SUMO modification of the neuroprotective protein TDP1 facilitates chromosomal single-strand break repair

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Breaking and sealing one strand of DNA is an inherent feature of chromosome metabolism to overcome torsional barriers. Failure to reseal broken DNA strands results in protein-linked DNA breaks, causing neurodegeneration in humans. This is typified by defects in tyrosyl DNA phosphodiesterase 1 (TDP1), which removes stalled topoisomerase 1 peptides from DNA termini. Here we show that TDP1 is a substrate for modification by the small ubiquitin-like modifier SUMO. We purify SUMOylated TDP1 from mammalian cells and identify the SUMOylation site as lysine 111. While SUMOylation exhibits no impact on TDP1 catalytic activity, it promotes its accumulation at sites of DNA damage. A TDP1 SUMOylation-deficient mutant displays a reduced rate of repair of chromosomal single-strand breaks arising from transcription-associated topoisomerase 1 activity or oxidative stress. These data identify a role for SUMO during single-strand break repair, and suggest a mechanism for protecting the nervous system from genotoxic stress.
**Results**

**TDPI is a substrate for modification by SUMO.** Yeast two-hybrid analyses conducted to identify novel TDP1 binding partners uncovered 14 independent clones encoding full-length UBE2L1, the human homologue of the yeast SUMO-conjugating enzyme UBC9 (Fig. 1a). These observations suggested a previously unanticipated role for the SUMO modification pathway during TDP1-mediated repair. To test whether TDP1 is a substrate for covalent SUMO conjugation, we first reconstituted SUMOylation reactions *in vitro* using recombinant human p53, a known SUMO target (Fig. 1b). Parallel reactions conducted with human recombinant TDP1 revealed a slower migrating band, as detected by anti-TDP1 or anti-SUMO1 antibodies (∗Fig. 1c). These products were absent from control reactions conducted in the presence of a SUMO1 mutant that is incapable of forming the covalent conjugation reaction. Furthermore, their appearance was dependent on ATP (Fig. 1d), confirming that they are covalent TDP1–SUMO1 conjugates. Subsequent comparison of the different SUMO isoforms suggested that TDP1 is preferentially modified by SUMO1 (Fig. 1e). As most SUMO target proteins are modified at very low steady-state levels *in vivo*, we ectopically expressed TDP1 and SUMO1 in mammalian cells and examined the possibility of covalent TDP1–SUMO1 conjugations. For these experiments, we transfected HEK293 cells with Myc–TDP1 and/or Myc–GFP–SUMO1 (Fig. 2a). We purified the acceptor lysines to arginine resulted in SUMOylated species with identical size to SUMOylated TDP1 (∗Fig. 2b). Superposition of the acceptor lysines to arginine and subjected the purified proteins to *in vitro* SUMOylation reactions. Notably, the N-terminal domain is only conserved in higher eukaryotes (Fig. 2b, dotted boxes) and database analyses revealed K111 to be conserved across vertebrates. Consistent with SUMOylation occurring in this domain, reactions conducted in the presence of TDP11–150 revealed a prominent slower migrating band (Fig. 2c, lane 1). This band was absent from reactions containing TDP11–150; K111R and instead a faster migrating band appeared with lower intensity, suggesting a secondary SUMOylation site that was picked up in the absence of K111 *in vitro* (Fig. 2c, lane 2). Mutation of K139 to arginine resulted in SUMOylated species with identical size to SUMOylated TDP11–150, albeit with higher intensity, suggesting that K111 is the main SUMOylation site and that K139 may contribute to SUMOylation of the truncated TDP11–150 *in vitro* (Fig. 2c, lane 3). Indeed, mutation of both K111 and K139 to arginine completely abrogated SUMOylation of TDP11–150 (Fig. 2c, lane 4). The slower migrating band was SUMOylated TDP1, as it was absent in reactions containing a mutant form of SUMO that cannot be covalently conjugated (Fig. 2c, lanes 5 and 6). We next compared full-length recombinant TDP1 and TDP1K111R for their ability to covalently conjugate SUMO1 *in vitro*. Mutation of lysine 111 to arginine was sufficient to abolish SUMO1 covalent attachment to full-length TDP1 (Fig. 2d). We next examined whether lysine 111 is the main SUMOylation site *in vivo*. For these experiments, we generated Myc–TDP1 and mutants in which the N-terminal, C-terminal or all predicted SUMO sites were mutated to arginine. Next, we tested whether endogenous TDP1 is covalently conjugated by SUMO1. For these experiments, we used HeLa cells stably expressing histidine (6×His)-tagged SUMO1. We purified histidine-tagged SUMO conjugates using nickel-charged beads and subjected the conjugation products to immunoblotting using anti-TDP1 antibodies. Endogenous TDP1–SUMO1 was enriched on nickel beads incubated with extracts expressing histidine-tagged SUMO1, but not with control HeLa cell extracts (Fig. 1h). That the enriched products correspond to TDP1–SUMO1 conjugates was confirmed by demonstrating TDP1 activity that releases tyrosine from a 3′-phosphotyrosine synthetic Top1 substrate, which mimics Top1-linked DNA breaks (Fig. 1i). To further confirm that TDP1 is covalently SUMOylated in mammalian cells, we examined the effect of sentrin/SUMO-specific protease-1 (SENPI) on human purified SUMO1–TDP1 conjugates. SUMOylation is a dynamic process and once attached the SUMO peptide can be removed from target proteins by SENPs. For these experiments, we transfected HEK293 cells with His–GFP–SUMO1 and/or Myc–TDP1 and purified histidine-tagged GFP–SUMO1 conjugate using nickel-charged beads. Conjugation products were then subjected to Myc–IPs to purify Myc–TDP1–SUMO1–His–GFP products (Fig. 1j). Anti-Myc immunoblotting revealed a discrete band in Myc–IPs from extracts coexpressing Myc–TDP1 and His–GFP–SUMO1 (Fig. 1k). Subsequent treatment of the Myc–IP with SENPI resulted in a band corresponding in size to Myc–TDP1 (∗Fig. 1l). Taken together, we conclude that TDP1 is covalently modified by SUMO1 in mammalian cells.

**TDPI is SUMOylated at lysine 111.** To study the consequences of TDP1 SUMOylation, we aimed at identifying SUMOylation site(s). Most SUMO target proteins contain a specific stretch of WKKF, where ψ is a large hydrophobic residue and K is the target lysine. *In silico* analyses revealed two potential SUMOylation sites in the N-terminal domain and three sites in the C-terminal domain (Fig. 2a). We mutated the predicted acceptor lysines to arginine and subjected the purified proteins to *in vitro* SUMOylation reactions. Notably, the N-terminal domain is only conserved in higher eukaryotes (Fig. 2b, dotted boxes) and database analyses revealed K111 to be conserved across vertebrates. Consistent with SUMOylation occurring in this domain, reactions conducted in the presence of TDP11–150 revealed a prominent slower migrating band (Fig. 2c, lane 1). This band was absent from reactions containing TDP11–150; K111R and instead a faster migrating band appeared with lower intensity, suggesting a secondary SUMOylation site that was picked up in the absence of K111 *in vitro* (Fig. 2c, lane 2). Mutation of K139 to arginine resulted in SUMOylated species with identical size to SUMOylated TDP11–150, albeit with higher intensity, suggesting that K111 is the main SUMOylation site and that K139 may contribute to SUMOylation of the truncated TDP11–150 *in vitro* (Fig. 2c, lane 3). Indeed, mutation of both K111 and K139 to arginine completely abrogated SUMOylation of TDP11–150 (Fig. 2c, lane 4). The slower migrating band was SUMOylated TDP1, as it was absent in reactions containing a mutant form of SUMO that cannot be covalently conjugated (Fig. 2c, lanes 5 and 6). We next compared full-length recombinant TDP1 and TDP1K111R for their ability to covalently conjugate SUMO1 *in vitro*. Mutation of lysine 111 to arginine was sufficient to abolish SUMO1 covalent attachment to full-length TDP1 (Fig. 2d). We next examined whether lysine 111 is the main SUMOylation site *in vivo*. For these experiments, we generated Myc–TDP1 and mutants in which the N-terminal, C-terminal or all predicted SUMO sites were mutated to arginine (Fig. 2e). We co-transfected HEK293 cells with the different forms of Myc–TDP1 and/or GFP–SUMO1, followed by immunoprecipitation using anti-Myc antibodies. A single amino-acid substitution of K111 to arginine was sufficient to abrogate the formation of TDP1–SUMO1 conjugates (Fig. 2e). Taken together, we conclude that K111 is the main SUMOylation site in TDP1.
SUMOylation-deficient mutant shows reduced rate of SSB repair.
To examine the consequences of TDP1 SUMOylation in vivo, we compared the ability of HEK293 cells ectopically expressing SUMO1 and TDP1 or TDP1K111R for their ability to repair camptothecin (CPT)-induced DNA damage using alkaline comet assay. These assays primarily measure repair of DNA SSBs, where they form and disappear with kinetics established for this repair process.4,5,21. As expected, HEK293 cells expressing TDP1 accumulated four- to fivefold fewer SSBs compared with control cells (Fig. 3a). Notably, cells expressing TDP1K111R accumulated two- to threefold more SSBs compared with TDP1-expressing cells ($P = 0.0169$; t-test). This defect was not due to differences in expression levels as confirmed by immunoblotting (Fig. 3b) and was evident from scatter plots of the raw data from individual experiments (Fig. 3c). The difference in breaks is notable in terms of SSB repair, as ~4-fold difference underlies the severe neurological demise observed in neural tissues lacking XRCC1, the core SSB repair protein.22. To examine whether the difference in repair was due to SUMOylation, we compared CPT-induced SSBs in the presence and absence of ectopically expressed SUMO1. While overexpression of SUMO1 had no impact on CPT-induced SSBs in control cells and in TDP1K111R-expressing cells (Fig. 3d,e; $P = 0.65$; t-test), it reduced the extent of SSBs in
TDP1-expressing cells (P = 0.007; t-test). These observations suggest that the difference in repair observed between TDP1<sub>K111R</sub> and TDP1 was due to SUMOylation of the latter. As TDP1 also repairs oxidative SSBs resulting from IR<sup>23</sup>, we compared TDP1- and TDP1<sub>K111R</sub>-expressing cells for their ability to repair IR-induced SSBs. While IR induced similar levels of SSBs in all three cell lines (Fig. 3), the kinetics of their removal was delayed in cells expressing TDP1<sub>K111R</sub> compared with TDP1-expressing cells (Fig. 3g), suggesting a role for TDP1 lysine 111 during the repair of IR-induced SSBs.

To further examine the consequences of SUMOylation in cells lacking endogenous TDP1, we infected Tdp1<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) with retrovirus particles containing vector alone (Tdp1<sup>−/−</sup> V) or encoding human TDP1 (Tdp1<sup>−/−</sup>–hTDP1) or Tdp1<sup>−/−</sup> K111R (Tdp1<sup>−/−</sup>–hTDP1<sub>K111R</sub>). During 30-min incubation with CPT, Tdp1<sup>−/−</sup> V accumulated approximately fivefold more SSBs compared with cells complemented with wild-type human TDP1, Tdp1<sup>−/−</sup>–hTDP1<sub>K111R</sub> (Fig. 3h). Importantly, Tdp1<sup>−/−</sup>–hTDP1<sub>K111R</sub> cells accumulated ~3-fold more breaks compared with Tdp1<sup>−/−</sup>–hTDP1<sub>K111R</sub> cells (P = 0.015; t-test). Consistent with the kinetics of SSB repair, subsequent incubation in CPT-free medium for short periods resulted in a decline in the level of SSBs observed in all three cell lines. Notably, levels of SSBs remained at a higher level in Tdp1<sup>−/−</sup>–hTDP1<sub>K111R</sub> compared with Tdp1<sup>−/−</sup>–hTDP1<sub>K111R</sub>. To examine whether unrepaired SSBs in Tdp1<sup>−/−</sup>–hTDP1<sub>K111R</sub> impacted on cellular survival, we compared the three cell types for their ability to form macroscopic colonies following exposure to CPT. Tdp1<sup>−/−</sup> V cells were very sensitive to CPT and, as expected, complementation with hTDP1 led to marked protection, whereas complementation with hTDP1<sub>K111R</sub> led to partial protection (Fig. 3i). Moreover, complementation of Tdp1<sup>−/−</sup>–K111R quiescent cortical neural cells with human TDP1 or TDP1<sub>K111R</sub> revealed a role for K111 to maintain cell viability following CPT (Fig. 3k). Taken together, these data suggest that mutating the acceptor lysine K111 to a non-SUMOylatable arginine results in defects in repairing SSBs.

**K111R does not alter TDP1 structure or catalytic activity.** Why does a TDP1 SUMOylation-defective mutant display attenuated rates of SSB repair? Although unlikely for missense point mutations, we tested the possibility that the K111R mutation might lead to a gross distortion of TDP1 structure. We subjected recombinant TDP1 and TDP1<sub>K111R</sub> produced in <i>Escherichia coli</i> to thermal denaturation experiments, a widely used technique to determine the stability of proteins. The thermal denaturation experiments revealed that TDP1<sub>K111R</sub> exhibited a similar thermal stability as wild-type TDP1, suggesting that the K111R mutation does not alter the structure of TDP1. Additionally, we assessed the catalytic activity of TDP1<sub>K111R</sub> using a recombinant system that measures the rate of SSB repair. The results indicated that TDP1<sub>K111R</sub> displayed similar catalytic activity as wild-type TDP1, confirming that the K111R mutation does not impair the catalytic function of TDP1.

**Figure 2 | TDP1 is SUMOylated at Lysine 111.** (a, b) BLAST analysis identifies TDP1 orthologues in plants, fungi and animals with an extended amino terminus in vertebrates ‘dotted boxes’. Sequence analysis of this domain identifies two putative SUMOylation sites. Alignment of sequences from human (Homo sapiens; NP_001008744), cattle (Bos taurus; NP_001180084; XP_784680), monkey (Pongo abelii; XP_002825063), frog (Xenopus tropicalis; NP_001039242), fish (Danio rerio; XP_700174) using UniProt software identifies K11 (highlighted in grey) as a conserved residue. (c) Purified human TDP1 encompassing the first 150 amino acids TDP1<sup>1–150</sup>–WT and equivalent versions, in which lysine 111, 139 or both were mutated to arginine ‘K111R’, ‘K139R’ and ‘K111R K139R’, respectively were subjected to SUMOylation reactions containing 50 nM SAE1/SAE2, 500 nM UBC9 and 5 mM ATP in the presence of 30 µM WT SUMO1 (lanes 1–4) or mutant SUMO1 (lanes 5 and 6). Reaction products were fractionated by SDS-PAGE and analysed by immunoblotting with anti-TDP1 (ab4166; Abcam) or anti-SUMO1 (Activemotif) antibodies. (d) Purified full-length human TDP1 ‘WT’ or TDP1<sub>K111R</sub> ‘K111R’ was subjected to SUMOylation reactions and analysed by immunoblotting. (e) HEK293 cells were transfected with ‘+’ or without ‘−’ GFP–SUMO1 and with wild-type Myc–TDP1 or a version harbouring substitution of K111 ‘N1’, K139 ‘N2’, K111 and K139 ‘N1/2’, K231, K417 and K527 ‘C’ or K111, K139, K231, K417 and K527 ‘NC’ to arginine. Total cell extract (~4×10<sup>6</sup> cells) was subjected to immunoprecipitation using anti-Myc monoclonal antibodies (981; Cell Signaling) and immunoprecipitates were fractionated by SDS–PAGE and analysed by immunoblotting. Molecular weight size markers (kDa) are depicted. MT, mutant; WT, wild-type.
To further examine the impact on protein folding and catalytic activity, we incubated purified recombinant TDP1 or TDP1K111R with oligonucleotide duplexes harbouring 3’-phosphotyrosine that mimic Top1-linked breaks and quantified the 3’-phosphate products. The reactions showed a comparable concentration-dependent conversion of 3’-phosphotyrosine to 3’-phosphate, suggesting no impact of the K111R mutation on catalytic activity (Fig. 4e,f). Taken together, we conclude that mutation of the SUMOylation site of TDP1 to a non-SUMOylatable version results in delayed rate of SSB repair without a measurable impact on structure or catalytic activity.

SUMOylation of TDP1 does not alter enzymatic activity. Next, we considered the possibility that SUMOylation may alter protein examine structural changes of proteins\(^{24}\). The K111R mutation had no detectable impact on the thermal stability or unfolding profile of TDP1, as determined by comparing the denaturation curves and their corresponding melting temperatures (Fig. 4a). We also compared the circular dichroism absorption spectrum of TDP1 and TDP1K111R (Fig. 4b–d). The two proteins gave spectral shapes with negative bands at ~210 and 220 nm, and positive bands at ~195 nm. Analyses of secondary structure by the variable selection algorithm (CDSSTR), which provides superior fits for globular proteins\(^{25–28}\), revealed no significant difference in \(\alpha\)-helical or \(\beta\)-sheet content (\(P > 0.5\), t-test), suggesting no apparent change in structure (Supplementary Table S1). This was also supported by using the CONTIN and K2D algorithms (Supplementary Figures S2 and S3).
function by introducing structural changes that result in changes in enzymatic activity. 29,30. To test this possibility, we subjected human recombinant TDP1 or TDP1K111R to SUMOylation reactions in the presence of wild-type SUMO1 or mutant SUMO1 (Fig. 4g) and incubated reaction products with the synthetic Top1 substrates (Fig. 4h–j). We also compared the effect of SUMOylation on TDP1 produced in mammalian cells (Fig. 5a,c,e,f). Furthermore, we subjected purified human SUMOylated TDP1 to SENP1 treatment and compared the activity of the resulting products to that of mock-treated fractions (Fig. 5b,d,g,h). Incubation of Myc–TDP1 with Top1–substrate mimics resulted in a dose-dependent conversion of 3'-phosphotyrosine to 3'-phosphate, indicating that TDP1 catalytic activity was not affected by Myc, His or GFP tags. Quantification of reaction products at different concentrations or at different time points revealed no marked impact of SUMOylation on TDP1 activity (Fig. 5e–h). We conclude from these experiments that SUMO1 conjugation to TDP1 does not affect its enzymatic activity. We also excluded the possibility that TDP1 SUMOylation might modulate interaction with DNA ligase III (Lig3α), a known binding partner and a component of the SSB repair machinery (Fig. 5a,b). Using western blotting, we could not detect a measurable increase of TDP1 SUMOylation after exposure to exogenous DNA damage (Supplementary Fig. S1). This suggests that TDP1 SUMOylation is a housekeeping modification that occurs, as is also the case with other SUMO1-modified targets such as fission yeast Top1 and human Kap1 (refs 31 and 32), at low steady-state levels where cycles of...
SUMOylation aids accumulation of TDP1 at sites of DNA damage. We next considered the possibility that TDP1 SUMOylation does not display measurable impact on catalytic activity. (a) Lysates from HEK293 cells expressing Myc–TDP1 alone or additionally expressing His–GFP–SUMO1 were incubated with Ni-beads to purify His-tagged proteins, followed by Myc-immunoprecipitation. Extracts were fractionated by SDS-PAGE and 1/10 serial dilutions analysed by immunoblotting using anti-Myc antibodies. ‘unb.’ is the unbound material. His-tagged products enriched on Ni-beads were eluted with imidazole and subjected to myc–immunoprecipitation. The immunoprecipitate was then incubated with buffer alone ‘ − ’ or buffer containing 1 µM SENP1 ‘ + ’. Purified Myc–TDP1–SUMO1–His–GFP and Myc–TDP1 were quantified by comparing with known concentrations of recombinant TDP1 analysed by immunoblotting. (b) Serial dilutions of Myc–TDP1 or Myc–TDP1–SUMO1–His–GFP from purifications shown in (a, b, respectively) were incubated with 32P-radiolabelled Top1–substrates and reaction products analysed by denaturing PAGE and phosphorimaging. Reaction products from experiments using purification, shown in (a), were quantified from Top1–DSBs (c) or Top1–SSBs (d) relative to total labelled substrate, and percentage conversion to 3′-P was calculated from n = 3 independent experiments ± s.e.m. Conversion to 3′-P was determined from the purification, shown in (b), at the indicated concentrations (g) or at 10 nM purified human TDP1 at the indicated time periods (h). Error bars, s.e.m of n = 3 independent experiments from n = 2 biological replicates.

Figure 5 | SUMOylation of TDP1 does not display measurable impact on catalytic activity. (a) Lysates from HEK293 cells expressing Myc–TDP1 alone or additionally expressing His–GFP–SUMO1 were incubated with Ni-beads to purify His-tagged proteins, followed by Myc-immunoprecipitation. Extracts were fractionated by SDS-PAGE and 1/10 serial dilutions analysed by immunoblotting using anti-Myc antibodies. ‘unb.’ is the unbound material. His-tagged proteins enriched on Ni-beads were eluted with imidazole and subjected to Myc-immunoprecipitation. The immunoprecipitate was then incubated with buffer alone ‘ − ’ or buffer containing 1 µM SENP1 ‘ + ’. Purified Myc–TDP1–SUMO1–His–GFP and Myc–TDP1 were quantified by comparing with known concentrations of recombinant TDP1 analysed by immunoblotting. (c, d) Serial dilutions of Myc–TDP1 or Myc–TDP1–SUMO1–His–GFP from purifications shown in (a, b, respectively) were incubated with 32P-radiolabelled Top1–substrates and reaction products analysed by denaturing PAGE and phosphorimaging. Reaction products from experiments using purification, shown in (a), were quantified from Top1–DSBs (c) or Top1–SSBs (d) relative to total labelled substrate, and percentage conversion to 3′-P was calculated from n = 3 independent experiments ± s.e.m. Conversion to 3′-P was determined from the purification, shown in (b), at the indicated concentrations (g) or at 10 nM purified human TDP1 at the indicated time periods (h). Error bars, s.e.m of n = 3 independent experiments from n = 2 biological replicates.

SUMOylation aids accumulation of TDP1 at sites of DNA damage. We next considered the possibility that TDP1 SUMOylation might affect its local concentration at sites of DNA damage. This would be an appealing mechanism to increase substrate spectrum, as in vitro data suggest a requirement for a high concentration of TDP1 to process SSBs16,23. The difference in un repaired SSBs that accumulate in TDP1K111R compared with TDP1–expressing cells could be attributed to differences in the local concentration of TDP1 at sites of DNA damage (Supplementary Fig. S2). To examine whether TDP1 SUMOylation fulfils this function, we compared the accumulation of GFP–TDP1 to that of GFP–TDP1K111R at sites of ultraviolet A laser-induced DNA damage in mammalian cells. Laser damage induces a mixture of nicks, gaps, SSBs and DSBs, all of which have been shown to trap Top1, resulting in Top1–DNA breaks33–35. In addition, a subset of these breaks may be direct substrates for TDP1 (refs 7,12 and 36). While GFP–TDP1 accumulated rapidly at sites of laser damage reaching a maximum level within 5 s, accumulation of GFP–TDP1K111R was slower and less extensive (Fig. 7a–c). Interestingly, accumulation of GFP–TDP1K111R continued to increase over the entire time course of the experiment, perhaps reflecting the need to continue protein accumulation to achieve levels similar to that seen with GFP–TDP1. 90 s after DNA damage, the local enrichment of GFP–TDP1 at laser stripes was ~150% compared with ~125% for GFP–TDP1K111R (Fig. 7a). This difference was not due to differences in TDP1 expression,
as the global expression was similar by western blotting (Fig. 7a, inset) and, more importantly, only cells that showed similar level of GFP–TDP1 total fluorescence were subjected to the tracking experiments (Fig. 7b). Notably, the K111R mutation did not ablate recruitment completely, suggesting that either recruitment is partially SUMOylation-dependent or other SUMO sites, although not detected biochemically, may contribute to the remaining fraction of enrichment.

If it is true that TDP1 SUMOylation promotes repair by facilitating its accumulation at sites of DNA damage, then depletion of UBE21 (UBC9), the obligate SUMO-conjugating enzyme, should similarly reduce accumulation of TDP1 at sites of DNA damage. To test this, we depleted UBC9 in MRC5 cells using short hairpin RNA and compared the accumulation of GFP–TDP1. As predicted, depletion of UBC9 led to a marked reduction of the ability of GFP–TDP1 to accumulate at sites of laser damage (Fig. 7b–f). Enrichment of GFP–TDP1 in UBC9-depleted cells displayed similar dynamics to that of GFP–TDP1 K111R, as it continued to increase during the time course of the experiment. Importantly, depletion of UBC9 did not result in further reduction in the
Figure 7 | TDP1 SUMOylation promotes its accumulation at sites of DNA damage. (a) Human mRC5 cells were plated onto glass-bottom dishes and transfected with pMCEGFP–TDP1 or pMCEGFP–TDP1K111R. Cells expressing similar total GFP signal were locally irradiated with a ultraviolet A laser, and GFP–TDP1 accumulation at the site of damage was quantified for the indicated time periods (seconds), where ‘C’ represents undamaged and ‘0’ the enrichment obtained immediately after damage. Data are plotted as the percentage increase in fluorescence (arbitrary units) at the site of ultraviolet A irradiation. Data are the average ± s.e.m. of ~60 cells measured from n = 6 biological replicates. The difference between the accumulation of GFP–TDP1 (yellow circles) and GFP–TDP1 K111R (blue circles) was statistically significant at all time points examined (P < 0.001, Student’s t-test). Global expression of GFP–TDP1 was analysed by immunoblotting using anti-TDP1 antibodies, inset. (b) Average total fluorescence ± s.e.m. from cells analysed in a showing comparable level of expression between GFP–TDP1 and GFP–TDP1K111R. (c) Representative images of cells from experiments presented in a are shown before irradiation ‘control’, immediately after ‘0 s’ and at the indicated time points ‘5–40 s’. Scale bar, 10 μm. (d) MRC5 cells were transfected with non-silencing control constructs ‘CT’ or UBC9-depleted ‘UBC9 KD’ cells were fractionated by SDS–PAGE and analysed by immunoblotting using anti-UBC9 (Abcam) or anti-actin antibodies. 

accumulation of GFP–TDP1K111R, indicating that the observed effect was due to SUMOylation. Taken together, these results indicate that SUMOylation of TDP1 at K111 facilitates its accumulation at sites of DNA damage, thereby enhancing its DNA repair capacity.

Contribution to the repair of transcription-associated SSBs. A major source of Top1-breaks is the collision of Top1 intermediates with elongating RNA polymerases during transcription. Inhibiting RNA polymerase II by 5,6-dichlorobenzimidazole 1-b-ribofuranoside (DRB) or α-amanitin has been shown to reduce the extent of CPT-induced DNA SSBs. We reasoned that if TDP1 SUMOylation contributes to its recruitment to sites of transcription-associated SSBs, then inhibiting transcription should result in reduced accumulation of TDP1. Consistent with this prediction, pretreatment of GFP–TDP1-expressing cells with the transcription inhibitor DRB led to reduction in TDP1 accumulation at sites of laser damage (Fig. 8a,b). The reduction in recruitment was less than that observed for mock-treated GFP–TDP1K111R, which could reflect a role for TDP1 SUMOylation during damage generated by DRB-resistant RNA polymerases. Alternatively, it could be due to roles for TDP1 SUMOylation unrelated to transcription. Importantly, DRB did not affect the initial recruitment of GFP–TDP1K111R, suggesting a role for TDP1 SUMOylation at K111 during the repair of transcription-blocking lesions. However, DRB ablated the time-dependent increase of GFP–TDP1K111R accumulation during the
subsequent 90-s observation period. We reason that inhibiting transcription may reduce the frequency of collision of Top1 intermediates with elongating RNA polymerase II, thereby reducing the need for recruiting more GFP–TDP1K111R. We next quantified the extent of transcription-associated SSBs using alkaline comet assays. For these experiments, we used HEK293 cells to achieve ~90% transfection efficiency. If the difference in repair capacity observed between TDP1 and TDP1K111R was, at least in part, due to a role for TDP1 SUMOylation in repairing transcription-associated SSBs, then inhibiting transcription should ablate or reduce this difference. While cells expressing TDP1K111R accumulated a higher level of SSBs compared with TDP1 expressing cells, they both decreased to a comparable level following incubation with DRB (Fig. 8ac,b; $P = 0.78$; t-test), suggesting that TDP1 SUMOylation contributes to the repair of transcription-associated SSBs. Taken together, these data suggest that TDP1 SUMOylation at K111 participates in the overall repair of transcription-dependent SSBs.

**Discussion**

Top1-linked DNA breaks underlie the clinical utility of an important class of anticancer drugs, and their progressive accumulation causes neurodegeneration in humans. A key factor in the repair of Top1-breaks is TDP1, mutation of which causes the demise of postmitotic tissue. Here we report that TDP1 is a substrate for SUMO modification, which occurs primarily at lysine 111 within the vertebrate-conserved N-terminal domain. SUMOylation promotes accumulation of TDP1 at sites of DNA damage, thereby accelerating the rate of SSB repair and contributes, at least in part, to the repair of transcription-generated SSBs, the major source of Top1-breaks in non-cycling cells. These observations raise the possibility of an important regulatory role in postmitotic tissue. Notably, ~4-fold difference in the rapid clearance of SSBs results in severe neurological dysfunction in murine SENPs model systems and has been linked to ataxia in humans.

Collision of elongating RNA polymerase II with Top1 intermediates results in stalling of transcription and triggers partial
degradation of Top1 to a small peptide. TDP1 can then access these structures to remove the stalled Top1 peptide from DNA termini. We propose that collision with RNA polymerases is a trigger to degrade Top1 and to subsequently enrich TDP1 at sites of DNA damage (Fig. 8e). The latter process is, at least in part, dependent on SUMOylation of TDP1 at lysine 111. We propose a further, evolutionarily conserved, layer of regulation via a SUMO–SIM interaction that maintains TDP1 at sites of DNA damage. There is precedence for the involvement of SUMO during Top1 repair. Top1 itself, the main source of DNA breaks that require TDP1, is also a substrate for SUMO conjugation. In fission yeast, the SUMO conjugation machinery also has a role for dealing with Top1-breaks. The Nse2 E3 SUMO ligase functions together with the fission yeast structure-specific nuclease Rad16-Swi10 to facilitate the repair of Top1-breaks, independently of Tdp1 (refs 32 and 48). Notably, lysine 111 is absent in fission yeast Tdp1, highlighting an evolutionarily driven SUMO regulation for human TDP1.

Mutation of the SUMO acceptor lysine 111 to arginine did not result in an apparent impact on the core structure of TDP1, as indicated by the thermal shift profiles, circular dichroism spectrograms and by comparing enzymatic activities of the recombinant proteins. While CD spectra showed no significant change in α-helical or β-sheet content, it pointed at a possible minor change in conformation, which is unlikely to be of biological relevance, as enzymatic activities were not affected. Moreover, cellular TDP1 and TDP1K111R interacted to the same extent with Lig3ε, which is known to bind to that domain, as measured by two independent assay methods (Fig. 6). Knockdown of UBC9 reduced recruitment of TDP1 to a level similar to that of TDP1K111R, but had no impact on TDP1K111R recruitment, indicating that the difference was due to SUMOylation. Furthermore, overexpression of SUMO1 facilitated repair of cells expressing TDP1, but not TDP1K111R further supporting that the observed phenotype was due to SUMOylation.

Crystal structure, cell-free and DNA binding studies suggest that high local concentrations of TDP1 are required to process Top1–SSBs. However, while vertebrate cells achieve this was not clear. Here we examined whether SUMOylation of TDP1 increased its catalytic activity, using the 3′-phosphotyrosine substrate to mimic Top1-linked DNA breaks. SUMOylated TDP1 prepared in vitro or purified from mammalian cells exhibited similar catalytic activity to the unSUMOylated version. It is possible that SUMOylation facilitates binding of Top1 peptide to the peptide-binding pocket of TDP1 and thus increases catalytic activity when using a peptide substrate instead of a phosphotyrosine substrate. However, we do not favour this possibility, as the peptide-binding pocket is distant from the SUMOylation site. Instead, our results indicated by the thermal shift profiles, circular dichroism spectrograms and by comparing enzymatic activities of the recombinant proteins that SUMOylation promotes TDP1 accumulation at sites of DNA damage, thereby facilitating the repair of SSBs. These data identify a molecular role for SUMOylation during TDP1-mediated SSB repair reactions and implicates this process in protecting postmitotic tissue from genotoxic stress.

Methods

**Yeast two-hybrid screen and analyses.** *Saccharomyces cerevisiae* Y190 cells (Clontech) were transformed with pGBK7-T–TDPI[K111R] and transfectants were selected on synthetic media plates (glucose and yeast nitrogen基). Recombinant proteins were dialysed in 30 mM sodium phosphate buffer, pH 7.4, and filtered immediately before the spectrum was measured using a JASCO J-715 spectropolarimeter (JASCO). The CD spectrum of the buffer alone was subtracted from that of the sample, and time constant was set to 4 s with a scan rate at 50 nm min⁻¹. The bandwidth was 1 nm and the sensitivity set to 0.5. Scans were performed from 260 to 190 nm with a 0.1-nm data pitch and continuous scan mode. A Peltier device was used to maintain a temperature at 10 °C. The buffer baselines were subtracted and data represent the average of four independent experiments ± s.e.m.

**Immunoprecipitation.** The generation and maintenance of mammalian cells are provided in Supplementary Methods. HEK293 (~1.5×10⁵) cells were transfected with pCMV vector encoding full-length human UBE2I according to the published DNA sequence. Cells were isolated and subjected to sequencing. We identified 14 independent clones (clone 0–11; SC–5308, 12,000). While both antibodies recognized the same SUMOylated species, the latter did not pick up the unmodified protein.

**Circular dichroism spectroscopy.** Recombinant proteins were dialysed in 30 mM sodium phosphate buffer, pH 7.4, and filtered immediately before the spectrum was obtained through 0.2-micron filters (Millipore). The concentration of recombinant proteins was accurately determined immediately before, during and after the CD scans, to ensure accurate secondary structure estimations. Samples were normalized to the lowest concentration (0.6 mg ml⁻¹), placed in a 0.2-mm quartz cuvette (Starna, Essex, UK) and measurements taken using a JASCO J-715 spectropolarimeter (JASCO). The CD spectrum of the buffer alone was subtracted from that of the sample, and time constant was set to 4 s with a scan rate at 50 nm min⁻¹. The bandwidth was 1 nm and the sensitivity set to 0.5. Scans were performed from 260 to 190 nm with a 0.1-nm data pitch and continuous scan mode. A Peltier device was used to maintain a temperature at 10 °C. The buffer baselines were subtracted and data represent the average of four independent experiments ± s.e.m.
Purification of SUMOylated TDP1 from human cells. Approximately 1x10⁹ HEK293 cells were transfected with plasmids encoding GFP–His–SUMO1 and Myc–TDP1 and lysed, as described above, using protease inhibitors and 30 mM imidazole in both the lysis and wash buffer. Following washing, SUMOylated proteins were eluted from the beads using three consecutive elutions with lysis buffer containing 250 mM imidazole. Elution fractions were pooled and SUMOylated TDP1 was purified using Myc-immunoprecipitation, as described above. Beads enriched in SUMOylated TDP1 were finally suspended in an equal volume of wash buffer. Where indicated, 80 µl bead suspension enriched with purified Myc-TDP1–SUMO1 was resuspended in 150 mM NaCl, 50 mM Tris, pH 7.5, 0.5 mM DTT. A volume of 40 µl bead suspension was then mock-treated or incubated with 0.2–1 µg of human sentrin/SUMO-specific protease-1 (SENPO1; Enzo Life Sciences) for 20 min at 37 °C. Following three consecutive washes with 150 mM NaCl, 50 mM Tris, pH 7.5, beads were subjected to in vitro activity assays or analysed by immunoblotting with anti-TDP1 or anti-Myc antibodies. Quantification of purified Myc–TDP1 and Myc–TDP1–SUMO1–His–GFp was conducted by comparing with known concentrations of recombinant His–TDP1 by western blotting. The latter was determined by Bradford assays, and by comparing with Coomassie-stained BSA standards. We estimate 2 ng µl⁻¹ bead suspension of purified human Myc–TDP1 or Myc–TDP1–SUMO1–His–GFp.

Cell-free DNA repair assays. Purified recombinant proteins were subjected to in vitro SUMOylation reactions and the appropriate dilutions were mixed with 50 mM 3²-¹²⁵I-labelled oligonucleotide duplexes (see Supplementary Methods for DNA sequence) in 25 mM HEPES, pH 8.0, 130 mM KCl and 1 mM DTT. Purified SUMOylated TDP1 from mammalian cells was quantified as described above and serial dilutions of beads were mixed with 50 mM 3²-¹²⁵I-labelled substrates. Reactions were incubated for 1 h at 37 °C, terminated by formamide loading buffer, fractionated by denaturing PAGE, and analysed by phosphorimaging.

Clonogenic survival and viability assays. HEK293 cells or Tdp1−/− MEFs containing empty vector or vectors expressing wild-type TDP1, TDP111118 or GFP–SUMO1 were subjected to 200 µM CPT (MEFs) or 50 µM CPT (HEKs) for 30 min at 37 °C, or exposed to 20 Gy IR (caesium 137, Cammell 1000) on ice. Where indicated, cells were subsequently incubated in drug-free media for the indicated repair period. DNA strand breakage was quantified by alkaline comet assays as described in Supplementary Methods.

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Acknowledgements
We thank Jo Morris and Alan Lehmann for advice and for the provision of reagents and cell lines. We thank Roger Philips for advice on live cell imaging and analyses; Louise Serpel, Alison Rodger and Antony Oliver for advice on thermal shifts and CD analyses. This work is funded by grants from the Wellcome Trust (085284 and 091043) to S.F.-K. O.S.W. and C.R. are additionally funded by studentships from the MRC.

Author contributions
S.-C.C. made the initial observation that led to the connection of TDP1 with the SUMO pathway. J.J.R.H. conducted the mammalian biochemical experiments for TDP1 SUMOylation and the immunofluorescence analyses on fixed cells. S.-C.C. performed the yeast experiments and established the murine cells. S.-C.C., J.J.R.H. and S.F.-K. conducted the cellular and cell-free DNA repair assays. O.S.W. prepared the recombinant proteins, conducted the thermal shifts and cell-free SUMOylation assays, performed and analysed the circular dichroism experiments. C.R. and S.-C.C. conducted the live cell imaging experiments and C.R. analysed the data. All authors designed the experiments and interpreted the data. S.F.-K. conceived the study, coordinated the project and wrote the manuscript.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Hudson, J. J. R. et al. SUMO modification of the neuroprotective protein TDP1 facilitates chromosomal single-strand break repair. Nat. Commun. 3:733 doi: 10.1038/ncomms1739 (2012).

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