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Epigenetic and genetic inactivation of tyrosyl-DNA-phosphodiesterase 1 (TDP1) in human lung cancer cells from the NCI-60 panel

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Abstract

Tyrosyl-DNA-phosphodiesterase 1 (TDP1) repairs 3'-blocking DNA lesions by catalytically hydrolyzing the tyrosyl-DNA-phosphodiester bond of trapped topoisomerase I (Top1) cleavage complexes (Top1cc). It also removes 3'-blocking residues derived from oxidative damage or incorporation of chain terminating anticancer and antiviral nucleosides. Thus, TDP1 is regarded as a determinant of resistance to Top1 inhibitors and chain terminating nucleosides, and possibly of genomic stability. In the 60 cell lines of the NCI Developmental Therapeutic Anticancer Screen (the NCI-60), whose whole genome transcriptome and mutations have recently been characterized, we discovered two human lung cancer cell lines deficient for TDP1 (NCI_H522 and HOP_62). HOP_62 shows undetectable *TDP1* mRNA and NCI_H522 bears a homozygous deleterious mutation of *TDP1* at a highly conserved amino acid residue (K292E). Absence of TDP1 protein and lack of TDP1 catalytic activity were demonstrated in cell lysates from both cell lines. Lack of TDP1 expression in HOP_62 was shown to be due to *TDP1* promoter hypermethylation. Our study provides insights into the possible inactivation of TDP1 in cancers and its relationship to cellular response to Top1-targeted drugs. It also reveals two TDP1 knockout lung cancer cell lines for further TDP1 functional analyses.

Keywords

TDP1; Topoisomerases; topotecan; CellMiner; promoter hypermethylation

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Introduction

TDP1 is a nuclear and mitochondrial enzyme conserved in eukaryotes [1–3]. It hydrolyzes phosphodiester bonds between DNA 3'-phosphates and the active site tyrosine residue of topoisomerase I (Top1) [2, 4, 5] as well as a variety of other DNA 3'-blocking lesions resulting from oxidative damage (3'-phosphoglycolates) [3, 6–8], alkylation damage [8] or incorporation of chain-terminating antiviral and anticancer nucleotide analogs [9]. TDP1 has also been implicated as a backup pathway for the repair of topoisomerase II cleavage complexes [8, 10] and as regulating the fidelity of nonhomologous end joining [11]. The biological significance of TDP1 is also emphasized by the fact that a TDP1 mutation causes the human genetic disease, spinocerebellar ataxia with axonal neuropathy (SCAN1) [12].

DNA topoisomerases are crucial for regulating the topology of the genome and removing DNA supercoiling resulting from transcription, replication and chromatin dynamics [13, 14]. They also are important targets for anti-cancer therapies. Top1 inhibitors represent widely used anticancer agents, and camptothecin (CPT) derivatives are prescribed for ovarian and small-cell lung cancers (topotecan) and for colorectal cancer (Irinotecan) [15]. These drugs act by trapping Top1-DNA complexes [14]. Top1-DNA complexes can also be trapped by a wide range of endogenous and exogenous DNA lesions [16]. Thus, the repair of Top1 cleavage complex by TDP1 is relevant for genomic stability and cancer therapy. TDP1 itself is a potential target for novel anti-cancer drugs, especially in the light of its relevance to tumor response to CPT derivatives. Efforts are ongoing to develop effective and safe inhibitors of TDP1 [17, 18] to augment tumor sensitivity to Top1 inhibitors.

The NCI-60 panel of human cancer cell-lines is derived from nine different tissues of origin. It was initially developed for anti-cancer drug efficacy screening by the Developmental Therapeutics Program (DTP) of the US National Cancer Institute. In addition, the NCI-60 has been extensively characterized in biological, molecular and pharmacological studies [19–22]. The complete NCI-60 genomic databases have recently been made publicly available including transcript levels across multiple platforms [21] and mutations of approximately 21,000 genes by whole human exome sequencing [22], enabling non-bioinformaticists to query the largest publicly available database of gene expression, mutations, microRNA, drugs and investigational compounds.

Here we examined the expression and genetic mutation profiles of *TDP1* in the NCI-60. With the availability of CellMiner (<http://discover.nci.nih.gov/cellminer/>), a web application for rapid retrieval of genetic and pharmacological data from the NCI-60 [21, 22], this task is made feasible and relatively easy. We demonstrate how integration of bioinformatics and biological investigation lead to the identification of two TDP1-deficient cell lines from the NCI-60 cell panel.

Material and methods

Cell lines and lysate preparation

Cells were cultured at 37°C with 5% CO₂ in medium supplemented with 10% fetal bovine serum. The NCI-60 cell lines were kindly provided by the Molecular Pharmacology Branch, Developmental Therapeutics Program at the National Cancer Institute. To prepare whole cell lysates, 10⁷ cells were lysed in 100 µl of CellLytic^M reagent (C2978; Sigma-Aldrich, MO) supplemented with Protease Inhibitor Cocktail (87786; Pierce, IL). After mixing and incubation at 4°C for 15 min, lysates were then sonicated and centrifuged at 16,000 rpm at 4°C for 30 min. Supernatants were collected, aliquoted, and stored at –80°C.

Colonogenic assay

Cells were plated at a density of 1000 and 2000 per well in six-well plates, incubated for 10 days in medium containing various concentrations of topotecan to allow formation of colonies. Cells were fixed with methanol, stained with 0.1% crystal violet (Sigma-Aldrich, MO) for 5 min and washed with distilled water. Colonies were counted after air-drying. Plating efficiency (PE) was defined as the number of colonies counted/the number of cells seeded. The survival fraction (SF) of untreated cells was defined as 100. SF was calculated as: PE treated/PE untreated $\times 100$.

Immunoblotting and Antibodies

Rabbit polyclonal anti-TDP1 antibody (ab4166) and mouse monoclonal anti- β -actin antibody (ab8226) were obtained from Abcam (Cambridge, MA). Rabbit polyclonal anti-TDP2 antibody (A302-737A) was from Bethyl (Montgomery, TX). Immunoblotting was carried out using standard procedures.

Preparation of DNA substrates and in vitro repair reactions

Oligonucleotides with 3'-phosphotyrosine linkages were synthesized by Midland Certified Reagent Co., Inc. (Midland, TX). The oligonucleotide (N14Y) and conditions used for TDP1 reaction are detailed in [23]. Samples were subjected to 16% denaturing PAGE. Gels were dried and exposed on PhosphorImager screens. Imaging and quantification were done using a Typhoon 8600 (GE Healthcare, UK) and ImageJ software (National Institutes of Health, Bethesda, MD).

Transfection and immunostaining

For complementation of TDP1 in HOP62 cell line, FLAG-TDP1 construct in mammalian expression vector was transfected using Lipofectamine 2000 (Invitrogen, NY) according to the manufacturer's protocol. 24 hours after transfection, cells were treated with 1 μ M of CPT or equal volume of DMSO for 1 hour. Following treatment, the medium was removed and the cells were washed in cold phosphate buffer saline (PBS). Cells were immediately fixed by incubating in 4% paraformaldehyde at room temperature for 15 minutes and permeabilized by treating for 10 minutes with PBT (0.1% Triton X-100 in PBS). Primary antibodies against γ H2AX and TDP1 were detected using anti-mouse or anti-rabbit IgG secondary antibodies labeled with Alexa Fluor 488/568 (Invitrogen, NY). Cells were mounted in anti-fade solution with DAPI (Vector Laboratories, CA) and examined using a laser-scanning confocal microscope (Zeiss 710) with a $\times 63$ oil objective. Images were collected and processed using the Zeiss AIM software and sized in Adobe Photoshop CS5.

High-throughput sequencing of the NCI-60 and data analysis and validation

High-throughput sequencing and data analyses were performed as described [24].

Methylation Analysis by Bisulfite Sequencing

Genomic DNA was isolated from HOP_62 and HCT116 cells. 500 ng isolated DNA for each cell lines were bisulfite modified using EpiTect Bisulfite Kit (59104, Qiagen, CA) following the manufacturer's protocol. The bisulfite modified DNA was eluted in 20 μ l elution buffer, and 4 μ l of the modified DNA was PCR amplified (30 cycles) using the bisulfite-sequencing PCR primers in PCR mix [200 μ M dNTP, 1.5 mM $MgCl_2$, 0.4 U Platinum Taq DNA Polymerase (10966, Invitrogen, NY), 200 pM of each primer in 20 μ l reaction volume]. PCR products were visualized by agarose-gel electrophoresis and extracted from gels using the MinElute Gel Extraction Kit (28604, Qiagen, CA). Gel extracted PCR products were used for Sanger sequencing with the same primers used for amplification. Sanger sequencing followed by direct PCR amplification of bisulfite modified

DNA determines the average level of cytosine methylation at each CpG site in the amplicon. Chromatograms were visually inspected using BioEdit Sequence Alignment Editor (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). The bisulfite PCR sequencing data analysis and lollipop diagram were generated using BIQ Analyzer (<http://biqanalyzer.bioinf.mpi-inf.mpg.de/>). The bisulfite conversion rate was >99%, as determined by calculating the C to T conversion rate for all cytosine bases that are not in the CpG dinucleotide context. The bisulfite specific primers used to amplify the CGI at the promoter region of TDP1 were designed using MethPrimer, a program for designing bisulfite-conversion-based Methylation PCR Primers [13]. The primer sequences used to amplify the region at chr14 (90,422,115-90,422,835) (hg19) containing the CpG Island (chr14:90,422,143-90,422,593) at the TDP1 promoter are: F: 5'-AGTTAGGAGAGATTAGGTTTTTTTAGTTT-3' and R: 5'-ACAACAACCTACTAACCTTACTACGTA-3'.

Statistical analyses

The significance of differences between means was assessed by Student's t-test. *P* values shown are two-tailed unless otherwise stated. All statistics and figures were constructed using Prism (GraphPad Software, CA).

Results

TDP1 transcript levels in the NCI-60 cancer cells

First, we used the NCI-60 transcriptome database and Cellminer tools [21] (<http://discover.nci.nih.gov/cellminer>) to determine the expression (mRNA) of *TDP1* in the NCI-60. The average intensity of *TDP1* transcripts varies from 4.02 to 8.23 in the Affymetrix Human Exon 1.0 ST microarray platform (Supplemental Table 1). Figure 1A shows a z-score representation [21] summarizing the various microarray platforms in the NCI database. Across 59 of the 60 cells, *TDP1* transcript levels exhibit moderate variation, with less than 3 standard deviations in z-score values. Most melanoma, lung, renal and CNS cancer cells are below average while leukemia, ovarian and prostate cancer cell lines are above average. Notably, a single cancer cell line, lung HOP_62, shows markedly low *TDP1* transcripts (> 4 standard deviations from the mean in the zscore chart; Fig. 1A).

Detailed analysis of *TDP1* transcripts using the Affymetrix GeneChip Human Exon 1.0 ST (GH Exon 1.0 ST) for the NCI-60 (Fig. 1B) shows that HOP_62 (red squares) has below threshold signal for all probe sets, confirming lack of detectable message across the whole *TDP1* transcript in HOP_62 cells.

TDP1 inactivation by homozygous deleterious mutation in NCI_H522 cells

Next, we used the NCI-60 whole exome sequencing (WES) database [22] to identify *TDP1* coding variants. Six exonic mutations in *TDP1* were found in eight cell lines (Fig. 2A). The A134T variant, which is present in the 1000 Genomes with a frequency of about 10%, is present in 3 cell lines (leukemia CCRF_CEM and K_562 and lung cancer NCI_H23). The other five variants (Y46C, V71A, N179S, K292E and E418K) have not been reported in normal samples. Among those variants, Y46C, N179S and K292E are predicted to be deleterious based on sequence homology and protein structure by SIFT and PolyPhen-2 programs, respectively [25] (Fig. 2A). We reasoned the K292E mutation may be significantly harmful because: 1) residue K292 is highly conserved across species (Fig. 2B); 2) the substitution of a lysine by a glutamic acid is likely to change the protein structure; and 3) it is homozygous in the lung cancer cell line NCI_H522¹. This mutation was validated by Sanger sequencing (Fig. S1).

Both lung cancer cells HOP_62 and NCI_H522 are TDP1 null

Next we investigated TDP1 protein levels and enzymatic activity in HOP_62 and NCI_522 in comparison with the other 6 lung cancer cell lines of the NCI-60 panel. Western Blotting showed undetectable TDP1 polypeptide in NCI_H522 and HOP_62 cells (Fig. 3A). On the other hand, relatively high protein levels were detected in the other cells, especially those over-expressing *TDP1* transcript, such as NCI_H23 (Fig. 3A and see Fig. 1A).

Because of the high specificity of TDP1 for 3'-tyrosyl oligonucleotides, the catalytic activity of TDP1 can be measured specifically in whole cell extracts [8] by conversion of a 3'-phosphotyrosine linked oligonucleotide to a 3'-phosphate product. As expected, the two cell lines with undetectable TDP1 protein (HOP_62 and NCI_H522) showed no TDP1 biochemical activity (Fig. 3B–C). All the other cell lines showed TDP1 activity with NCI_H23 and NCI_H460 being the most proficient (Fig. 2B–C). Plotting protein levels and catalytic activities of TDP1 showed a significant correlation across the panel of lung cancer cells ($p = 0.0055$, Fig. 3D).

TDP1 inactivation by promoter hypermethylation in HOP_62 cells

One plausible explanation to the silencing of *TDP1* in HOP_62 cells is hypermethylation of the *TDP1* promoter. Hypermethylation of CpG islands in promoters is typically associated with gene inactivation. CpG islands are defined as 200-bp regions of DNA with a G content greater than 50% and an observed over-expected CpG dinucleotide ratio above 0.6 [27]. According to this criterion, CpG islands in the chromatin landscape within 5 kb near *TDP1* promoter were identified using BIQ Analyzer (<http://biq-analyzer.bioinf.mpi-inf.mpg.de/>) [10]. We analyzed 72 CpG islands in the *TDP1* promoter (Fig. 4A, gray rectangles). Bisulfite sequencing showed 70 hypermethylated CpGs in the 657 base pairs region of *TDP1* promoter in HOP_62 cells, but not in HCT116 cells, a human colon carcinoma cell line used as control (Fig. 4B, and Figs. S2 and S3). Taken together, these results indicate that hypermethylation of the *TDP1* promoter in HOP_62 cells is responsible for deficiencies of TDP1 mRNA, protein and activity. Treatment with 0.5 μ M 5-azacytidine for 48 hours restored TDP1 expression in HOP_62 cells (Fig. 4C), further providing evidence in support of promoter hypermethylation as the mechanism for *TDP1* silencing in HOP_62 cells.

TDP1 ectopic-expression protects cells against CPT-induced DNA damage in HOP62 cells

TDP1-deficient cells are hypersensitive to CPT and exhibit elevated DNA damage with increased levels of CPT-induced γ H2AX foci [8, 28–30]. To test the biological significance of TDP1 in rescuing HOP62 cells from CPT-induced DNA damage, we transiently expressed Flag-tagged TDP1 in HOP62 cells. Reduction of CPT-induced γ H2AX foci was seen in the cells expressing ectopic FLAG-TDP1 (Fig. 5A). Quantification revealed that the average CPT-induced γ H2AX foci in HOP62 cells expressing ectopic FLAG-TDP1 was 6.3 ± 3.6 , while the untransfected cells generated 23.3 ± 2.6 foci per cell ($P = 0.0012$, Fig. 5B). These results are in keeping with the previous reports [8, 28–30], showing that TDP1 is a key enzyme for Top1cc repair and sensitivity to CPT.

Lack of TDP1 is only a partial pharmacodynamic determinant of sensitivity to topotecan across non isogenic cancer cell lines

Given the importance of TDP1 expression for cellular survival to CPT derivatives in isogenic model systems, we tested whether TDP1 could be a pharmacodynamic biomarker

¹The complete list of genomic data is retrieved from CellMiner [26] U.T. Shankavaram, S. Varma, D. Kane, M. Sunshine, K.K. Chary, W.C. Reinhold, Y. Pommier, J.N. Weinstein, CellMiner: a relational database and query tool for the NCI-60 cancer cell lines, BMC genomics, 10 (2009) 277.: <http://discover.nci.nih.gov/cellminer/>.

for Top1-targeted anticancer drugs by looking at the correlation between TDP1 protein levels and sensitivity to topotecan in the NCI-60 lung cancer cell panel. Figure 6A shows that, in this set of non-isogenic cell lines, TDP1 protein levels are not correlated with topotecan antiproliferative activity. The GI₅₀s of topotecan were confirmed by clonogenic assays. Among the 8 cell lines assayed, HOP_62, A549 and H460 formed reliable colonies after 10 days incubation. The GI₅₀ of topotecan is 14 nM for HOP_62, 8 nM for H460 and 26 nM for A549. The correlation between GI₅₀ of topotecan versus TDP1 protein levels for the three cell lines is plotted in Fig. 6B. The lack of correlation suggests that TDP1 is only a partial determinant of cellular sensitivity to Top1 inhibitors in cancer cells with various non-isogenic backgrounds.

Discussion

Our study shows genetic inactivation of TDP1 in two out of eight lung cancer cell lines from the NCI-60 panel. To our knowledge, this is the first report showing TDP1 can be totally inactivated in human cancer cells. A prior study had shown that TDP1 tends to be overexpressed in lung cancers [17]. Notably, we did not observe TDP1 overexpression in the lung cancer panel compared to the 8 other tissues of origin that constitute the NCI60 (see Fig. 1).

TDP1 inactivation in cancer cells growing normally in tissue culture conditions is consistent with the fact that TDP1 is a non-essential gene based on the normal growth of yeast TDP1 knock out [1, 2, 4, 11, 31], or mice TDP1 knockout and their derived fibroblasts [24, 32]. Whether knocking out TDP1 provides a selective advantage to cancer cells still needs to be established. Further studies are also warranted to elucidate whether TDP1 contributes to genomic stability. In favor of this possibility, a recent study indicates that, in yeast, TDP1 might contribute to genomic stability by increasing the fidelity of non-homologous end joining [11].

Our finding that promoter hypermethylation totally inactivates TDP1 transcription in HOP_62 cells reveals the potential importance of DNA methylation as a regulator for TDP1 expression. Notably, *TDP1*'s direct neighbor *EFCAB11* is also selectively down regulated in HOP_62 cell line (Fig. S4). *EFCAB11* and *TDP1* form a bidirectional gene pair, whose transcription start sites are neighboring and directed away from each other (see Figs. 1 and S4). However, little is known about *EFCAB11*, except it codes for EFhand calcium binding domains, which are found in structurally and functionally diverse proteins. Thus, we conclude that both genes are silenced by hypermethylated CpG islands in their shared bidirectional promoter. Although the biological significance of hypermethylated bidirectional promoter is unclear, such situation occurs for other DNA repair genes [33] and has been suggested to play a role in tumorigenesis [34, 35].

TDP1 inactivation in NCI_H522 is linked to a genetic mutation that changes lysine 292 into a glutamic acid. Although *TDP1* transcripts were readily detected in H522 cells, TDP1 mRNA does not appear to be translated into protein as H522 cells fail to show detectable TDP1 protein or enzymatic activity. As suggested by Anfinsen [36], during protein translation, the amino acid chain spontaneously folds into the thermodynamically most stable form to achieve the lowest energy state possible. More recent studies have suggested that proper protein folding begins with interaction of a relatively small number of residues to form a folding core for the remainder of the structure to rapidly condense on [37]. Therefore, the folding is likely to be determined primarily by the pattern of hydrophobic and polar residues that favor preferential interactions of specific residues as the structure becomes increasingly compact. Furthermore, the secondary structure, the α - helices and β -sheets, is stabilized fundamentally by hydrogen bonding between the amide and carbonyl groups of

the main chain [38]. Therefore, the substitution of a positively charged, hydrophobic lysine to a negatively charged, hydrophilic glutamate residue in H522 cells most likely disrupts TDP1 protein folding. Misfolded proteins that are useless or even toxic and disease causing, including many notorious infectious and neurodegenerative diseases [39], are subjected to strictly controlled mechanisms to target them for degradation. This would explain the absence of TDP1 in H522 cells. Treatment with a clinically used proteasome inhibitor bortezomib did not restore TDP1 expression in H522 cell line (Fig. S5), suggesting the protein is either never made or degraded very early during synthesis.

Although TDP1 is a well-established pathway for the excision of trapped Top1 cleavage complexes [40, 41], it is clear from studies performed in yeast [2, 31] and human cells [42–44] that alternative endonuclease pathways including XPF-ERCC1 and Mus81 are critical to for removing by endonucleolytic cleavage of the 3'-DNA segment covalently attached to Top1. In addition, both HOP_62 and H522 cells express TDP2 protein, which might be compensatory given its reported role in resolving Top1cc in the absence of TDP1 [28, 45] (Fig. S6). This may contribute to our finding that TDP1 protein and activity levels are not closely correlated with response to topotecan across the eight lung cancer cell lines of the NCI-60 (see Fig. 5). Hence, our study demonstrates that TDP1 should be viewed as a potential pharmacodynamic biomarker for anticancer response to Top1-targeted drugs in the context of multivariate analyses. Our study also reveals two TDP1 knockout cell lines (HOP62 and NCI_H522) that can be used for complementation and functional studies of TDP1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Genetic inactivation of TDP1 in 2 out of 8 lung cancer cell lines from the NCI-60 panel.
- This is the first report showing TDP1 can be inactivated in human cancer cells.
- The mechanism for NCI_H522 TDP1 deficiency is a point mutation K292E that leads to unstable protein.
- Promoter hypermethylation causes down regulation of TDP1 in Hop62 cell line.
- TDP1 should be viewed as a potential pharmacodynamic biomarker for anticancer response to Top1-targeted drugs in the context of multivariate analyses.

Figure 1. TDP1 gene expression and mutations for the NCI-60 human tumor cell lines. **A)** Relative TDP1 gene expression profile. Bars to the right show increased expression, bars to the left show decreased expression relative to the expression mean. Expression values are normalized as z-scores (described in [21]). The horizontal axis is marked in standard deviations from the mean. Right blue box: average probe intensity calculated from Affymetrix HG-U95, HG-U133, HG-U133 plus 2.0 and HuEx 1.0 microarrays. Data are accessible at <http://discover.nci.nih.gov/cellminer>. **B)** TDP1 mRNA expression for all probe sets from the Affymetrix GeneChip Human Exon 1.0 ST (GH Exon 1.0 ST) for the NCI-60

cells (gray squares). Red squares with value at background level are for HOP_62. Reference gene structures are shown at the bottom as blue ribbons.

A

TDP1 exonic mutations

AA impact	Y46C	V71A	A134T	N179S	K292E	E418K
CO:HCT_15	-	-	-	-	-	34
LE:CCRF_CEM	-	-	45	-	-	-
LE:K_562	-	-	100	-	-	-
LE:MOLT_4	-	18	-	-	-	-
LC:NCI_H23	-	-	44	-	-	-
LC:NCI_H522	-	-	-	-	100	-
OV:IGROV1	-	-	-	44	-	-
OV:SK_OV3	39	-	-	-	-	-
SIFT	0.02	0.12	0.6	0	0	0.22
Polyphen-2	0.992	0.001	0	0.999	0.999	0.091
1000 Genomes	0	0	0.09	0	0	0

B

Human	286	HADWHQKTQGIWL	298
Cow	290	REDWHQKTQGIWL	302
Mouse	287	REDWHQKTQGIWL	299
Arabidopsis	259	HVDWNNKSQGLWM	271
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Figure 2.

TDP1 mutations across the NCI-60. **A**) Deleterious mutations as predicted by SIFT and PolyPhen-2 programs [22] are in black boxes. Numbers indicate the percent of reads with the indicated variant. For the 1000 Genomes, black boxes indicate variants that are absent in normal samples (0). The lung cancer cell line NCI_H522, which is homozygous for K292E mutation, is indicated in bold. **B**) Conservation of K292 amino acid residue in TDP1 across species.

Figure 3.

TDP1 protein levels and catalytic activities across the NCI-60 lung cancer cells. **A**) TDP1 protein levels determined by Western blotting with 10⁶ cells loaded in each lane. **B**) TDP1 catalytic activity from the same cell lysates using a single-stranded 14-mer oligonucleotide with a phosphotyrosine attached on the 3'-terminus [8, 9, 24]. Reactions were carried out at 25°C for 30 minutes with serial dilutions of the cell lysates. **C**) Quantification of the 3'-end-processing data shown in panel B. **D**) Correlation between TDP1 catalytic activities (Y-axis) and protein levels (X-axis). The catalytic activity at 1:1000 dilution was used for calculation. Protein levels were quantitated using NCI_H23 set as 1. Pearson $r = 0.8654$ (95% CI, 0.4116 to 0.9753); $p = 0.0055$ (two-tailed test).

Figure 4.

TDP1 promoter hypermethylation in HOP_62 cells. **A)** Schematic representation of the *TDP1* promoter region. Two CpG islands are within 5 kb in the *TDP1* promoter. The CpG island smaller than 300 bases is shown at left. The chromosomal region (Chr14: 90422126–90422782) containing the larger CpG island (shown at right) was sequenced (see Fig. S1).

B) Sequencing results showing hypermethylation of the genomic region from *TDP1* promoter to the first intron in HOP_62 cells. 72 CpG islands are present within 657 bases, of which 70 islands are methylated in HOP_62. **C)** HOP62 cells were treated continuously with 0.5 μ M of 5- azacytidine for 48 and 72 h. Cell lysates were cleared, separated by SDS-

PAGE and Western blotted with anti-TDP1 antibody indicating rescue of TDP1 protein expression in HOP_62 cells. Actin served as loading control.

Figure 5.

TDP1 expression protects cells from DNA damage induced by camptothecin (CPT) in HOP_62 cell line. **A)** Representative immunofluorescence images showing reduction of CPT-induced γ H2AX foci in cells overexpressing TDP1. Cells were transiently transfected with Flag-tagged TDP1 driven by pCMV promoter. TDP1 overexpressing cells are marked out by dashed line circles and indicated by arrows. **B)** Quantitation of γ H2AX foci per cell. The intensity of γ H2AX levels in pan-staining cells were compared to those with countable foci numbers and arbitrarily assigned to 50 foci per cell. Number of cells analyzed: NT, TDP1 untransfected: $n = 17$; NT, TDP1 transfected: $n = 9$; CPT, TDP1 untransfected, $n =$

42; CPT, TDP1 transfected, $n = 13$. Standard t-tests were used for statistical analyses; N.S., no significant difference.

Figure 6.

A) Relationship between TDP1 protein levels and topotecan activity in the lung cancer cells. Protein levels were determined as shown in Fig. 3. Topotecan growth inhibitory 50 (GI₅₀) concentrations were determined by cologenic assay. **B)** Validation of GI₅₀ of topotecan in HOP_62, A549 and H460 cell lines. Correlation of topotecan growth inhibition is plotted against TDP1 protein levels.