Aberrant topoisomerase-1 DNA lesions are pathogenic in neurodegenerative genome instability syndromes

Sachin Katyal1,2, Youngsoo Lee1,3, Karin C Nitiss4, Susanna M Downing1, Yang Li1, Mikio Shimada1, Jingfeng Zhao1, Helen R Russell1, John H J Petrini5, John L Nitiss4 & Peter J McKinnon1

DNA damage is considered to be a prime factor in several spinocerebellar neurodegenerative diseases; however, the DNA lesions underpinning disease etiology are unknown. We observed the endogenous accumulation of pathogenic topoisomerase-1 (Top1)-DNA cleavage complexes (Top1ccs) in murine models of ataxia telangiectasia and spinocerebellar ataxia with axonal neuropathy 1. We found that the defective DNA damage response factors in these two diseases cooperatively modulated Top1cc turnover in a non-epistatic and ATM kinase–independent manner. Furthermore, coincident neural inactivation of ATM and DNA single-strand break repair factors, including tyrosyl-DNA phosphodiesterase-1 or XRCC1, resulted in increased Top1cc formation and excessive DNA damage and neurodevelopmental defects. Notably, direct Top1 poisoning to elevate Top1cc levels phenocopied the neuropathology of the mouse models described above. Our results identify a critical endogenous pathogenic lesion associated with neurodegenerative syndromes arising from DNA repair deficiency, indicating that genome integrity is important for preventing disease in the nervous system.

The DNA damage response is essential for maintaining genome integrity and preventing various human diseases, many of which are characterized by pronounced neuropathology1–3. Although most components of this signaling pathway have been identified, their tissue-specific function for preventing characteristic disease-related pathology is unclear, as are the precise DNA lesions underpinning the etiology of these syndromes. The nervous system is particularly at risk from DNA damage, and endogenous DNA breaks occur spontaneously during development and in the mature brain5–7. One instance of defective DNA damage signaling is ataxia telangiectasia (A-T), a neurodegenerative syndrome associated with cancer susceptibility, immunodeficiency and radiosensitivity8–10. A-T results from dysfunction of ATM (ataxia telangiectasia, mutated), a serine/threonine protein kinase, which is required for the activation of cell cycle checkpoints, chromatin remodeling, DNA repair and apoptosis after DNA double-strand breaks8,10. This is reinforced by the A-T–like neuropathology1–3. Although most components of this signaling pathway have been identified, their tissue-specific function for preventing characteristic disease-related pathology is unclear, as are the precise DNA lesions underpinning the etiology of these syndromes. The nervous system is particularly at risk from DNA damage, and endogenous DNA breaks occur spontaneously during development and in the mature brain5–7. One instance of defective DNA damage signaling is ataxia telangiectasia (A-T), a neurodegenerative syndrome associated with cancer susceptibility, immunodeficiency and radiosensitivity8–10.

A-T results from dysfunction of ATM (ataxia telangiectasia, mutated), a serine/threonine protein kinase, which is required for the activation of cell cycle checkpoints, chromatin remodeling, DNA repair and apoptosis after DNA double-strand breaks8,10. This is reinforced by the A-T–like neuropathology present when Mre11 is partially inactivated (resulting in A-T like disease, ATLD)9,11,12; this factor is a component of the ATM-activating Mre11-Rad50-Nbs1 (MRN) DNA double-strand break sensor9,11,13,14. ATM activation via the MRN complex is required for apoptosis of immature neural cells after DNA damage via p53 and Chk2 phosphorylation5,13,15, and failure to eliminate these damaged neuroprogenitors could predispose mature A-T tissue to later neurodegeneration. However, ATM’s full neuroprotective repertoire in the nervous system remains elusive16.

Two neurodegenerative syndromes similar to A-T, ataxia with oculomotor apraxia (AOA1) and spinocerebellar ataxia with axonal neuropathy (SCAN1), result from defects in the DNA repair enzymes aprataxin (APTX) and tyrosyl-DNA phosphodiesterase 1 (TDPI), respectively. APTX and TDP1 function primarily during DNA single-strand break repair11,17–19; APTX is an adenyl hydrolase that resolves 5′-adenylation intermediates during DNA ligation, whereas TDP1 cleaves and processes 3′-end covalent Top1-DNA intermediates and DNA lesions formed by oxidative damage11,12,19. To investigate etiologic connections between these diseases and A-T, we considered whether ATM function intersects single-strand break repair disorders. Because ATM has been implicated in the response to Top1 adducts20–22, which could increase levels of DNA damage, particularly DNA single-strand breaks, we determined whether ATM regulates Top1-induced damage in neural tissue. We found that a key function of ATM is to avert detrimental DNA lesions in both the developing and mature nervous system by preventing the accumulation of Top1ccs. This involved ubiquitination and sumoylation-mediated turnover of Top1 to resolve Top1ccs, and was ATM kinase independent. Our results further implicate defective Top1 processing and the accumulation of neural DNA damage as causative for neuropathology in multiple neurodegenerative syndromes arising from mutation of DNA damage response factors.

RESULTS
ATM regulates Top1cc in neural tissue
Throughout neural development, cells encounter a variety of events that compromise genome integrity, among which is endogenous damage via Top1 malfunction during DNA replication and transcription1,12,23–25.

1Department of Genetics, St. Jude Children’s Research Hospital, Memphis, Tennessee, USA. 2Department of Pharmacology and Therapeutics, University of Manitoba and Manitoba Institute of Cell Biology, CancerCare Manitoba, Winnipeg, Manitoba, Canada. 3GIRC, Ajou University School of Medicine, Suwon, Korea. 4Department of Biopharmaceutical Sciences, University of Illinois-Chicago, Rockford, Illinois, USA. 5Molecular Biology Program, Memorial Sloan-Kettering Cancer Center and Cornell University Graduate School of Medical Sciences, New York, New York, USA. Correspondence should be addressed to S.K. (sachin.katyal@umanitoba.ca) or P.J.M. (peter.mckinnon@stjude.org).

Received 7 February; accepted 9 April; published online 4 May 2014; doi:10.1038/nn.3715
Top1 alters DNA topology and relaxes DNA supercoiling by breaking and rejoining one strand of DNA, and thousands of these transient Top1ccs form during normal cellular function. However, a portion of these can persist in genomic DNA, and when trapped, a Top1cc includes a DNA strand break, which is a direct threat to cell survival12,15,23. The anticancer agent camptothecin (CPT) is effective at killing replicating cells because it promotes accumulation of Top1ccs that are converted into lethal DNA double-strand breaks following collision with replication forks23,24. CPT-induced Top1cc and associated DNA breaks during transcription can activate ATM to initiate a DNA damage response (DDR), which is important to prevent genome damage to cells during proliferation21,22,26. Thus, removal of trapped Top1 is critical to avoid DNA damage accumulation and resultant sequel.

Loss of TDP1, whose function is required to repair Top1-DNA complexes via cleavage of the covalent Top1-DNA phosphodiester bond, leads to the neurodegenerative disease SCAN1. Thus, we examined brain tissue from Tdp1−/− mice27 to measure the endogenous levels of Top1cc in a setting in which Top1cc removal from DNA is compromised. We observed high levels of Top1cc in Tdp1−/− neural tissue compared with control tissue, particularly during early development (Fig. 1). Because of phenotypic similarities between SCAN1 and A-T1, we assessed Top1cc levels in the nervous system of Atm−/− mice. We found that, at embryonic day 12.5 (E12.5), a highly proliferative stage in embryonic brain development, ATM-deficient neural tissue had accumulated substantial Top1cc levels (Fig. 1c). Although Top1cc levels were increased during early brain development in both Tdp1−/− and Atm−/− embryos, they were reduced after E18.5, suggesting these complexes increase during rapid embryonic proliferation rates. Following birth and into adulthood, elevated Top1cc levels persisted in the nervous system (Fig. 1d). At 1 year of age, both Atm−/− and Tdp1−/− cerebellum showed higher levels of Top1cc, indicating that loss of either of these factors perturbs the steady-state levels of Top1-DNA complexes (Fig. 1e).

**Atm−/− neural cells accumulate Top1cc-associated DNA damage**

Top1ccs involve DNA strand breaks, and the persistence of this complex predicts increased amounts of DNA damage23,30. To determine whether Top1cc accumulation is associated with increased DNA strand breaks, we compared DNA repair rates in Atm−/− and Tdp1−/− cells under conditions that promote the formation of these complexes, using the Top1 poison camptothecin (CPT)26,31. To measure levels of DNA damage, we used the alkaline comet assay. We found that quiescent Atm−/− astrocytes accumulated Top1-dependent breaks at levels threefold higher than wild-type cells after CPT treatment, although at lower levels than Tdp1−/− astrocytes (Fig. 2a). Because cells are non-replicating, DNA breaks from trapped Top1cc are mostly single-stranded breaks. In contrast, astrocytes deficient in the ATM-related DNA-dependent protein kinase, catalytic subunit (DNA-PKcs, Prkdc), repaired Top1-induced DNA damage at similar levels as wild-type astrocytes, indicating the relative specificity of the Atm−/− defect (Fig. 2a). In addition, cells derived from a mouse model of ATLD (Mrc1ATLD/ATLD) also failed to show a repair defect after CPT treatment, indicating that the role of ATM in preventing Top1-associated strand breaks is not Mrc1-dependent (data not shown). We also confirmed that DNA breaks in these non-replicating cells were a result of transcription-associated Top1 damage21,22,32, as the transcription inhibitor 5,6-dichloro-1-β-d-ribofuranosylbenzimida/zole abrogated CPT-induced DNA damage (Supplementary Fig. 1).

**ATM is implicated in the regulation of Tdp1 activity**33,34. We therefore assessed Tdp1 enzymatic activity in wild-type and Atm−/− brains. Quantification of the cleavage rate of an oligonucleotide substrate that mimics the Top1 protein-linked DNA 3′-terminus revealed that there was no difference in Tdp1-dependent product formation in Atm−/− cerebellar extracts compared to wild type, indicating that ATM does not affect Tdp1 enzymatic activity (Supplementary Fig. 2). This suggests that the role of ATM in regulating Top1cc is not mediated by Tdp1.
The kinase activity of ATM is critical for phosphorylation of various substrates that activate cell cycle arrest or apoptosis after DNA damage. We determined whether ATM kinase activity was necessary to prevent Top1cc-associated DNA strand breaks. Using the KU55933 ATM inhibitor (ATMi), we compared Atm−/− astrocytes to ATMi-treated wild-type astrocytes in the presence of CPT. Unlike Atm−/− astrocytes, ATMi-treated wild-type astrocytes showed normal DNA repair profiles (Fig. 2b). Inhibition of ATM kinase activity in these experiments was confirmed by abrogation of post-translational modification of Chk2 and p53 after DNA damage (Fig. 2b). These data imply that an aspect of the ATM protein, independent of ATM kinase activity, mediates ATM-dependent Top1cc repair in DNA repair.

In addition to the specificity of CPT toward Top1, various DNA lesions including single-strand breaks and abasic sites can also result in increased Top1cc levels that are irreversible. We compared the relative effects of other genotoxins toward Top1cc accumulation and found that the loss of ATM or Tdp1 also increased Top1cc formation after ionizing radiation (IR), H2O2 or methylmethane sulfonate (MMS; Fig. 2c). Correspondingly, DNA damage in Atm−/− or Tdp1−/− astrocytes was increased compared with wild-type cells after treatment with these genotoxins (Fig. 2d). Atm−/− cerebellar granule neurons also showed an approximately threefold higher level of unrepaired DNA breaks after IR than did wild-type counterparts (Fig. 2e). Tdp1−/− granule neurons also showed elevated DNA breaks after IR, although the levels were relatively less than the comparative threefold increase observed over Atm−/− astrocytes (Fig. 2d); this possibly reflects intrinsic cell-type differences or cell culture effects. These data indicate that various types of DNA damage, including oxidative lesions, can increase Top1cc formation, compromising DNA integrity after ATM or Tdp1 loss. Notably, the antioxidant N-acetylcysteine reduced the levels of DNA breaks after CPT in either Atm−/− or Tdp1−/− astrocytes, suggesting that oxidative stress–induced DNA breaks can exacerbate damage by trapping Top1ccs (Supplementary Fig. 3).
**Figure 3** ATM modulates Top1 turnover after CPT treatment. (a) Top1 levels were higher in *Atm<sup>−/−</sup>* astrocytes than in wild-type cells after CPT treatment. The proteasome inhibitor MG132 inhibited Top1 turnover after CPT treatment in control cells. Top1cc levels, as determined by the ICE assay, corresponded to CPT-induced trapping of Top1-DNA and reduced Top1 turnover. Top1 and Top1cc quantitation was normalized to untreated control levels. (b) Treatment with MG132 increased the levels of DNA damage, particularly in control cells. Error bars represent s.e.m. (c) MG132 also dampened ATM-dependent signaling, as shown by decreased ATM and Kap1 phosphorylation. Total Kap1 and Gfap levels revealed equal protein loading. (d) Poly-ubiquitin immunoblots of immunoprecipitated Top1 showed that ubiquitination of Top1 was markedly reduced by either CPT or KU55933 treatment. Top1 immunoprecipitated from CPT-treated control cells and blotted with Top1 antibodies revealed that Top1 migrated as a collection of higher molecular weight species (red asterisks), reflecting Top1 post-translational modification (Top1<sup>PTM</sup>). Reduced amounts of post-translationally modified Top1 were found in Top1 immunoprecipitates from *Atm<sup>−/−</sup>* lymphoid cells after CPT-treatment. Similar to CPT-treated control cells, a comparable amount of Top1 was immunoprecipitated from CPT/KU55933 co-treated control lymphoblasts. Immunoblots of extracts before immunoprecipitation showed reduced total Top1 expression in CPT-treated control cells (arrowheads) compared with other cell types and treatments. (e) High molecular weight Top1 bands from Top1 immunoprecipitates were immunoreactive to antibody to SUMO1 (red asterisks), indicating that Top1 undergoes poly-sumoylation (Top1<sup>pSUMO1</sup>). In *Atm<sup>−/−</sup>* cells, Top1<sup>pSUMO1</sup> was reduced after CPT treatment, whereas, similar to CPT-treated control cells, a comparable amount of Top1<sup>pSUMO1</sup> was immunoprecipitated from CPT/KU55933 co-treated control lymphoblasts. (f) Western blots indicate absence of ATM in A-T cells, whereas KU55933 treatment of control cells resulted in defective Chk2 phosphorylation after CPT treatment, indicating effective ATM inhibition. Full-length western blots are presented in Supplementary Figure 11.

**ATM regulates DNA damage–dependent Top1 degradation**

Removal of trapped Top1cc is associated with proteasome-mediated Top1 degradation, which involves protein modification by ubiquitination and sumoylation, with Tdp1 cleaving the Top1 peptide from DNA<sup>21,23,30,36</sup>. To determine whether ATM modulates Top1cc levels via Top1 degradation, we examined the effect of the proteasome inhibitor MG132 after CPT in ATM-deficient and control cells. We found that total Top1 levels were decreased in control cells after CPT, but less so in *Atm<sup>−/−</sup>* cells (Fig. 3a). Consistent with the CPT-induced decrease in Top1 being proteasome dependent, co-treatment with MG132 and CPT elevated Top1 and Top1cc levels in control cells by 4–5-fold (Fig. 3a). In contrast, Top1 and Top1cc levels in *Atm<sup>−/−</sup>* cells were only marginally increased after MG132 treatment, suggesting that ATM modulates proteasome-mediated Top1 turnover (Fig. 3a). Accordingly, under these experimental conditions, elevated Top1cc levels after MG132 and CPT treatment resulted in a marked increase in DNA breaks in control astrocytes (Fig. 3b). Notably, MG132 treatment resulted in levels of DNA damage in control cells that were similar to those in *Atm<sup>−/−</sup>* cells. These data imply that ATM has a predominant role in directing proteasome-mediated turnover of Top1 in trapped Top1cc. In addition, proteasome inhibition also attenuated ATM signaling in wild-type cells after CPT, as indicated by reduced ATM (p-ser-1987) and Kap1 (p-ser-824) phosphorylation (Fig. 3c).

We also compared the above results (in murine cells) to human A-T cells. We again found a marked reduction in total Top1 protein in CPT-treated control cells, compared with an approximately fourfold higher level of Top1 in the CPT-treated A-T counterparts, consistent with a requirement for ATM in Top1 turnover (Supplementary Fig. 4). Consistent with these results, ICE assays revealed that more Top1ccs were trapped in A-T cells than in wild-type controls (Supplementary Fig. 4). Co-treatment of control cells with ATMi and CPT resulted in Top1 downregulation comparable to CPT-only treatment alone, indicating that, similar to our earlier data (Fig. 2), Top1 downregulation is not ATM kinase dependent (Supplementary Fig. 4). Collectively, these data support ATM as a key regulator of Top1 degradation, acting in a kinase-independent manner after CPT.

To understand how ATM modulates Top1 turnover, we examined the ubiquitination and sumoylation status of Top1, as both modifications are important for turnover of this protein<sup>21,36,37</sup>. Analysis of Top1 immunoprecipitates from CPT-treated cells revealed ubiquitinated Top1 in control cells (Fig. 3d), but a marked reduction of ubiquitinated Top1 in A-T cells. Furthermore, Top1 immunoprecipitates showed a characteristic Top1-immunoreactive ladder in control cells (Fig. 3d,e), reflecting post-translationally modified Top1 (Top1<sup>PTM</sup>), whereas this laddering was reduced in A-T cells. These high molecular weight Top1 bands also bound antibody to SUMO1 after CPT treatment, and, similarly, this Top1<sup>pSUMO1</sup> immunoreactivity was markedly reduced in A-T cells (Fig. 3e). Co-treatment of control cells with ATMi and CPT revealed similar Top1 laddering, confirming that ATM kinase activity is dispensable for these modifications (Fig. 3d–f). We also found that knockdown of ATM, but not TDP1, reduced sumoylation of Top1 after CPT treatment in human cells.
**Figure 4** ATM is essential for DNA damage signaling after CPT treatment. (a) Primary Atm<sup>−/−</sup> astrocytes formed few γH2AX foci after CPT treatment (5 μM for 60 min), whereas abundant γH2AX foci were seen in wild-type (WT) and Tdp1<sup>−/−</sup> astrocytes. All genotypes showed equivalent levels of γH2AX after IR. Upper panels show immunofluorescence analysis of typical γH2AX foci, which are quantified in the graphs below, as are the foci observed after IR. (b) Similar to astrocytes, MEFs also exhibited ATM-dependent γH2AX after CPT. 53BP1 foci also showed a similar induction after CPT and colocalized with γH2AX foci; these data are quantified in the adjacent graphs. dKO, Atm<sup>−/−</sup>; Tdp1<sup>−/−</sup>. (c) In contrast with Atm<sup>−/−</sup> cells (A–T), the loss of NBS1 did not affect γH2AX foci formation following CPT treatment. (d) Although bleomycin (Bleo) treatment (10 μg ml<sup>−1</sup>, 30 min) induced γH2AX foci at similar levels in wild-type, Atm<sup>−/−</sup> and Tdp1<sup>−/−</sup> cells, pre-treatment with CPT prevented bleomycin-induced γH2AX foci formation in Atm<sup>−/−</sup> and Atm<sup>−/−</sup>; Tdp1<sup>−/−</sup> cells. The ATM inhibitor (ATMi) KU55933 prevented CPT-induced γH2AX foci formation, indicating that ATM kinase activity is required for H2AX phosphorylation. (e) Quantitation of γH2AX foci per cell for the different treatments and genotypes presented in d. (f) When CPT-treated (5 μM CPT, 60 min) Atm<sup>−/−</sup> or Atm<sup>−/−</sup>; Tdp1<sup>−/−</sup> cells MEFs were subsequently incubated with CPT-free media, DNA damage signaling was activated in a Prkdc-dependent manner, as γH2AX foci failed to form in the presence of the DNA-PKcs inhibitor, NU7441 (2 μM). For all foci quantification experiments, 30 cells for each cell line and corresponding treatment were counted and experiments were repeated in quadruplicate (total n = 120 independent cells measured per line per treatment). Bar graphs represent mean cellular foci values of all replicates, error bars represent s.e.m. and P values were calculated using Student’s unpaired t test. The y axes of the graphs in b and f are nonlinear to indicate increased foci number in specific genotypes after DNA damage.

(Supplementary Fig. 5). Collectively, these results indicate that ATM regulates Top1 degradation in response to DNA damage in a manner independent of ATM kinase activity or TDP1 to attenuate Top1cc levels, thereby preventing DNA damage.

**ATM is essential for DNA damage signaling from trapped Top1**

Given that a prime role of ATM is as a serine/threonine protein kinase<sup>8,9</sup>, it was unexpected that this enzymatic activity was dispensable for Top1cc regulation and DNA strand break repair. A prominent feature of DNA damage signaling is the phosphorylation of histone H2AX on serine 139 by ATM or other related kinases (for example, Prkdc)<sup>10</sup> as an early event in establishing the DDR. Thus, we monitored the formation of phosphorylated histone H2AX (γH2AX) in response to CPT and other genotoxins using quiescent primary cells to avoid replication-associated damage. We found a pronounced defect in the induction of γH2AX after CPT treatment specifically in Atm<sup>−/−</sup> cells compared with Tdp1<sup>−/−</sup>, Prkdc<sup>−/−</sup> or wild-type counterparts. This indicates that ATM is uniquely required for DNA damage signaling after CPT (Fig. 4a); comparable results using CPT have also been observed in postmitotic neurons<sup>21</sup>. In contrast to CPT, IR induced similar levels of γH2AX in all of the genotypes examined (Fig. 4a). CPT-induced γH2AX foci also did not form in Atm<sup>−/−</sup>/murine embryonic fibroblasts (MEFs) or A-T fibroblasts (Fig. 4b and Supplementary Fig. 6). In addition, analysis of Atm<sup>−/−</sup>; Tdp1<sup>−/−</sup> double knockout cells revealed that γH2AX formation in response to CPT is dependent on ATM, even after Tdp1 inactivation (Fig. 4b). However, CPT-induced γH2AX occurred at similar levels as in controls in cells from the A-T-related disease Nijmegen Breakage syndrome (NBS; Fig. 4c and Supplementary Fig. 6) and Mre11<sup>ATLD</sup> ATLD (data not shown), indicating that ATM-dependent phosphorylation of H2AX after CPT is not MRN dependent.

We also examined γH2AX formation in Atm<sup>−/−</sup> cells after treatment with dual genotoxins by co-treating with CPT and bleomycin. Despite bleomycin activating γH2AX in Atm<sup>−/−</sup> cells, pre-treatment with CPT resulted in minimal bleomycin-induced γH2AX formation, implying that CPT-induced damage suppresses DNA damage signaling from ATM-related kinases (Fig. 4d,e). However, after CPT washout (a 30-min CPT pulse followed by a 120-min recovery), γH2AX formed in Atm<sup>−/−</sup> cells and at higher levels in Atm<sup>−/−</sup>; Tdp1<sup>−/−</sup> astrocytes, and these foci were suppressed by the Prkdc inhibitor NU7441 (Fig. 4f). Because replication-associated breaks don’t occur in these quiescent cells, the DNA-dependent protein kinase (Prkdc) likely becomes activated as Top1cc lesions are processed. Collectively, our findings suggest a bi-modal response of ATM to CPT-induced DNA damage; ATM kinase activity is essential for DNA damage signaling, as shown by γH2AX formation after CPT treatment, whereas DNA repair (resolution of...
Synthetic lethality occurs after dual inactivation of ATM and Tdp1

Although the Atm−/− brain accumulates Top1cc, these mice retain apparently normal neurological function and exhibit no overt neuropathology39,40. A similar situation is found with Tdp1−/− mice27. We reasoned that if ATM and Tdp1 independently regulate Top1cc, intercrossing Atm−/− and Tdp1−/− mice would increase the levels of trapped Top1cc in the brain, and this should be an effective means to assess the in vivo effect of this lesion. We observed a >90% reduction in viability of Atm−/−; Tdp1−/− compound mutant mice (Table 1 and Supplementary Table 1). Analysis during development revealed that most Atm−/−; Tdp1−/− embryos were smaller than controls and lost viability between E13.5 and E16.5, a stage at which Top1cc levels are maximal (Fig. 1c). Given that SCAN1 and A-T are neurodegenerative syndromes, we determined the extent to which ATM and Tdp1 loss specifically affects the nervous system. To do this, we used mice in which Atm was inactivated in neural progenitors using Nestin-cre and a conditional Atm allele39 to generate AtmNes-cre mice. AtmNes-cre; Tdp1−/− mice showed a similar phenotype to Atm−/−; Tdp1−/− mice, with premature lethality between E13.5–16.5, indicating that the lethality following co-inactivation of ATM and Tdp1 was related to effects on the nervous system (Table 1 and Supplementary Table 1).

To further understand the basis for the genetic interaction between ATM and Tdp1, we considered whether the phenotype of the double mutant mice might actually reflect the established role of ATM in responding to DSBs8,9,10, rather than a specific function in regulating Top1cc-induced DNA damage. We also generated Top1cc−/−; Mre11ATLD/ATLD and Top1cc−/−; Lig4Nes-cre double mutant mice to determine whether the MRN complex, which is important for ATM activity in the context of Tdp1 inactivation. However, in both cases, the double mutant mice were born at normal Mendelian ratios and did not show any overt phenotype (Table 1 and Supplementary Table 1). We also generated Top1cc−/−; Mre11ATLD/ATLD and Top1cc−/−; Nbs1AB/AB double mutant mice to determine whether the MRN complex, which is critical for ATM activation8–11, is important for ATM activity in the context of Tdp1 inactivation. However, we again found that MRN and Tdp1 double mutants were born at Mendelian frequencies without any apparent phenotype (Table 1 and Supplementary Table 1). Collectively, these data indicate that the neuroprotective synergy observed between ATM and Tdp1 likely reflects a role of ATM that is different to its established function in DNA double-strand break signaling and activation by the MRN complex.

ATM and Tdp1 are required for CNS development

To understand how dual loss of ATM and Tdp1 affects the nervous system, we examined mutant embryos at various developmental stages. Immunohistochemical analyses of Atm−/−; Tdp1−/− and AtmNes-cre; Tdp1−/− embryos revealed substantial levels of DNA damage, as indicated by γH2AX-positive cells throughout the developing forebrain, midbrain and cerebellum (Fig. 5a and Supplementary Figs. 7 and 8). Consistent with increased DNA damage, widespread p53 immunostaining and apoptosis was localized primarily in the proliferative ventricular zone (Fig. 5b and Supplementary Figs. 7 and 8). This contrasts with observations in wild-type, Atm−/− or Tdp1−/− embryos, where only sporadic apoptosis and no γH2AX- or p53-immunopositive cells were present (Fig. 5a,b and Supplementary Figs. 7 and 8). Although the appearance of γH2AX in Atm−/−; Tdp1−/− neural tissues seems to contradict our results showing that ATM is essential for γH2AX formation after CPT (Fig. 4), these in vivo results reflect Top1cc collision with replication forks, which generates DNA double-strand breaks, and the subsequent activation of other DNA damage-responsive kinases such as Atr. We also found high levels of apoptosis during early neurogenesis, as revealed by active caspase-3 and TUNEL staining in E12.4 Atm−/−; Tdp1−/− embryos (Fig. 5c). DNA damage or apoptosis was absent outside of the Atm−/−; Tdp1−/− and AtmNes-cre; Tdp1−/− embryonic nervous system (data not shown).

Given the synthetic lethality between ATM and Tdp1, and an absence of an overt phenotype after loss of Tdp1 and double-strand break repair factors or regulators of ATM activation, we considered it likely that toxic Top1cc accumulation accounted for the Atm−/−; Tdp1−/− phenotype. We found substantially increased Top1cc levels in the double mutants compared to single mutants and controls (Fig. 5d), despite normal levels of Top1cc in the double mutant embryos (Fig. 5e). Given that Top1cc levels were elevated in Atm−/− and Tdp1−/− neural tissues without associated pathology, our data suggest a DNA damage threshold may exist in neurons.

To directly test the pathogenic consequence of elevated Top1cc, we exposed developing embryos to the CPT analog topotecan (TPT). We found a reduction in embryo size and widespread apoptosis throughout the nervous system in response to TPT administration, which was strikingly reminiscent of the Atm−/−; Tdp1−/− phenotype (Fig. 5f). Notably, despite systemic exposure of the embryos to topotecan and its wide bioavailability, we found that apoptosis was almost exclusively confined to the nervous system, underscoring the sensitivity of neural development to this DNA lesion (Supplementary Fig. 9).

Because apoptosis can potentially result in Top1cc accumulation12,23, we generated Atm−/−; Top1cc−/− mice in which p53 was also inactivated, which blocks p53-dependent apoptosis after DNA damage in the nervous system. Although the loss of one or both copies of p53 effectively blocked apoptosis (Fig. 5g) and rescued lethality (data not shown), these triple mutants showed similar Top1cc levels to the Atm−/−; Tdp1−/− mice (Fig. 5g). These data confirm that elevated Top1cc levels in the double mutants result from the inactivation of ATM and Tdp1 and are not an outcome of apoptosis.

Top1ccs occur after endogenous DNA damage

Top1ccs can also accumulate when these normally transient complexes encounter various types of DNA lesions, including strand breaks and

---

**Table 1** ATM loss exacerbates Tdp1 deficiency

| Genotype                  | Expected N | Observed N |
|---------------------------|------------|------------|
| Atm−/− × Tdp1−/−          | Control    | 969        | 1,083      |
|                           | Mutant     | 125        | 11         |
| AtmNes-cre × Tdp1−/−      | Control    | 360        | 474        |
|                           | Mutant     | 120        | 5          |
| Mre11ATLD/ATLD × Tdp1−/−  | Control    | 105        | 108        |
|                           | Mutant     | 15         | 12         |
| Lig4Nes-cre × Tdp1−/−     | Control    | 284        | 283        |
|                           | Mutant     | 9          | 10         |
| PrKdc−/− × Tdp1−/−        | Control    | 166        | 166        |
|                           | Mutant     | 9          | 10         |

Genetic analysis shows synthetic lethality after coincident loss of Tdp1 and ATM. Compound mutants involving DNA double-strand break repair (Lig4 or PrKdc) or MRN (Mre11ATLD/ATLD) deficiency do not affect the expected rate of generation of compound mutant mice. Mouse genetic inheritance ratios were calculated using standard rules for Mendelian genetic inheritance: expected and observed ratios were calculated on the basis of parental genotypes and the number of live-born mice.

DNA strand breaks associated with Top1cc is kinase independent. Thus, these data show that ATM has a unique role, independent of Tdp1, in responding to Top1cc-induced DNA damage.
Compound Atm−/− and Tdp1−/− mutants are usually lethal during development. 

(a) Analysis of the E14.5 Atm−/−; Tdp1−/− developing nervous system revealed an accumulation of DNA breaks (γH2AX, arrowheads). In the double-deficient embryonal brain, pronounced γH2AX foci (red signal demarcated by yellow arrowheads) were noted in the forebrain, whereas few foci were noted in the Atm−/− control brain. PCNA immunostaining (green) identified proliferating cells in the ventricular zone of the neocortex. (b) Similarly, abundant p53 immunoreactivity (arrowheads) was present in the ventricular zone of Atm−/−; Tdp1−/− embryonal brain, but was absent in the Atm−/− control. (c) Apoptosis occurred early during neurogenesis in the Atm−/−; Tdp1−/− forebrain, as activated caspase-3 staining and TUNEL (white arrows) was abundant at E12.5. (d) ICE analysis of Top1ccs in the E14.5 embryonic brain. 20 μg of whole-cell extract derived from E14.5 brain tissue were blotted to normalize total topoisomerase-1 protein content amongst the E14.5 CNS tissue. The remaining three rows were blotted with increasing amounts (μg) of genomic DNA isolated from these tissues. Top1cc were immunodetected in gDNA using an antibody to Top1. Relative Top1cc levels in Atm−/−; Tdp1−/− embryonal brain compared with controls (control, Atm−/− and Tdp1−/−) are listed below the blot. Embryonic tissues used in ICE assays were derived from gDNA pooled from three independent embryos. (e) Western blot analysis of E14.5 Atm−/−; Tdp1−/− neural tissue used for ICE bioassay confirming tissue genotypes and the equivalent amounts of Top1 protein amongst the genotypes for ICE analysis. Full-length western blots are presented in Supplementary Figure 11. (f) Exposure of E12.5 embryos to topotecan resulted in apoptosis throughout the developing nervous system by E14.5. The graph shows levels of TUNEL-positive cells in wild type and the relative increase associated apoptosis after coincident ATM and Tdp1 deletion was prevented when p53 was attenuated via single or dual allele inactivation. Arrows indicate TUNEL-positive cells. PCNA immunostaining identified proliferating cells, whereas Tuj1 immunostaining identified differentiating neurons. Top1cc levels in the E14.5 brain were not different in the presence or absence of apoptosis. *P < 0.001.

abasic sites12,23. We asked whether Top1cc accumulation might be a pathologic lesion in other disease-relevant scenarios. To do this, we examined mice with defective Xrc1, a factor that is central for DNA repair via single-strand break/base excision repair (BER)15. Loss of Xrc1 in the nervous system leads to defective DNA repair and resulting DNA damage accumulation40. Conspicuously, increased levels Top1cc were present in Xrc1Nes-cre proliferative neural tissue at E14.5, reflecting ATM or Tdp1 loss (Fig. 6a), further supporting the notion that DNA damage can result in trapped Top1ccs. Notably, we also examined Aptx−/− embryos, in which the adenyl hydratase Aptx responsible for preventing AOAA1 is inactivated, but Top1cc levels were not increased compared to controls (Fig. 6a). These findings indicate that the type of DNA damage influences Top1cc accumulation. Xrc1 serves a critical scaffold function during DNA repair and its loss leads to marked destabilization and loss of the repair factor DNA ligase III (Lig3). Because TDP1 is known to interact with Xrc1, we examined levels of the various BER components to determine whether Xrc1 deletion results in destabilization of TDP1 (or other repair factors), as it does Lig3. If so, this could explain elevated Top1cc levels after Xrcc1 loss. However, although Lig3 expression was decreased in Xrcc1Nes-cre brain, all of the other BER factors that we examined showed normal protein levels after Xrcc1 loss, indicating that increased Top1cc in Xrcc1Nes-cre tissue did not result from destabilization of TDP1 (Fig. 6b).

We next generated (Atm; Xrcc1)Nes-cre mice to determine the consequences of coincident ATM and Xrcc1 inactivation toward Top1cc occurrence. Although Xrcc1Nes-cre mice show a mild reduction in cerebellar size40, dual inactivation of these factors led to marked neural development defects, involving marked cerebellar atrophy and associated Top1cc accumulation in cerebellar tissue (Fig. 6c,d). Levels of Top1cc were higher during neural development than in the P16 cerebellum (Fig. 6d), suggesting that, in Xrcc1Nes-cre tissue, these lesions are detrimental during development. Accordingly, in (Atm; Xrcc1)Nes-cre neural tissue, we observed pronounced apoptosis that commenced from ~E12.5 onwards (data not shown). Although Top1cc levels were elevated after Xrcc1 inactivation, it is likely that the effects of
Figure 6 Top1cc can arise in the nervous system in response to DNA damage. (a) Neural inactivation of Xrcc1, but not Apx1, resulted in elevated Top1cc during development. Values for the ICE assay indicate relative signal compared with wild type. Loss of Xrcc1, but not ATM or Apx1, resulted in increased γH2AX foci, although these were not associated with widespread apoptosis. PCNA immunostaining identified proliferating cells, whereas TuJ1 immunostaining identified differentiating neurons. (b) Western blot analysis of BER factors revealed that, although Xrcc1 loss led to destabilization of Lig3, other factors, such as Tdp1 and Top1, were present at normal levels. Other mutant genotypes serve as controls for protein immuno-detection. Ctx, P5 cortex; Ce, P5 cerebellum. Full-length western blots are presented in Supplementary Figure 11. (c) The P16 (Atm; Xrcc1)Nes-cre cerebellum was markedly affected during neural development (arrow). Nissl staining showed general cerebellar morphology, whereas calbindin immunostaining identified Purkinje cells. Bottom, a more lateral section of the cerebellum. (d) Dual inactivation of ATM and Xrcc1 resulted in elevated Top1cc levels in the P16 cerebellum. Values for the ICE assay indicate relative signal compared with wild type.

Direct DNA strand breaks after Xrcc1 loss also exacerbates the (Atm; Xrcc1)Nes-cre phenotypic abnormalities. These data suggest that Top1cc may be a general DNA lesion that affects the nervous system in DNA repair-deficient human diseases.

Discussion

The precise DNA lesions responsible for neurodegeneration in a spectrum of human genome instability diseases remain unknown. A prime example is that of A-T, a debilitating childhood neurodegenerative syndrome that is associated with defective DNA damage signaling6. We found that, in the nervous system, in addition to its activation by DNA double strand breaks8–10, ATM is required to regulate Top1cc, a transient topoisomerase-DNA intermediate that occurs frequently during normal cellular function. Our data indicate that ATM functions in a non-epistatic manner to TDP1 to regulate the fate of trapped Top1cc. However, these factors cooperatively function to reduce Top1cc levels, as indicated by the synergistic rise in Top1ccs in the absence of both proteins. Although Tdp1 has specific enzymatic activity to cleave Top1-DNA adducts, ATM regulates the SUMO/ubiquitin-mediated turnover of Top1. In this regard, the later onset of neurodegeneration in SCAN1 may indicate that Tdp1 function in vivo is comparatively more critical for preventing transcription-associated Top1cc accumulation in post-mitotic tissue.

Aberrant accumulation of Top1cc must be prevented, as the DNA strand break formed during topoisomerase activity is potentially detrimental to a cell. For example, during proliferation, replication fork collision with trapped Top1ccs can lead to DNA double-strand breaks and cell death. In addition, Top1ccs can be trapped by proximal oxidative DNA breaks that enhance their conversion into DNA damage24,41,42. Because oxidative stress potentially results in thousands of DNA single-strand breaks each day15,43, it may be a major contributor toward elevation of Top1cc levels in the nervous system, a tissue that carries a high oxidative load. Thus, DNA lesions associated with Top1cc are pervasive throughout the life of the nervous system, and regulation of this topoisomerase complex is a critical genome maintenance requirement in this tissue.

The canonical role of ATM is regulating the response to DNA double-strand breaks10,16. It is likely that this aspect of ATM operates in parallel with Top1cc regulation (Supplementary Fig. 10). In the nervous system, DNA double-strand breaks can result in ATM-dependent apoptosis5,38, in which ATM activation occurs via the double-strand break–sensing MRN complex5,11. Mutation in the Mre11 component of the MRN complex can lead to ATLD12, a neurodegenerative syndrome that is similar to A-T. Because ATLD reflects the importance of the MRN-ATM axis in responding to DNA double-strand breaks, ATM regulation of Top1cc levels implicates an additional prevalent genotoxic lesion in A-T compared with ATLD. The more severe phenotype of A-T compared with that of ATLD, despite both syndromes having an early onset44, is consistent with the occurrence of this additional detrimental DNA lesion.

Our study points to an important neuroprotective role for regulation of Top1cc in the nervous system, thereby broadening the understanding of the etiologic lesions that contribute to neurodegenerative syndromes. In addition to A-T and SCAN1, this lesion may have a substantial pathogenic effect in other syndromes associated with DNA repair deficiency and genome instability. Given the requirement for other classes of topoisomerases for multiple DNA transactions24,45, it seems likely that defects in these or factors that modulate their activity will affect neural function. For instance, defective topoisomerase activity has been implicated in autism spectrum disorder46,47, possibly as these enzymes are important...
for transcriptional regulation of long genes linked to autism\textsuperscript{48}. Furthermore, deficiency in Top3β has recently been identified as a RNA topoisomerase that is important for the function of the FRMP, a factor that prevents Fragile X mental retardation syndrome\textsuperscript{49,50}. 

\section*{METHODS}

Methods and any associated references are available in the online version of the paper.

\section*{ACKNOWLEDGMENTS}

We thank E. Soans and M. Mishina for assistance with the ICE bioassay, B. Kuzio for general technical assistance, F. Alt (Children’s Hospital of Boston) for Prkdc\textsuperscript{--/--} mice, K. Caldecott and S. El-Khamisy (U. Sussex) and R. Klein-Geltink (St. Jude Children’s Research Hospital) for helpful discussions and S. Foster (Memorial Sloan-Kettering Cancer Center) for help analyzing the mice. We also thank the St. Jude Children’s Research Hospital Animal Resource Center and the Transgenic Core Unit for support with mouse work. P.J.M. is supported by the US National Institutes of Health (NS-37956, CA-96832), the CCSG (P30 CA21765), and the American Lebanese and Syrian Associated Charities of St. Jude Children’s Research Hospital. J.L.N. is supported by the National Cancer Institute (CA25814 and CA28313). H.J.P. is supported by the US National Institutes of Health (GM59413), the Goodwin Foundation and the Goodwin Foundation. Y. Lee is supported by the SFC program (2011-030833). S.K. is a Neoma Broadway AP Endowed Fellow and is supported by grants from the University of Manitoba, CancerCare Manitoba and a Manitoba Health Research Council Establishment award.

\section*{AUTHOR CONTRIBUTIONS}

S.K. and P.J.M. conceived and planned all of the experiments and produced the final version of the manuscript. S.K. performed all of the experiments with contributions from K.C.N. (in vitro TDP1 cleavage assay), Y. Lee, M.S. and H.R.R. (generation of the mutant mice and additional technical support), S.M.D. and Y. Li (processing tissue for ICE bioassay and mouse colony management), and J.Z. (ATm\textsuperscript{S317}cre and ATM immunoblotting experiments). H.J.P. contributed critical reagents and experimental results. J.L.N. contributed to experimental design and the interpretation of results and the preparation of the final version of the manuscript.

\section*{COMPETING FINANCIAL INTERESTS}

The authors declare no competing financial interests. 

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.
**ONLINE METHODS**

**Mice.** Generation of the germline Tdp1−/−, Atm−/−, Mre11ATL1/ATL2, Nbs1ATB/ATB, and conditional ATM, Xrcx1, p53 mouse lines have been previously described. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility with a 12-h day/night cycle. A maximum of five adult animals per cage were allowed. All animal experiments were carried out in accordance with US National Institutes of Health regulations and were approved by the St. Jude Children’s Research Hospital animal care and use committee.

**Isolation of primary cells.** Primary astrocytes were prepared from P2 mouse brains as described previously. Cortices were dissociated by passage through a 5-mi pipette and cells were resuspended in Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12:1 (1:1 DMEM/F12, Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, vol/vol), 1× glutamax, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin and 20 ng ml−1 epidermal growth factor (EGF, Millipore). Primary astrocytes were established in Primaria T-25 tissue culture flasks (Falcon) at 37 °C in a humidified CO2-regulated (5%) incubator.

Neurosphere cultures were prepared using the Neurocult neural stem cell proliferation kit (STEMCELL Technologies) according to the manufacturer’s protocol. Briefly, E14.5 embryos were mechanically dissociated by trituration and then passed through a 40-µm cell strainer. Neural stem cells were then cultured in Neurocult neurobasal media supplemented with proliferation supplements and 20 ng ml−1 EGF (STEMCELL Technologies) in upright T-25 tissue culture flasks (Falcon) at 37 °C in a humidified CO2-regulated (5%) incubator until neurospheres were established. For neurosphere growth assays, whole neurospheres were mechanically dissociated, counted, and seeded at 5 × 105 cells per well in a 24-well plate and incubated for 5 d at 37 °C.

Primary MEFs were prepared from E13.5 embryonic mesenchyme. Tissue was minced using dissection scissors, trypsinized and resuspended in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 1× glutamax, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin and β-mercaptoethanol and established in T-25 tissue culture flasks (Falcon) at 37 °C in a humidified CO2-regulated (5%) incubator until neurospheres were established. For neurosphere growth assays, whole neurospheres were mechanically dissociated, counted and seeded at 5 × 105 cells per well in a 24-well plate and incubated for 5 d at 37 °C.

**Cell survival assays.** Mouse embryonic stem cells, neural stem cells (NSCs) and MEFs were plated in replicates of 8 onto 96-well plates (5,000 cells per well). Following CPT treatment, WST-1 cell proliferation reagent (10 µl per well) was added, incubated at 37 °C for 2 h and read with a plate-reader at 450 nm (Bio-Rad). Cell survival was tabulated and graphed relative to untreated controls. Experiments were repeated in triplicate.

**Cell treatments.** For comet analysis, cells were treated with either 150 µM H2O2 (Thermo Fisher) for 10 min on ice, 14 µM CPT (Calbiochem) for various times at 37 °C, MMS (Sigma) for 10 min at various concentrations at 37 °C, bleomycin (Bedford Laboratories) at 40 µg ml−1 for 30 min at 37 °C, or γ-irradiation (20 Gy using 325Cs). Following H2O2, bleomycin and IR treatments, cells were incubated for various times in drug-free medium at 37 °C before analysis, whereas, following CPT and MMS treatments, cells were immediately harvested for analysis. For transcriptional inhibition analysis, cells were pre-incubated with media containing 50 µM 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB, Sigma) for 30 min at 37 °C followed by replacement of media containing both 14 µM CPT and 50 µM DRB for 30 min at 37 °C. For ATM analysis, cells were pre-incubated with media containing 10 µM KUS5933 (Calbiochem or Selleck chem) for 60 min at 37 °C, followed by either irradiation (10 Gy with 2 h recovery at 37 °C; for western analysis) or replacement of media containing both 14 µM CPT and 10 µM KUS5933 for 60 min at 37 °C (for comet assay and ICE-T). For foci analysis (γH2AX/53BP1), confluent cells were treated with CPT (5 mM, 60 min, 37 °C), IR (2 Gy, 60 min recovery at 37 °C), bleomycin (10 µg ml−1, 60 min at 37 °C) or H2O2 (150 mM in phosphate-buffered saline (PBS) on ice, 60 min recovery in media at 37 °C) and underwent immunofluorescence analysis (below). For DNA-PKI analysis, following CPT treatment (5 µM CPT, 30 min at 37 °C), cells were washed three times and incubated with 2 µM Nu7441 (2 h, 37 °C) followed by immunofluorescence analysis. For proteasomal analysis, cells were pre-treated with 10 µM MG132 (1 h at 37 °C) before CPT co-treatment. Similarly, for NAC analysis, cells were pretreated with 30 mM NAC (1 h at 37 °C) before CPT co-treatment.

**Alkaline comet analysis.** Cells were resuspended in pre-chilled 1× PBS, mixed with an equal volume of 1.2% low melting point agarose (Invitrogen) and maintained at 42 °C and immediately layered onto frosted glass slides (Fisher) pre-coated with 0.6% agarose and maintained in the dark at 4 °C for all further steps. Slides were immersed in pre-chilled lysis buffer (2.5 M NaCl, 10 mM Tris-Cl, 100 mM EDTA (pH 8.0), 1% Triton X-100 (vol/vol), 3% DMSO (vol/vol), pH 10) for 1.5 h, washed with pre-chilled distilled water (twice for 10 min each) and placed into pre-chilled alkaline electrophoresis buffer (50 mM NaOH, 1 mM EDTA, 1% DMSO) for 45 min. Electrophoresis was carried out at 95 mA for 25 min, followed by neutralization in 0.4 M Tris-Cl (pH 7.0). Comets were stained with SYBR Green (1:10,000 in 1× PBS, Sigma) for 10 min. A minimum of 100 comet tail moments were measured using the Comet Assay IV system (Perceptive Instruments) coupled to an Axioskop2 plus microscope (Zeiss) at 20× magnification. Experiments were repeated in triplicate and the mean comet tail moments were calculated and graphed (n = 3, 300 independent comet tail moments measured per line per treatment). Experimental samples were assigned randomized identity and analysis was performed blind.

**For in vivo comet analysis, mice were irradiated (individually) with 15 Gy and cerebella were harvested (into cold calcium- and magnesium-free PBS) either immediately or after 30 min of recovery. Granule neurons were isolated via a percoll gradient as previously described.** Agarose-embedded granule neurons were subjected to comet analysis as described above. Experiments were repeated in duplicate, with each replicate also performed in duplicate (n = 4, 400 independent comet tail moments measured per line per treatment). Experimental samples were assigned randomized identity and analysis was performed blind.

**Tdp1 cleavage assay.** Wild-type and Atm−/− cerebellar tissue were homogenized (30 strokes with a dounce homogenizer) in cleavage lysis buffer (20 mM Tris-Cl (pH 7.5), 10 mM EDTA, 1 mM EGTA, 100 mM NaCl, 1% Triton X-100 and complete protease inhibitor (Roche)). Soluble cell extracts were recovered by centrifugation at 14,000g for 5 min at 4 °C. Protein concentrations were determined by Bradford assay (Bio-Rad). A 3-linked biotinylated oligo (5′-AGGCCGCGGACGCGTCCGAG-3′) mimicking a Tdp1 substrate containing a phosphorosyrl bond was end labeled with [32P] using T4 polynucleotide kinase, gel purified and incubated with cerebellar protein extracts, as previously described. Enzymatic products were separated by 10% SDS-urea PAGE and visualized and quantified by phosphorimaging analysis (Molecular Dynamics).

**Immunodetection of Top1-DNA covalent complexes (ICE assay).** Top1-DNA covalent complexes were isolated using the ICE bioassay as previously described. Briefly, cells or tissues were lysed in 1% sarkosyl (wt/vol) with dounce homogenization (ten strokes for cells, 40 strokes for embryonic CNS tissue). DNA was sheared through a 26G needle (ten strokes) and cell lysates were gently layered onto a CsCl cushion and centrifuged in a NVT 90 rotor at 122,000g for 20 h at 25 °C (Beckman Coulter). The resulting pelleted covalent DNA-protein complexes were washed, resuspended in TE, and aliquots were diluted with 25 mM sodium phosphate buffer (pH 6.5) and applied to a nitrocellulose membrane by using a slot-blot vacuum manifold (Bio-Rad). Total protein extract was applied to adjacent slots as a control for total Top1 levels amongst tissues. Top1 protein and Top1-DNA complexes were immunodetected using a polyclonal antibody to Top1 (rabbit, 1:1,000, Bethyl, cat# 302-590A), followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody and detection using ECL Prime on X-ray film. ICE blot bands were subsequently probed with [32P]-labeled mouse (embryonic stem cells) or human (293T) gDNA to control for relative DNA loading. For astrocyte ICE assays, densitometric analysis of the Top1-DNA signal was performed using ImageJ. Experiments were performed at least in duplicate.

**Western blot analysis.** Protein extracts (cells or tissue) were prepared by using lysis buffer (50 mM Tris-Cl, 200 mM NaCl, 0.2% NP-40 (vol/vol), 1% Tween-20 (vol/vol), 100 µg ml−1, 60 min at 37 °C).
were incubated with 1,000 units of S7 nuclease (Roche) on ice for 20 min and, antibody to pATMS1981 (10H11.E12, 1:1,000, Abcam, cat# ab36810), antibody to ATM (MAT3, mouse, 1:1,000, Sigma; D2E2, rabbit, 1:2,000, Cell Signaling, cat# 2873), antibody to pATMS1981 (10H11.E12, 1:1,000, Abcam, cat# ab36810), antibody to Top1 (mouse, 1:1,000, Abnova, cat# H00055775-A01), antibody to topoisomerase 1 (1:1,000, Bethyl, cat# 302-590A) and antibody to phospho-Chk2 S624 (rabbit, 1:1,000, Bethyl, cat# A300-767A), followed by appropriate horseradish peroxidase–conjugated secondary antibodies (1:2,000, catalog #NA934V, GE Healthcare) and detected using ECL or ECL Prime chemiluminescence reagent (catalog #RPN2106, GE Healthcare). Antibody to actin (goat, 1:500, Santa Cruz Biotech, clone I-19, cat# sc-1616) and Ponceau staining of the transferred membrane were used as protein-loading controls. Densitometric analysis of the Top1 signal (following CPT treatment) was performed using ImageJ. Experiments were performed in duplicate. Full-length blots are shown in Supplementary Figures 11 and 12.

Top1 immunoprecipitations. Control and A-T human lymphoblasts were used for Top1 immunoprecipitation, as we could not immunoprecipitate Top1 from mouse cells. Cells were pre-incubated with media containing inhibitors (mock/DMSO, 50 µM DRB or 10 µM KU55933, 60 min at 37 °C) followed by mock (DMSO) or camptochein treatment (14 µM, 60 min at 37 °C). Cells were collected, washed in 1× PBS, lysed (200 mM NaOH, 2 mM EDTA) and neutralized (100 mM HCl, 60 mM Tris-HCl) on ice and underwent sonication (3 × 10-s bursts, setting 5, 550 sonic dismembrator, Fisher Scientific) in 1× SSN nuclease buffer (5 mM MgCl2, 5 mM CaCl2, 0.5 mM DTT, 0.1 mM EDTA, 20 mM N-ethylmaleimide, 1× complete protease inhibitors (Roche). Cell lysates were incubated with 1,000 units of SSN nuclease (Roche) on ice for 20 min and, centrifuged at 10,000 g for 30 min at 4 °C to remove genomic DNA. 1 mg of protein extract (resulting supernatant) underwent Top1 immunoprecipitation (2 h, 4 °C) in radioimmunoprecipitation buffer (RIP buffer; 0.5% NP-40, 0.25% sodium deoxycholate, 0.05% SDS (wt/vol), 0.5% PBS, 20 mM N-ethylmaleimide, 1× complete protease inhibitors (Roche). Cell lysates were washed 3× with 1× RIP buffer and immunoblotted with antibody to Top1 (Bethyl, cat# 302-590A) and 50 µL Proteins A/G PLUS agarose beads (Santa Cruz, sc-2003). Immunoprecipitations were washed 3× with RIP buffer and immunoblotted with antibody to Top1 (1:1,000), antibody to SUMO1 (1:1,000, Abcam, cat# ab23058), antibody to ubiquitin (1:1,000, Santa Cruz, cat# sc-8017). Experiments were performed in triplicate.

TDP1 overexpression and ATM/TDP1 knockdown analysis. 293T cells were transfected (using FuGene 6) with short-hairpin RNA constructs (Sigma) corresponding to mouse ATM (shATM; 5′-CCGGGCGGCTACACTGAGATGA CGTCATGCTGCTACTGAAAGGTTTG-3′ and 5′-CCGGCC TTTCATTCAACACTGTTCAAGAGTGGTTAAGGTTT TA AAGGTT-3′) or a “scrambled” control (shScrambled). 24 h following transfection, cells underwent puromycin selection (2 µg ml−1) with a media change every 24 h for 10 days. Stable-transfectants were then re-transfected with shScrambled or shATM constructs along with either pFlag vector control, pFlag-TDP1 or an shRNA targeting human TDP1 (shTDP1; 5′-CCGGGCGGCTACACTGAGATGA CGTCATGCTGCTACTGAAAGGTTTG-3′). Following CPT treatment, cells were lysed and underwent Top1 immunoprecipitation to assess levels of Top1 post-translational modification.

Histology. Pregnant female mice underwent transcardial perfusion with 4% buffered paraformaldehyde (wt/vol, PFA) and harvested embryos were cryoprotected in buffered 25% sucrose (wt/vol) solution. Embryos sectioned sagitally at 10 µm using an HSM500M cryostat (Microman). Immunostaining was carried out with the antibodies listed below. For colorimetric visualization of positive signals, sections were incubated with antibodies overnight at room temperature after quenching endogenous peroxidase using 0.6% H2O2 (vol/vol) in methanol. Slides were washed with PBS three times, followed by incubation with biotinylated secondary antibody and avidin–biotin complex (Vectastain Elite kit, Vector Labs). Antibodies were used after citrate buffer–based antigen retrieval. Immunoreactive signals were visualized with the VIP substrate kit (Vector Labs) using the manufacturer’s protocol. Sections were counterstained with 0.1% methyl green (wt/vol), dehydrated, and mounted in DPX (Fluka). For fluorescent detection of immunoreactivity, FITC- or Cy3-conjugated secondary antibodies (Jackson Immunologicals) were used and counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories). For immunohistochemistry and immunocytochemistry, we used antibodies to H2AX (Ser-139, rabbit, 1:200, Cell Signaling, cat# 2577), p33 (clone CM5, rabbit, 1:1,000, Vector Laboratories, cat# VP-P956), PCNA (clone PC10, mouse, 1:500, Santa Cruz, cat# sc-56), TuJ1/β-tubulin III (mouse, 1:1,000, Covance, cat# MMS-435P), Calbindin (mouse, 1:2,000, Clone CB955, Sigma, cat# C9848). Apoptosis was detected without antigen retrieval using antibody to ssDNA (rabbit, 1:300, IBL, cat# 18731). Experiments were performed in triplicate.

For fluorescent labeling of quiescent cortical astrocytes, human fibroblasts or MEFs, cells were grown to confluence on glass coverslips, fixed with 4% PFA in PBS for 10 min and permeabilized for 5 min in 0.5% Triton X-100 PBS. Cells were immunostained with antibodies diluted in PBS/0.3% BSA and incubated in PBS for 10 min and, permeabilized for 5 min in 0.5% Triton X-100/PBS. Cells were incubated with antibodies overnight at room temperature, washed, incubated in secondary antibodies (1:200, Catalog #NA934V, GE Healthcare) and mounted in ProLong Gold Antifade Reagent with DAPI (Invitrogen). For analysis of cells transfected with shATM or scrambled control, cells were fixed and immunostained as above following CPT treatment. For immunostaining, serial sections were collected, washed in 1× PBS, lysed (200 mM NaOH, 2 mM EDTA) and neutralized (100 mM HCl, 60 mM Tris-HCl) on ice and underwent sonication (3 × 10-s bursts, setting 5, 550 sonic dismembrator, Fisher Scientific) in 1× SSN nuclease buffer (5 mM MgCl2, 5 mM CaCl2, 0.5 mM DTT, 0.1 mM EDTA, 20 mM N-ethylmaleimide, 1× complete protease inhibitors (Roche). Cell lysates were incubated with 1,000 units of SSN nuclease (Roche) on ice for 20 min and, centrifuged at 10,000 g for 30 min at 4 °C to remove genomic DNA. 1 mg of protein extract (resulting supernatant) underwent Top1 immunoprecipitation (2 h, 4 °C) in radioimmunoprecipitation buffer (RIP buffer; 0.5% NP-40, 0.25% sodium deoxycholate, 0.05% SDS (wt/vol), 0.5% PBS, 20 mM N-ethylmaleimide, 1× complete protease inhibitors (Roche). Cell lysates were washed 3× with 1× RIP buffer and immunoblotted with antibody to Top1 (Bethyl, cat# 302-590A) and 50 µL Proteins A/G PLUS agarose beads (Santa Cruz, sc-2003). Immunoprecipitations were washed 3× with RIP buffer and immunoblotted with antibody to Top1 (1:1,000), antibody to SUMO1 (1:1,000, Abcam, cat# ab23058) or antibody to ubiquitin (1:1,000, Santa Cruz, cat# sc-8017). Experiments were performed in triplicate.

Statistics. Bar and line graphs represent mean values of all replicates, error bars represent s.e.m. and P values were calculated using Student's t test. For comet assays, a minimum of 100 cells for each treatment were counted and experiments were repeated in triplicate (in vitro comet) or quadruplicate (in vivo comet); exact cell numbers/comets are indicated in their respective figure legends. Mean comet tail moments were calculated and graphed. Experimental samples were assigned randomized identity and analysis was performed blind. For foci quantification experiments, a minimum of 30 cells for each treatment were counted and experiments were repeated in quadruplicate. Mouse genetic inheritance ratios were calculated using standard rules for Mendelian genetic inheritance. Expected and observed genetic ratios were calculated based upon the actual number of live-born mice (indicated by n). No statistical methods were used to pre-determine sample sizes, but are similar to those generally employed in the field. Data distribution was assumed to be normal, but this was not formally tested. A Supplementary Methods Checklist is available.