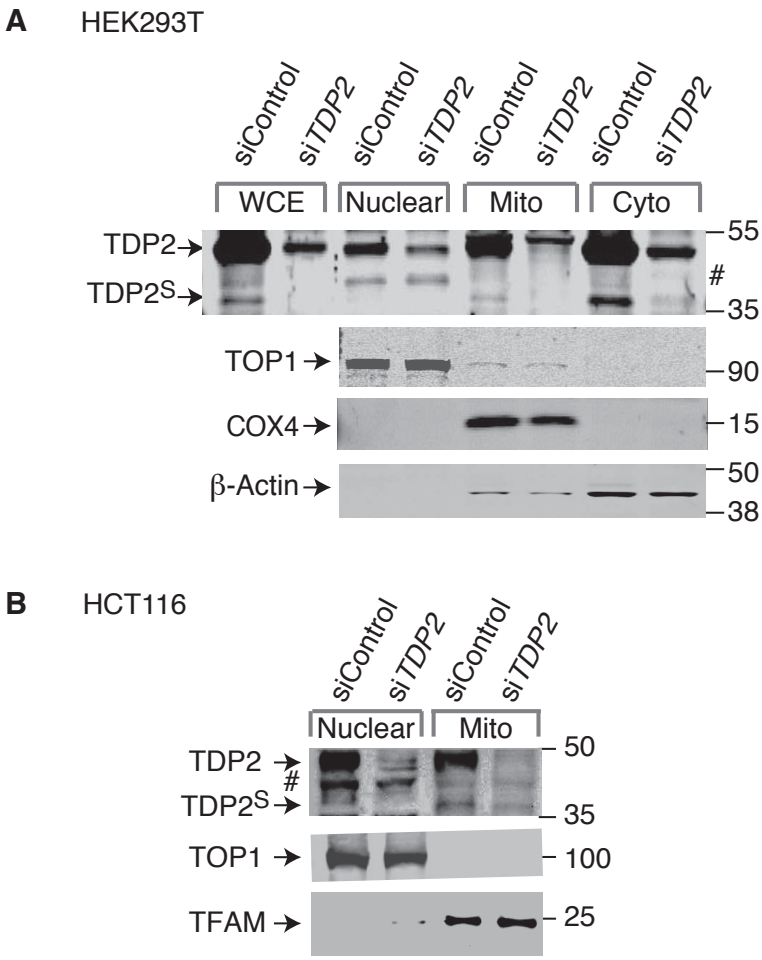
Appendix Fig S2. Sanger sequencing of *TDP2*^{S} transcript and comparison of transcript levels for *TDP2* and *TDP2*^{S} in lung cancer cells panel.

A PCR products using cDNA purified and constructed from HCT116 as template and isoform specific primers for *TDP2* and *TDP2*^{S} are of expected lengths, as resolved on a 1% agraose gel, demonstrating the specificity of each set of PCR primers.

B The PCR product using cDNA purified and constructed from HCT116 as template and *TDP2*^{S} isoform-specific primers was verified by sanger sequencing. The chromatogram of the sequence is shown below the corresponding region of the transcript

for *TDP2*^{S}, where the unique protein-coding exon of the transcript for *TDP2*^{S} is shaded in gray and the exon junction unique to *TDP2*^{S} is indicated.

B Computed mRNA counts (Fragments per kilobase per million reads) mapped to the two *TDP2* isoform from RNA sequencing data for the panel of lung cancer cells. RNA was quantified and treated with DNAse according to the manufacturers protocol (Qiagen, Inc). Total RNA libraries were generated using TotalScript™ RNA-Seq Kit (Epicentre) without normalization. Library Sequencing was done using the HiSeq 2000 (Illumina) with paired end 100bp reads using the TruSeq Cluster Kit v3 (Illumina) at the Center for Cancer Research Sequencing facility. Data conversion to fastq and alignment to human genome assembly 19 was done with the STAR split-read aligner. Data quality was determined using RNAseQC. From the sequenced and mapped reads, we selected reads mapping to the *TDP2* locus using *SAMtools* and computed the number of reads mapping to each nucleotide using the R Bioconductor package *Rsamtools*.

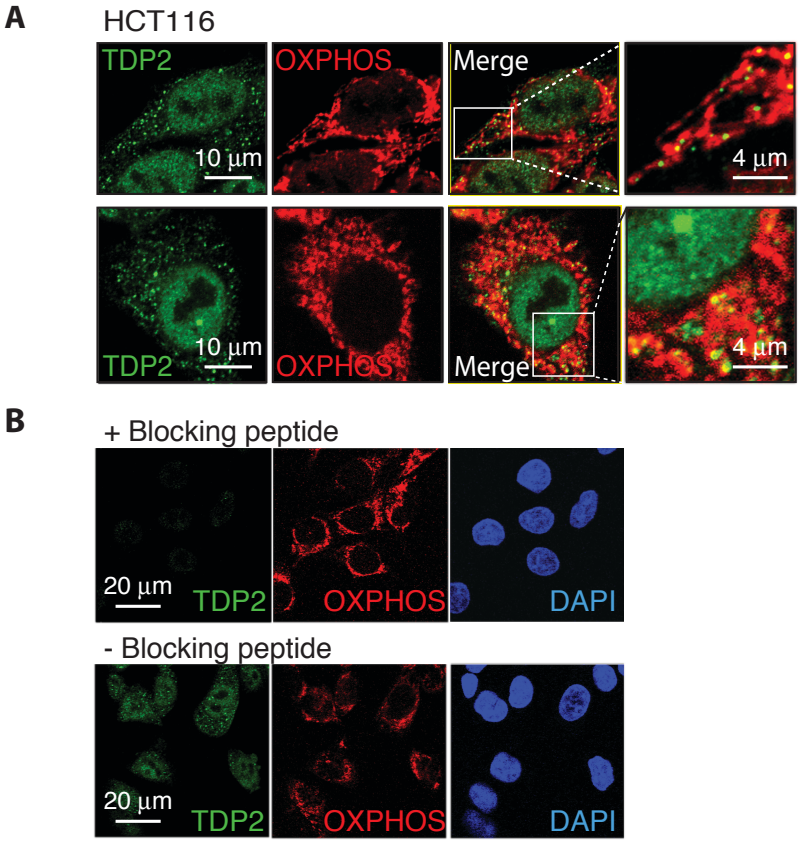


Appendix Fig S3. Cellular fractionation of human cell lines shows that TDP2 localize to both the nuclear and mitochondrial compartments.

A Representative immunoblot of cellular fractions from HEK293T cells. HEK293T cells were transfected with either siControl or si*TDP2* for 48 hours before the cells were fractionated into nuclear, mitochondrial and cytosolic fractions (30 μg protein per lane). Whole cell extract was also included as a control (30 μg protein per lane). Top panel probes for TDP2, while TOP1, COX4 and β-Actin serve as markers for the nuclear, mitochondrial and cytosolic fractions, respectively. In the nuclear fraction, only the full-length TDP2 was noticeably knocked-down by si*TDP2*. In the mitochondrial and cytosolic fractions, both TDP2 and $TDP2^S$ were knocked-down by si*TDP2*. Non-specific bands are denoted by #.

B Representative immunoblot of cellular fractions from human colon cancer HCT116 cells. HCT116 cells were transfected with either si*TDP2* or siControl for 48 hours before the cells were fractionated into nuclear and mitochondrial fractions (30 μg protein was loaded in each lane). The top panel probes for TDP2, while the TOP1 and TFAM serves

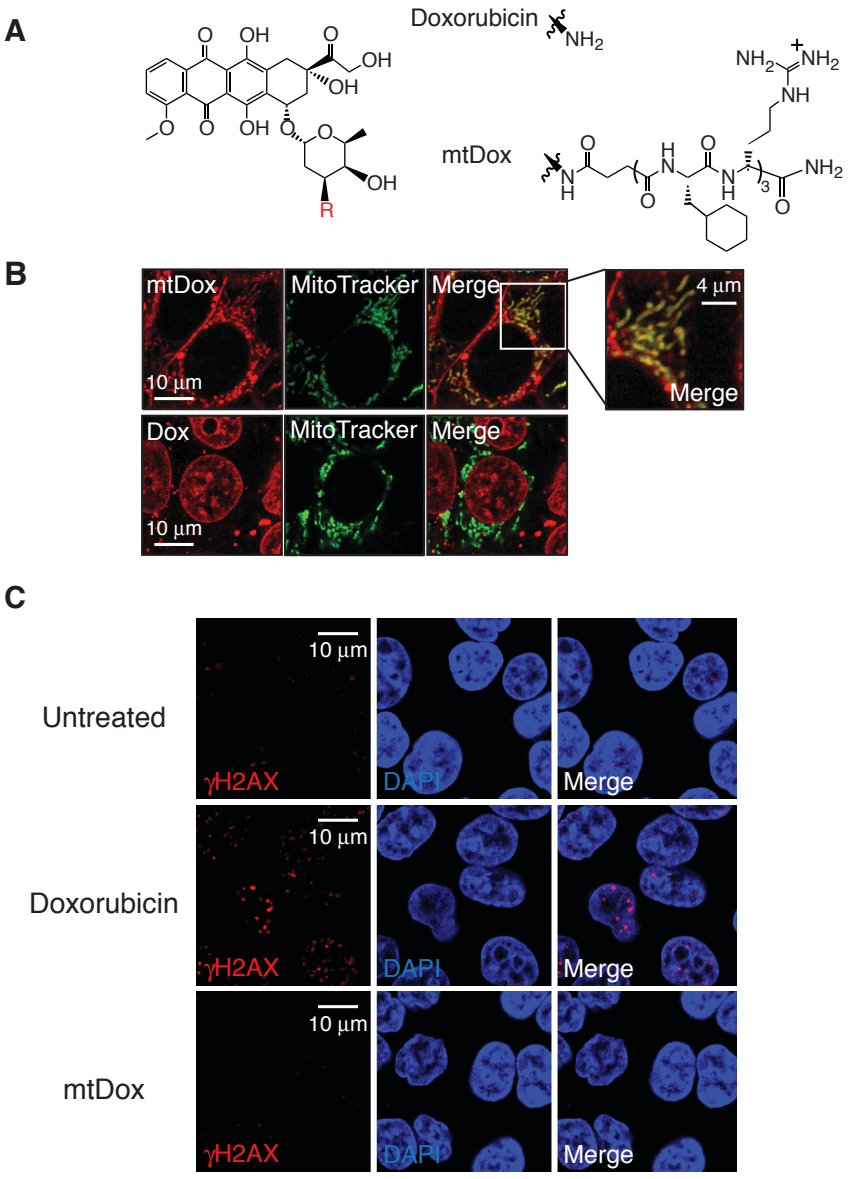
as marker for the nuclear and mitochondrial markers, respectively. In the nuclear fraction, only the full-length TDP2 was noticeably knocked-down by si*TDP2*. In the mitochondrial fraction, both TDP2 and TDP2^{S} were noticeably knocked-down by si*TDP2*.



Appendix Fig S4. TDP2 localizes to mitochondria in human cells.

A Representative immunofluorescence confocal microscopy images of HCT116 cells. Fixed HCT116 cells were incubated for two hours at room temperature with rabbit polyclonal anti-TDP2 (Bethyl, Montgomery, TX, dilution 1:50, left column in green) and mouse monoclonal anti-OXPHOS (Life Technologies, Carlsbad, CA, dilution 1:200, second column in red). After three washes with PBS, cells were counterstained with Alexa Fluor® 488 anti-rabbit and Alexa Fluor® 568 anti-mouse secondary antibodies where indicated (Life Technologies, Carlsbad, CA, dilution 1:500). Merged images are shown in the third column, with the zoomed-in area shown in the last column.

B Representative immunofluorescence confocal microscopy image of HCT116 cells, where anti-TDP2 antibody (Bethyl, Montgomery, TX, dilution 1:50) was preincubated with (upper panels) or without (lower panels) its blocking peptide at 4°C overnight (Bethyl, Montgomery, TX, 2:1 ratio). First column on left in green shows staining for anti-TDP2. Second column from left in red shows staining for oxidative phosphorylation (OXPHOS) complexes, and the last column in blue shows staining with DAPI.

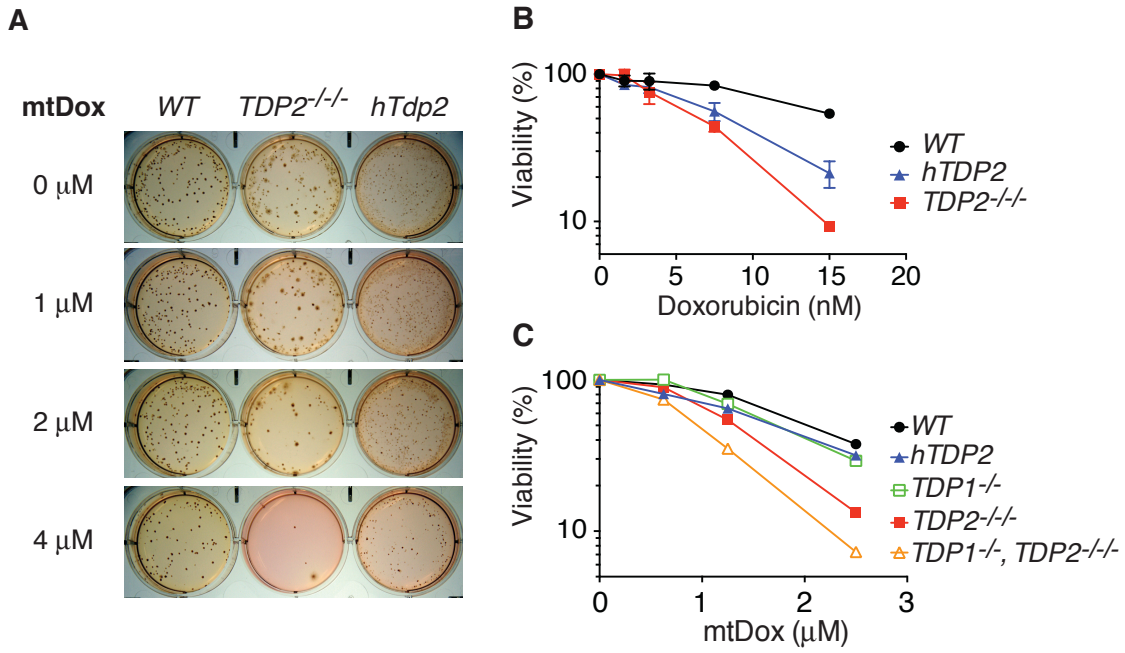


Appendix Fig S5. Mitochondrial-targeted doxorubicin conjugate (mtDOX) specifically translocates to the mitochondria without inducing nuclear DNA damage.

A Structures of doxorubicin and mitochondrial-targeted doxorubicin (mtDox).

B Live-cell fluorescence confocal microscopy of HCT116 cells treated with 5 µM mtDox or doxorubicin (Dox) for 24 hours. HCT116 cells were cultured on Nunc™ Lab-Tek™ chamber slides (Thermo Scientific, Waltham, MA) and imaged on a confocal microscope (LSM 710 or LSM 780, Zeiss, Oberkochen, Germany). Left panels show the intrinsic fluorescence of the drugs, middle panels show MitoTracker® Green staining, and merged images in the right panels, with an enlarged area.

C Immunofluorescence confocal microscopy of HCT116 cells treated with 40 nM doxorubicin or 1 μM mtDox for 16 hours before fixation and immunostaining with antibodies for γH2AX (Millipore), a nuclear DNA damage marker (shown in left panels). Images were collected using a confocal microscope (LSM 710 or LSM 780, Zeiss, Oberkochen, Germany).

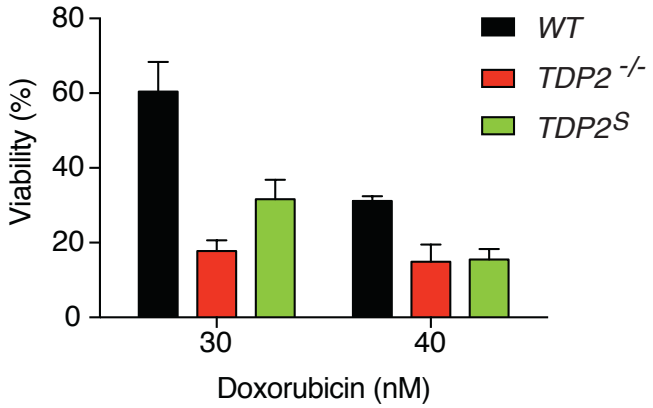


Appendix Fig S6. TDP2 protects against mitochondrial and nuclear TOP2 poisoning.

A Representative clonogenic assays of wild type (*WT*), *TDP2* knockout (*TDP2*$^{-/-/-}$), and human *TDP2*-recomplemented (*hTDP2*) DT40 cells. Quantification is shown in Fig 4D (n=2).

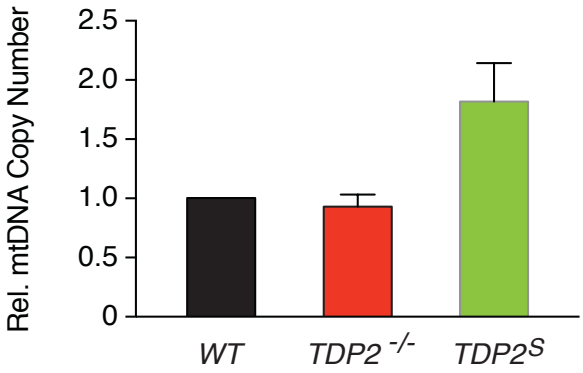
B The isogenic DT40 cells with the indicated genetic deficiencies were treated with the indicated concentration of doxorubicin for 72 hours in triplicates then assayed with the ATPlite 1-step kit (PerkinElmer, Waltham, MA, USA). Black closed circles are wild type (*WT*) cells, red closed squares are *TDP2* knockout (*TDP2*$^{-/-/-}$), and blue closed triangles are *TDP2*$^{-/-/-}$ cells complemented with human *TDP2* (*hTDP2*). Viability was normalized to untreated control cells (n=3, error bars represent SEM).

C Lack of TDP2 and not TDP1 sensitizes DT40 cells to mtDox. The isogenic DT40 cells with the indicated genetic deficiencies were treated with the indicated concentration of mtDox for 72 hours in triplicates before assayed with the ATPlite 1-step kit (PerkinElmer, Waltham, MA, USA). Black closed circles are wild type (*WT*) cells, red closed squares are *TDP2* knockout (*TDP2*$^{-/-/-}$), blue closed squares are *TDP2*$^{-/-/-}$ cells recomplemented with *hTDP2*, (*hTDP2*), green open squares are *TDP1* knockout (*TDP1*$^{-/-}$), and orange open triangles are *TDP1* and *TDP2* double knockout (*TDP1*$^{-/-}$, *TDP2*$^{-/-/-}$) cells. Survival was normalized to untreated control cells (n=2).



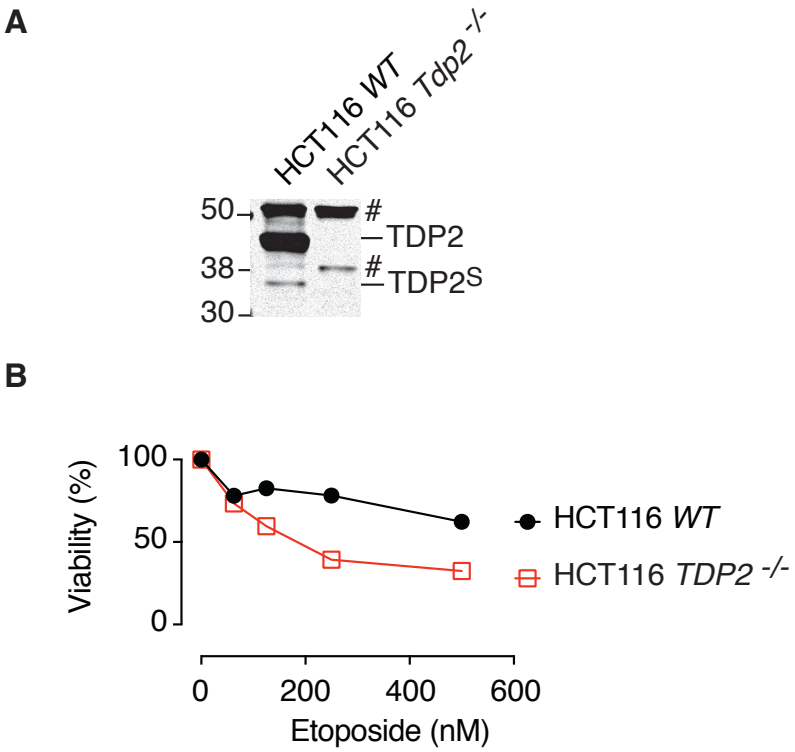
Appendix Fig S7. Lack of full-length TDP2 results in sensitivity to doxorubicin and is not rescued by TDP2^{S}.

Viability assays of wild type (*WT*), *TDP2* knockout (*TDP2*$^{-/-}$) and *TDP2*^{S} isoform-specific (*TDP2*^{S}) TK6 cells treated with doxorubicin. Cells were treated with the indicated concentration of doxorubicin for 72 hours in triplicates before cells were assayed with the ATPlite 1-step kit (PerkinElmer, Waltham, MA, USA). Survival was normalized to untreated control cells (n=3, error bars represent SEM).



Appendix Fig S8. Relative mtDNA copy numbers of *WT*, *TDP2*$^{-/-}$ and *TDP2*^{S} TK6 cells.

Genomic DNA of *WT*, *TDP2*$^{-/-}$ and *TDP2*^{S} TK6 cells was extracted using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) and used as templates in quantitative real-time PCR to measure relative mtDNA copy numbers. Quantitative real-time PCR was carried out with FastStart Universal SYBR Green Master Mix (Roche, Indianapolis, IN) in quintuplets on an ABI 7900 thermocycler (Life Technologies, Carlsbad, CA). The primer sequences are as follows: *β2 microglobulin* (*β2M*) as the nuclear housekeeping gene for normalization (5'-tgctgtctccatgtttgatgtatct [sense] and 5'-tctctgctccccacctctaagt [antisense]) and *ND1* for relative mtDNA copy number (5'-aagtcaccctagccatcattctac [sense] and 5'-gcaggagtaatcagaggtgttctt [antisense]). The Ct value for mitochondria genome is referenced to the Ct value of the *β2M* for each sample (ΔCt). Then the ΔCt for each cell line is in turn referenced to that of the *WT* cells (ΔΔCt), which is set at 1. The relative mtDNA copy number for each cell line is calculated based on its ΔΔCt values (n=3, error bars represent SEM).



Appendix Fig S9. Verification of *TDP2* knockout by CRISPR in human colon cancer HCT116 cells.

A Immunoblotting of whole cell extracts from HCT116 *WT* and *TDP2*$^{-/-}$ cells (30 µg per lane). Non-specific bands are marked by #.

B Viability assays of wild type (*WT*) and *TDP2* knockout (*TDP2*$^{-/-}$) HCT116 cells treated with etoposide, confirming that lack of TDP2 results in hypersensitivity to TOP2 poisons. Cells were treated with the indicated concentration of etoposide for 72 hours in triplicates before cells were assayed with the ATPlite 1-step kit (PerkinElmer, Waltham, MA, USA). Survival was normalized to untreated control cells (n=2).