Mechanism of repair of 5′-topoisomerase II–DNA adducts by mammalian tyrosyl-DNA phosphodiesterase 2

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The topoisomerase II (topo II) DNA incision-and-ligation cycle can be poisoned (for example following treatment with cancer chemotherapeutics) to generate cytotoxic DNA double-strand breaks (DSBs) with topo II covalently conjugated to DNA. Tyrosyl-DNA phosphodiesterase 2 (Tdp2) protects genomic integrity by reversing 5′-phosphotyrosyl–linked topo II–DNA adducts. Here, X-ray structures of mouse Tdp2–DNA complexes reveal that Tdp2 β–2-helix–β DNA damage–binding ‘grasp’, helical ‘cap’ and DNA lesion–binding elements fuse to form an elongated protein-DNA conjugate substrate-interaction groove. The Tdp2 DNA-binding surface is highly tailored for engagement of 5′-adducted single-stranded DNA ends and restricts nonspecific endonucleolytic or exonucleolytic processing. Structural, mutational and functional analyses support a single–metal ion catalytic mechanism for the exonuclease-endonuclease-phosphatase (EEP) nuclease superfamily and establish a molecular framework for targeted small-molecule blockade of Tdp2-mediated resistance to anticancer topoisomerase drugs.

To relieve DNA topological strain and facilitate cellular DNA and DNA-RNA transactions, type II topoisomerases metabolize DNA topoisomers by incising DNA, gating passage of a second DNA duplex through a topo II–linked DSB and religating the DNA break. The reversibility of topo II DNA-cleavage reactions is facilitated by the formation of covalent enzyme-phosphotyrosyl linkages between the 5′-phosphate ends of the incised duplex and an active site topo II tyrosine. Although topo II–DSB intermediates are transient, genetic and environmental perturbations can accelerate topo II DNA cleavage or stall topoisomerase religation1–3, shifting DNA cleavage-ligation equilibrium toward production of excessive DSBs that retain topo II–DNA adducts from polynucleotides to prevent inappropriate endo- or exonucleolytic cleavages and how this specificity and activity might be regulated. To clarify Tdp2 functions in genomic maintenance and cellular cancer-therapeutic resistance, we report combined structural and functional characterization of Tdp2 catalytic activity and enzymatic selectivity.

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RESULTS
Tdp2 domain mapping and catalytic activity
We used limited trypsin proteolysis (Supplementary Fig. 1), truncation mutagenesis, sequence analysis (Supplementary Fig. 2) and small-angle X-ray scattering (SAXS) coupled to measurement of Tdp2 5′-tyrosyl-phosphodiesterase activity (Fig. 1c–e) to identify the minimal catalytically active domain (referred to as Tdp2cat hereafter) from human (hTdp2cat, residues 108–362) and mouse (mTdp2cat, residues 118–370) Tdp2 (Supplementary Fig. 2). Analysis of the SAXS electron pair distribution function and solution scattering parameters (Supplementary Fig. 3 and Supplementary Table 1) shows that full-length hTdp2 (hTdp2FL) adopts an elongated structure (maximum particle dimension, 102 Å) that is susceptible to trypsin protease digestion (Supplementary Fig. 1). In contrast, a truncated, trypsin-stable hTdp2cat fragment is globular, on the basis of the SAXS analysis (Supplementary Fig. 3). Thus, the Tdp2 N-terminal UBA domain (Fig. 1b), which may interface with the cellular signaling apparatus and ubiquitination machinery16,17, appears to be flexibly linked to the folded C-terminal EEP domain core.

Purified hTdp2cat and mTdp2cat proteins (Fig. 1c) have Mg2+-dependent (Supplementary Fig. 4a) activity on 5′-phosphotyrosylated (5′-Y) termini of single-stranded DNA or on duplex substrates with 5′ overhangs of 1–4 nucleotides (nt) as well as on the small-molecule 5′-Y mimic thymidine 5′-monophosphate p-nitrophenyl ester (T5PNP) (Fig. 1d–g and Supplementary Fig. 4b). Blunt or

Figure 1. Tdp2 catalytic activity. (a) Poisoned topo II results in a tyrosine covalently linked to the 5′-phosphate of a DSB with 5′ overhangs. The 5′-Y bond is cleaved by Tdp2. (b) Domain structure of mammalian Tdp2 homologs showing mouse and human (bracketed) amino acid domain boundaries. (c) SDS-PAGE of purified human (h) and mouse (m) Tdp2 proteins used in structural and activity assays. (d) Tdp2 hydrolyzes T5PNP to produce p-nitrophenolate (PNP). (e) Catalytic activity of Tdp2 proteins assayed using the T5PNP reagent. Error bars indicate the s.d. from three independent measurements. (f) Structures of assayed substrates with varied 5′ and 3′ modifications. (g) Catalytic activity of Tdp2 on 5′-Y substrates in the context of indicated secondary structures, analyzed by denaturing gel electrophoresis. (h) 5′- versus 3′-phosphotyrosine cleavage assayed using 1 µM synthetic oligonucleotides containing a phosphotyrosine at the indicated terminus with 10 nM hTdp2cat, analyzed as in g. (i) Tdp2cat activity assayed on suboptimal substrates using 1 µM synthetic oligonucleotides containing the indicated terminal phosphate modification with 2 µM hTdp2cat, analyzed as in g.
Crystallization of Tdp2–DNA complexes

Mouse Tdp2cat X-ray crystal structures were determined in four distinct states, including three DNA–protein complexes (Online Methods): (i) DNA complex I, an mTdp2cat–5′-PO4-DNA–Mg2+ catalytic-product complex (at 1.5 Å); (ii) DNA complex III, an mTdp2cat–DNA–Mg2+ complex, with ssDNA excluded from the active site (at 1.85 Å); and (iv) an mTdp2cat–Mg2+-5′-dAMP complex (at 2.55 Å) (Table 1). An 8-base-pair DNA duplex with 1-nucleotide overhangs derived from a self-complementary oligonucleotide (Supplementary Table 2) was used for crystallization of the nonhydrolyzable substrate analog 5′-N adduct (complex I) and the 5′-PO4 reaction-product complex (complex II) (Fig. 2 and Supplementary Fig. 5). Notably, Tdp2 is catalytically active on 1-nucleotide-overhanging substrates (Supplementary Fig. 4b), though activity is decreased, probably owing to reduced accessibility of the 5′ terminus in these substrates, as compared to preferred 2- or 4-nucleotide overhangs. In all three DNA-complex structures, Tdp2 uses conserved protein side chains to extensively engage the terminal nucleotides of the DNA 5′-adduct strand (Fig. 2a–c, blue DNA strands and Supplementary Fig. 6). In contrast, there is a dearth of DNA–protein contacts made to the 3′ end of the complementary strand of the duplex DNA (Fig. 2a–c, gray DNA strands) employed as a vehicle for protein–DNA co-complex crystallization. Thus, the use of two-ended crystallization substrates has trapped protein–DNA interactions relevant to Tdp2 DNA processing and specificity for 5′-strand overhanging ends (Fig. 1g), which is consistent with a biological role for Tdp2 processing of topo II–linked DSBs.

Table 1 Data collection and refinement statistics

| Data collection | mTdp2cat–Mg2+-dAMP | mTdp2cat–DNA complex I | mTdp2cat–DNA complex II | mTdp2cat–DNA complex III | Selenomethionine mTdp2cat–Ba2+-dAMP |
|-----------------|---------------------|------------------------|------------------------|------------------------|-----------------------------------|
| Space group     | P2₁2₁2₁             | P2₁2₁2₁                | P2₁2₁2₁                | P2₁                    | P2₁2₁2₁                           |
| Cell dimensions | a, b, c (Å)          | 85.47, 169.60, 185.51  | 114.72, 118.79, 160.47 | 54.60, 68.60, 166.03   | 60.48, 42.97, 107.95              |
| α, γ (°)        | 90, 90, 90          | 90, 90, 90             | 90, 90, 90             | 90, 95.89, 90            | 90, 90, 90                        |
| Wavelength (Å)  | 0.9795              |                        |                        |                        |                                   |
| Resolution (Å)  | 50–2.55 (2.64–2.55) | 50–2.10 (2.18–2.10)    | 50–1.5 (1.55–1.50)     | 50–1.85 (1.92–1.85)     | 50–2.9 (3.0–2.9)                  |
| Completeness (%)| 97.7 (92.1)         | 97.9 (99.0)            | 98.9 (99.0)            | 97.4 (93.6)             | 99.9 (99.5)                       |
| Redundancy      | 5.3 (4.7)           | 3.0 (2.9)              | 4.4 (4.5)              | 3.1 (2.3)               | 3.8 (3.5)                         |
| Refinement      |                     |                        |                        |                        |                                   |
| Resolution (Å)  | 50–2.55             | 50–2.10                | 50–1.5                 | 50–1.85                | 50–2.9                            |
| No. reflections | 84,882              | 130,977                | 105,826                | 46,631                 |                                   |
| Rwork / Rfree  | 0.191 / 0.220       | 0.160 / 0.193          | 0.145 / 0.175          | 0.205 / 0.245          |                                   |
| No. atoms       | Protein             | 17,574                 | 12,056                 | 4,140                  | 4,017                             |
|                 | 149                 | 1691                   | 388                    | 490                    |
|                 | Water               | 397                    | 1041                   | 472                    | 437                               |
|                 | B factors           | 52.1                   | 42.6                   | 30.7                   | 25.5                              |
|                 | Protein             | 70.1                   | 61.2                   | 51.9                   | 40.7                              |
|                 | Ligand/ion          | 40.8                   | 52.5                   | 45.8                   | 35.1                              |
| R.m.s. deviations | Bond lengths (Å)    | 0.004                  | 0.005                  | 0.009                  | 0.010                             |
|                 | Bond angles (°)     | 0.6                    | 0.9                    | 1.3                    | 1.0                               |

Each dataset was collected from a single crystal. Values in parentheses are for highest-resolution shell.
two-fold topological pseudosymmetry relates the two halves of the Tdp2cat β-sandwich. Tdp2 directly engages and directs the 5′ terminus into the active site, with 14 residues mediating sequence-independent DNA contacts to the three terminal nucleotides of the 5′ strand (Fig. 2a–c, blue DNA in complexes I and II and Supplementary Fig. 6). By comparison, direct protein contacts to the 3′ end of the complementary strand are limited to a DNA base-stacking interaction with the exposed 3′ terminus and are mediated by nonconserved amino acids (Supplementary Fig. 6), which suggests that these contacts are not relevant to Tdp2–DNA interactions in solution.

Eight highly conserved Tdp2 motifs (M1–M8, Fig. 3a–c and Supplementary Figs. 2 and 6) define a contiguous surface of the protein mediating (i) Mg2+ coordination and active site chemistry (motifs M1, M2, M5, M6 and M8), (ii) DNA binding (motifs M5, M6 and M7) and (iii) a hydrophobic 5′-adduct recognition groove (motifs M1, M3 and M4). Regions M5–M7 assemble to form the Tdp2 DNA-binding groove. M7 adopts a previously unrecognized β2-β-helix–β (β2Hβ) DNA-binding fold that projects outward from the Tdp2 catalytic core to envelop the exposed 5′ DNA end. An extended network of conserved hydrophobic residues (Trp307, Leu315, Ile317, Tyr321 and Phe325) fuse and form a platform for DNA base stacking and deoxyribose-sugar interactions that ‘grasp’ the DNA 5′ terminus (Fig. 3a,c and Supplementary Fig. 6). Proximal to the active site, three hydrophobic side chains (Phe325, Leu315 and Trp307) form a groove that makes van der Waals contacts to the 5′-terminal sugar and interact nonspecifically with the cytosine 1 pyrimidine base (Fig. 3c).

Along with the β2Hβ grasp, motif M5 forms a helical DNA-binding ‘cap’ on the opposite side of the cleft to orient the 5′ terminus for its approach into the active site. M5-cap interactions from Arg241 and the cap’s 310-helix main chain amide directly bind to the DNA sugar-phosphate backbone of the approaching 5′ strand (Fig. 3c). Between the grasp and the cap, three residues from motif M6 (Arg276, Asn274 and Asp272) form the ‘floor’ of this highly conserved DNA-interaction groove (Fig. 3a–c). In complex with the DNA-substrate analog, regions M1, M3 and M4 form a pocket binding the 5′-N alkylamine adduct (Fig. 3a,b).

5′-DNA-end-binding motifs
To test roles for Tdp2-DNA interfaces observed in our mTdp2cat structures, we evaluated the impact of amino acid substitutions on the strictly conserved DNA-binding groove on human Tdp2cat catalytic activity, using three substrates: (i) p-nitrophenyl phosphate (PNPP), (ii) T5PNP and (iii) the DNA substrate 4-nt–5′-Y (Fig. 4a). Our goal in analyzing the activity of mutant hTdp2cat proteins (Supplementary Fig. 7) on this set of chemically related substrates was to dissect structure-activity relationships of the Tdp2 DNA-binding surface (Fig. 4a–c). We hypothesized that mutations affecting the basic DNA-binding groove would impair catalysis on DNA substrates but not significantly alter processing of T5PNP. In support of roles for orienting the 5′ terminus of the 4-nt–5′-Y substrates, substitution of two conserved cap and floor-DNA-binding arginine side chains R266EhTdp2 (R276mTdp2) and R231EhTdp2 (R241mTdp2) reduced activity on 4-nt–5′-Y (Fig. 4c); lanes 3–5 compared to lanes 20–22.

Figure 2 Structures of the mTdp2cat–DNA complexes. DNA crystallization constructs are diagrammed showing the regions of DNA that contact Tdp2 (blue). Extensive DNA contacts made by conserved Tdp2 side chains are made to the terminal 5′ nucleotides (Complex I and II) or six terminal 5′ nucleotides (Complex III). (a) DNA 5′-N substrate analog–bound complex (Complex I) displayed with 5′-N (red) and a cartoon representation with bound DNA (blue), Tdp2 helices (orange) and β-strands (yellow). (b) Tdp2–DNA product complex (Complex II). (c) Excluded ssDNA complex (Complex III).
and 37–39) but had modest impacts on hydrolysis of T5PNP (Fig. 4b).

At the DNA 5′ terminus, two β2Hβ-motif hydrophobic residues (Trp307 and Phe325) cup the terminal deoxyribose sugar. Consistent with important roles for orienting the DNA terminus for catalysis, the substitution of these β2Hβ hydrophobic side chains severely impairs hydrolysis (<5% of wild-type activity) of both 4-nt–5′-Y and T5PNP (Fig. 4b,c; lanes 23–25 and 40–42). Notably, the F315AΔTdp2 (F325MTdp2) and W297AΔTdp2 (W307MTdp2) mutants both retain >45% of wild-type activity on PNPP, a substrate lacking a 5′ nucleoside (Fig. 4a,b). Overall, observations from mutagenesis and activity studies are consistent with the polarity of DNA binding in the Tdp2 DNA interaction groove and support the structural observations that sequence-nonspecific DNA-binding contacts from the cap, floor and grasp orient the 5′ terminus to promote catalysis with combined substrate–sugar-phosphate binding.

5′-adduct-binding site

The position of the entire 5′-N alkylamine adduct is clearly visible in five of the six Tdp2 molecules in the crystallographic asymmetric unit and is well defined for the first three methyl groups in the remaining molecule (Fig. 5). The overall trajectory of the C1 and C2 methyl carbons is consistent. Structural overlays of the six DNA–protein complexes show that although the flexible adducts adopt variable structures, they all occupy a hydrophobic groove created by Leu134 (motif M1), Tyr188 (motif M3) and Met214 (motif M4) (Fig. 5a).

To better understand how Tdp2 might bind a cognate 5′-Y substrate, we assessed possible tyrosine-binding positions in this hydrophobic pocket, on the basis of binding of 5′-N. We positioned the tyrosine hydroxyl to be anchored at a bridging oxygen position, and we posit that 5′-Y could bind analogously to the alkyl chains occupying the hydrophobic 5′-N-binding groove (Fig. 5a). Modeling of the 5′-Y into this pocket reveals van der Waals interactions between the protein surface and the tyrosine aromatic ring. Consistent with this model, alamine substitutions of any of the hTdp2cat pocket residues, or of an adjacent residue stabilizing the conformation of Met214 (Met215) (hTdp2cat mutations, M204A, M205A, L124A, and Y178A), all confer catalytic defects for processing of 5′-Y (Fig. 4c; lanes 9–11, 12–14, 43–35 and 46–48) as well as the small-molecule substrates PNPP and T5PNP (Fig. 4b).

Tdp2 catalytic mechanism

The Tdp2 substrate-analog- and product-complex structures provide a high-resolution view of the Tdp2 active site in the absence of mutagenic perturbation. A structural superposition of DNA end–bound conformations in complexes I and II highlights a trajectory for a reaction coordinate for the adduct hydrolysis reaction that is characterized by inversion of configuration about the 5′-phosphorus (Fig. 5b,c). In the substrate analog–bound structure (a Mg2+-free crystal form), a bound water molecule (Fig. 5b–e; Nuc) is positioned appropriately for in-line nucleophilic attack ~180° opposite of the P-O bond of the 5′-N adduct. Although 5′-Y substrates were included in the crystallization solution of the product complex (a Mg2+-bound crystal form), enzymatic processing occurred in the crystallization drop, and the DNA of complex II is unambiguously bound as a 5′′-PO4 reaction product (Fig. 5b–f). No electron density is visible for the cleaved tyrosine moiety, which suggests that it does not remain bound to Tdp2 after phosphotyrosine-bond hydrolysis. In the product state, the single bound Mg2+ ion coordinates a water molecule (Fig. 5b,f; blue sphere) occupying a position analogous to the bridging oxygen of the 5′-adduct leaving group. Similarly, the proposed nucleophile in the substrate analog–bound complex occupies the approximate position of the 5′-PO4 product oxygen generated by phosphate inversion (Fig. 5b,d).

On the basis of geometry of the bound substrates and products, we propose a catalytic mechanism for Tdp2 DNA-adduct processing (Fig. 5c). In both the product and substrate complexes, Asp272 is best positioned to act as a catalytic base to activate a water molecule for nucleophilic attack in an S N2 displacement reaction, and it forms close hydrogen bonds (average distance of 2.45 Å for the six...
monomers in the crystallographic asymmetric unit) to the proposed water nucleophile or to the 5'-PO₄ product oxygen (average distance 2.48 Å for the two monomers in the crystallographic asymmetric unit). Magnesium is absent from our substrate analog–bound complex; however, Mg²⁺ directly interacts with the 5'-PO₄ in the product structure. Bound Mg²⁺ along with three additional conserved residues (His236, His359 and Ser239) binding 5'-PO₄ are positioned to interact with the substrate phosphate moiety and stabilize a pentacovalent reaction transition state in this scheme (Fig. 5c,d,f).

Supporting their predicted roles in tyrosyl phosphodiester hydrolysis, mutation of the presumed conserved catalytic base Asp272 (Fig. 4c; lanes 34–36) or transition state–stabilization ligands Ser239 or His359 impairs catalytic activity on all three substrates (Fig. 4b,c; lanes 31–33 and 26–28). On the basis of structural and mutagenesis data, our data are most consistent with a single-metal-ion mechanism (Fig. 5c) that is similar to a mechanism originally proposed for the related EEP nuclease Ape1 (ref. 15).

**Tdp2 DNA-processing specificity**

Overall, the architecture of the Tdp2 DNA-binding groove formed by the M6–M8 motifs explains the exquisite *in vitro* Tdp2 DNA structure–dependent DNA-substrate specificity (Fig. 1g–i). Consistent with the substrate-cleavage preference for 5'-Y overhangs, the dimensions of the substrate-binding groove proximal to the active center appear too small to efficiently accommodate double-stranded DNA without distortion of the enzyme active site and/or terminal DNA base-pair unwinding, which provides a possible explanation for reduced efficiency of processing on blunt or recessed 5' termini. In addition, Tdp2 displays a marked preference for 5'-Y over 3'-Y substrates (Fig. 1h,i and ref. 8). On the basis of structures and mutagenesis, substrate interactions with the DNA 5' end are facilitated by van der Waals contacts from the β2Hβ grasps (Trp307, Phe325) to the terminal C4' and C5' methyl groups (Fig. 6a). We posit that substrate interactions may be suboptimal for binding a 3'-Y DNA end where the phosphate is more closely juxtaposed to the deoxyribose sugar (Fig. 6b).

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**Figure 5** Tdp2 active site and catalytic mechanism. (a) Six positions of the 5'-N adduct in the crystallographic asymmetric unit are displayed, showing the position of the 5'-adduct–binding site. Proposed position of a 5'-Y substrate (model) is shown as a purple stick representation. (b) Structural overlay of product (blue DNA) and substrate-analog (red DNA) complexes illustrating inversion of configuration about the 5'-phosphorus during the reaction. (c) Proposed substrate structure–based catalytic mechanism. Tdp2 residues are colored as in Figure 2a. (d) Stereo view of the substrate-analog active site with 5'-N displayed in red. Tdp2 residues are colored as in Figure 2a. (e) A 2.1-Å resolution electron map (blue mesh, contoured at 1.0σ) is displayed overlaid on the substrate-analog structure (red). Nuc indicates the position of the proposed water nucleophile. (f) Stereo view of the product-complex active site. Interactions important for Mg²⁺-ion coordination and catalytic activity are indicated with gray dashed lines. The trajectory of the hydrolyzed bond between the 5'-phosphorus (orange) and the water that occupies the position of the leaving group (blue) is indicated with a dashed red line. (g) Experimental electron density of the product-complex active site. Final 1.5-Å electron map displays density for the DNA (blue, contoured at 1.7σ), active site residues of mTdp2 (orange) and the magnesium ion (purple) with its octahedrally coordinated waters (gray, red).
Whereas Tdp2 rapidly processes 5′-Y, a single-stranded 5′ end of the DNA is not further processed nucleolytically by Tdp2 in solution, even at high protein concentration (Fig. 1h), which underscores the precise selectivity of the Tdp2 active site for 5′-Y reversal. In accordance with this selectivity, we determined an additional structu..
However, despite global differences in DNA-binding mode, the approach of the scissile DNA-phosphotyrosyl linkage (Tdp2) or DNA-phosphodiester linkage (Ape1) within the EEP active site is quite similar. Collectively, these observations highlight that divergent EEP-surface DNA-interacting recognition elements have evolved for tailored protein–DNA adduct processing (Tdp2) and DNA-damage (Ape1) recognition with a common hydrolytic enzymatic processing mechanism.

Structures of several DNA-repair end-cleaving enzymes are now available, including those for DNA polymerase β, a DNA ligase, and the Flap endonuclease Fen1 (ref. 23). A common feature of DNA–end–binding recognition in these enzymes is the deployment of hydrophobic α-helical DNA-base platforms to sequester exposed DNA termini. Our X-ray structures further expand the repertoire of DNA-end recognition mechanisms to include the β2Hβ grasp and carboxyl-terminal DNA-binding domain of Tdp2. By engulfing DNA termini, these DNA-end damage-processing enzymes may protect exposed damage termini from inappropriate 5′ or 3′ exonucleolytic degradation. The observation that Tdp2 extensively engages the three terminal nucleotides of a 5′-ssDNA and DNA adducts by hydrolyzing the 5′-phosphotyrosine bond, yielding 5′-phosphorylated DNA termini that are repaired by the cellular DSB-repair machinery. (b) Substrate interactions by Tdp2 involves structure-specific recognition of 5′-phosphotyrosine–linked protein-adducted DNA termini, and Tdp2 employs single-metal-ion catalysis to reverse DNA damage.

Figure 8 Model for removal of 5′-phosphotyrosine–linked top II adducts from DNA by Tdp2. (a) Stalled top II cleavage complexes are degraded in a proteasome-dependent manner. Tdp2 encounters and removes the top II peptides by hydrolyzing the 5′-phosphotyrosine bond, yielding 5′-phosphorylated DNA termini that are repaired by the cellular DSB-repair machinery. (b) Substrate interactions by Tdp2 involves structure-specific recognition of 5′-phosphotyrosine–linked protein-adducted DNA termini, and Tdp2 employs single-metal-ion catalysis to reverse DNA damage.

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The authors declare no competing financial interests.

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ONLINE METHODS

Expression and purification of Tdp2 proteins. Human Tdp2 (hTdp2) and mouse Tdp2 (mTdp2) were expressed at 17°C from pMCSG9 (ref. 35) in BL21 Rosetta2 Escherichia coli (EMBE Biosciences). Following lysis by sonication in lysis buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 1 mM Tri(b-carboxyethyl)phosphine (TCEP) and 1 mM phenylmethylsulfonyl fluoride with the addition of 0.1 mg ml^{-1} lysozyme), maltose-binding protein (MBP)-tagged protein was affinity purified on amylose resin (New England Biolabs) and eluted in lysis buffer plus 10 mM maltose. MBP-Tdp2 fusion proteins were purified by size-exclusion chromatography on a 16/60 S-200 column (GE Life Sciences) in size-exclusion column buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 1 mM TCEP). For biochemical assays, the MBP-tagged proteins were concentrated and used without further purification. For mTdp2^{245} crystallization, the MBP tag was removed by TEV protease digestion, and mTdp2^{245} was purified further with butyl-Sephrose hydrophobic-interaction chromatography (GE Life Sciences) followed by dialysis into size-exclusion buffer.

Small-angle X-ray scattering. For SAXS studies, Tdp2 variants were buffer exchanged into SAXS buffer (15 mM Tris, pH 7.5, 300 mM NaCl, 1 mM TCEP, 2 mM MgCl2 and 1% glycerol) and concentrated in Amicon 3K 0.5-ml centrifugal concentrators (Fisher) immediately before SAXS data collection. SAXS data collection was performed at 3, 1.5 and 0.75 mg ml^{-1} protein concentration, and SAXS data were analyzed by using the ATSAS suite of SAXS data analysis tools (http://www.embio-hamburg.de/biosaxs/software.html).

Crystallography of Tdp2 and Tdp2–DNA complexes. Crystals of Tdp2–DNA complexes were grown by sitting-drop vapor diffusion by mixing 200 nl of precipitant with 200 nl of Tdp2 protein–DNA complex. mTdp2^{245} nucleotide and DNA complexes contained a final protein concentration of 8 mg ml^{-1} and (i) for SeMet-AMP-complex crystals, 10 mM dAMP, (ii) for DNA complex I, a 1.2-fold molar excess of 5′-9SA oligonucleotide and 1 mM MgCl2, (iii) for DNA complex II, a 1.2-fold molar excess of 5Y-9SA oligonucleotide and 1 mM MgCl2 or (iv) for DNA complex III, a 1.2-fold molar excess of 12SA oligonucleotide (Supplementary Table 2) and 1 mM MgCl2. For the DNA-bound complexes I, II and III, protein-DNA complex crystallization at 2:1 protein:DNA stoichiometry was promoted with the use of oligonucleotide substrates that are self-complementary through an 8-base-pair duplex region and bear double-ended overhangs (Supplementary Fig. 5 and Supplementary Table 2). To facilitate observation of Tdp2 bound to a 5′-adduct substrate analog without mutation of the Tdp2 active site, we used the hydrolysis-resistant 5′-N DNA (Fisher) immediately before SAXS data collection (Figs. 1 and 2a). SeMet-derivatized mTdp2^{245}–dAMP complex crystals were grown with a precipitant containing 100 mM MES, pH 6.5, 20 mM Bacle and 10% PEG 20,000, then cryoprotected in liquid nitrogen in the crystallization mother liquor supplemented by 25% PEG 20,000, 8% glycerol and 5% glucose. Crystals of mTdp2^{245} bound to a 5′-N DNA (complex I) were grown with a precipitant containing 100 mM sodium acetate, pH 4.5, 5% PEG 1000 and 50% ethylene glycol. Crystals of mTdp2^{245} bound to a product DNA (complex II) were grown with a precipitant containing 100 mM HEPES, pH 7.5, 20% PEG 3350, 200 mM sodium acetate and 10 mM MgCl2, then cryoprotected in the crystallization mother liquor supplemented by 25% PEG 3350, 8% glycerol and 5% glucose. DNA Complex III crystals mTdp2^{245} were grown with a precipitant containing 100 mM Tris, pH 8.5, 25% PEG 3350, 250 mM magnesium formate, then cryoprotected in the crystallization condition with 8% glycerol and 5% glucose.

X-ray diffraction data collection, phasing and refinement. X-ray data (Table 1) were collected at 105 K on beamline 22-1D of the Advanced Photon Source at a wavelength of 1.000 Å for the mTdp2^{245}–Mg^{2+}–5′-S′–dAMP complex, Tdp2–DNA complex I and Tdp2–DNA complex III. Tdp2–DNA complex I was collected at 0.826 Å, and the SeMet mTdp2^{245}–Ba^{2+}–dAMP data set was collected at the experimentally determined Se-K edge of 0.979 μm. X-ray diffraction data were processed and scaled using the HKL2000 suite36. The SeMet-derivatized mTdp2^{245}–dAMP complex crystals were phased using a single-wavelength anomalous dispersion (SAD) data set (Table 1). Positions of the 36 Se atoms of the nine Tdp2 monomers in the crystallographic asymmetric unit were located and refined with SOLVE37,38. An initial model built with RESOLVE39,39 was improved by iterative rounds of manual fitting in Coot and refinement against the mTdp2^{245}–Mg^{2+}–dAMP data set by using the program PHENIX38. Chain A of this model was used as the search model for determination of the mTdp2^{245}–DNA complexes I, II and III by molecular replacement in PHASER40. All crystallographic refinement and data analysis was performed in PHENIX37. The final models display excellent geometry. MOLPROBITY38 Ramachandran statistics: mTdp2^{245}–Mg^{2+}–dAMP complex, 96.9% favored, 0.0% outliers; mTdp2^{245}–5′-S′–N DNA complex I, 98.4% favored, 0.0% outliers; mTdp2^{245}–product DNA complex II, 98.9% favored, 0.0% outliers; and mTdp2^{245}–excluded DNA complex III, 97.2% favored, 0.0% outliers.

T5PNP and PNPP cleavage assays. Enzymatic reactions were performed in clear 96-well plates in assay buffer containing 100 mM NaCl, 20 mM Tris, pH 7.5, and 2 mM MgCl2. Reactions consisted of 2 mM T5PNP or PNPP and 1 μM Tdp2 proteins. Increase in absorbance at 415 nm was monitored for 60 min in a POLARStar Omega plate reader (BMG Labtech). Data were analyzed in Microsoft Excel.

Preparation of oligonucleotide substrates. Oligonucleotide substrates and construction are summarized in Supplementary Tables 2–4. 5′- or 3′-modified oligonucleotides were purchased from Midland Certified Reagent Company, and unlabeled DNA oligonucleotides were purchased from Integrated DNA. Oligonucleotides were resuspended in distilled water and diluted to a working concentration in buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mM EDTA) and either used directly in reaction assays or crystallization trials or annealed to a 1.2-fold excess of the indicated complimentary nucleotide in buffer (10 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mM EDTA), heated to 70°C and slowly cooled to room temperature before using in reaction assays.

Tdp2 oligonucleotide-substrate assays. Purified Tdp2 proteins (10 nM) were incubated with 1 μM FITC-labeled DNA substrates in reaction buffer containing 100 mM NaCl, 20 mM Tris, pH 7.5, and 2 mM MgCl2. Samples were removed at the indicated time points, and the reaction was stopped by adding a four-fold excess of Novex TBE-Urea loading buffer (Invitrogen). Reactions were resolved on Novex 15% TBE-Urea PAGE gels (Invitrogen). The FITC-labeled oligonucleotides were imaged by using a Typhoon 9000 imager with an excitation wavelength of 488 nm and a band pass emission filter set at 520 nm (GE Healthcare Life Sciences). ImageQuant software was used to quantify band intensities.

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