Deletion of Topoisomerase 1 in excitatory neurons causes genomic instability and early onset neurodegeneration

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Topoisomerase 1 (TOP1) relieves torsional stress in DNA during transcription and facilitates the expression of long (>100 kb) genes, many of which are important for neuronal functions. To evaluate how loss of Top1 affected neurons in vivo, we conditionally deleted (cKO) Top1 in postmitotic excitatory neurons in the mouse cerebral cortex and hippocampus. Top1 cKO neurons develop properly, but then show biased transcriptional downregulation of long genes, signs of DNA damage, neuroinflammation, increased poly(ADP-ribose) polymerase-1 (PARP1) activity, single-cell somatic mutations, and ultimately degeneration. Supplementation of nicotinamide adenine dinucleotide (NAD+) with nicotinamide riboside partially blocked neurodegeneration, and increased the lifespan of Top1 cKO mice by 30%. A reduction of p53 also partially rescued cortical neuron loss. While neurodegeneration was partially rescued, behavioral decline was not prevented. These data indicate that reducing neuronal loss is not sufficient to limit behavioral decline when TOP1 function is disrupted.
normal physiological processes, including oxidative stress, neuronal activation, and gene transcription, can damage DNA in neurons\textsuperscript{1–4}. If not precisely repaired, DNA damage creates permanent lesions in the neuronal genome, referred to as somatic mutations. Recent single-cell sequencing studies report that every neuron in the mammalian brain may contain unique somatic mutations\textsuperscript{5–7}.

DNA mutations are more abundant in individuals with neurodegenerative disorders\textsuperscript{1–12}, and reviewed in ref.\textsuperscript{13}. In fact, one common phenotype across numerous neurodegenerative disorders, including Alzheimer’s disease (AD), Parkinson’s disease (PD), amyotrophic lateral sclerosis, and ataxia-telangiectasia, is an abnormal accumulation of DNA damage in neurons\textsuperscript{13–16}. DNA damage-induced neurodegeneration is frequently associated with hyperactivation of the nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) consuming enzyme PARP1 (poly(ADP-ribose) polymerase 1), which leads to energetic breakdown and cell death\textsuperscript{17–20}. A greater understanding of the genes and the molecular mechanisms that maintain genomic integrity in postmitotic neurons could thus have therapeutic relevance for multiple neurodegenerative diseases.

Decades of research in yeast and mitotic mammalian cells support a role for topoisomerase 1 (TOPI) in gene transcription and maintenance of genomic integrity\textsuperscript{21,22}. Recent studies suggest that transcriptional stress-driven DNA damage is also prevalent in postmitotic neurons and has the potential to disrupt genes that neurons depend on most\textsuperscript{2}. We recently identified TOPI as a key transcriptional regulator in postmitotic neurons\textsuperscript{23,24}. TOPI relieves DNA supercoiling generated during transcription by catalyzing a single-strand DNA cleavage\textsuperscript{22}. During this reaction, TOPI transiently binds to the DNA forming a cleavage complex (TOP1cc). Acute depletion of TOPI or acute treatment with topotecan, a TOPI inhibitor, resulted in the downregulation of long genes (>100 kb) in cultured cortical neurons\textsuperscript{23}. TOPI-dependent and -independent mechanisms were implicated in these transcriptional responses\textsuperscript{24}. Long genes, as a class, are disproportionately associated with neuronal functions\textsuperscript{23,25}, and acute inhibition of TOPI impaired long genes associated with synaptic function in neuronal cultures\textsuperscript{26}. Using a single-cell RNA-sequencing (RNA-seq) approach, we also found that excitatory neurons in lower cortical layers expressed long genes to a greater extent when compared to excitatory neurons in upper cortical layers in embryonic and early postnatal mice\textsuperscript{27}.

Based on these observations, we hypothesized that TOPI might maintain genomic integrity and/or maintain transcriptional output in the central nervous system and that deletion of TOPI would have the greatest impact on lower layer excitatory cortical neurons. Here, we tested this hypothesis by conditionally deleting TOPI in postmitotic excitatory neurons in the mouse cerebral cortex and hippocampus.

**Results**

**Top1 cKO mice show motor deficits and early death.** To study the requirement for TOPI in postmitotic excitatory neurons of the cerebral cortex and hippocampus, we crossed Top1 conditional knockout mice (Top1\textsuperscript{fl/fl})\textsuperscript{24} with the Neurod6\textsuperscript{cre} mouse line\textsuperscript{28}. Neurod6 expression begins at embryonic day 11.5 and is maintained throughout maturation in postmitotic excitatory neurons of the mouse cerebral cortex and hippocampus, except for the dentate gyrus (DG), where Neurod6 is only transiently expressed postnatally\textsuperscript{28}. Both homozygous Top1-knockout mice (Neurod6\textsuperscript{Cre+/-}:Top1\textsuperscript{fl/fl}, referred to as Top1 cKO) and heterozygous (HET) Top1 KO mice (Neurod6\textsuperscript{Cre+/-}:Top1\textsuperscript{fl+/-}, referred to as Top1 cHET) were generated. Neurod6\textsuperscript{Cre+/-}:Top1\textsuperscript{fl+/-} or Top1\textsuperscript{fl+/-} mice were used as a control (wild-type (WT)). To confirm TOPI deletion, we examined TOPI protein levels at postnatal day 0 (P0) through immunostaining. TOPI was ubiquitously expressed in WT cHET and cKO mice at different time points. WT: P0 = n = 10, P7 = n = 41, P15 n = 37; cHET: P0 n = 6, P7 n = 27, P15 n = 20; cKO: P0 n = 6, P7 n = 17, P15 n = 12. *p < 0.05; **p < 0.001. WT vs. cHET p values were not significant in all comparisons. One-way ANOVA with Dunnett’s multiple comparison test for each time point. Kaplan-Meier survival curve. Mantel-Cox test. WT vs. cHET p > 0.05. n = 24 mice per genotype.

**Fig. 1 Deletion of Top1 in cortical and hippocampal neurons impairs motor function and causes premature death.** a TOPI and (inset) NEUN immunostaining of P0 WT and Top1 cKO somatosensory cortex (Ctx) and hippocampus (Hip). Scale bar = 300 μm, inset scale bar = 50 μm. Images are representative of two independent experiments. b Body weight of WT Top1 cHET and cKO mice at different time points. WT: P0 = n = 10, P7 = n = 41, P15 n = 37; cHET: P0 n = 6, P7 n = 27, P15 n = 20; cKO: P0 n = 6, P7 n = 17, P15 n = 12. WT vs. cHET p values were not significant in all comparisons. One-way ANOVA with Dunnett’s multiple comparison test for each time point. c Kaplan-Meier survival curve. WT vs. cHET p > 0.05. n = 24 mice per genotype. d Righting and geotaxis assays performed on P7 (WT = 13, cHET = 13, cKO = 13 mice) and P12 (WT = 10, cHET = 10, cKO = 9 mice). WT vs. cHET p values were not significant (p > 0.05). One-way ANOVA with Dunnett’s multiple comparison test. Dashed lines represent cutoff time. Values are mean and error bars are ±SEM.

The body weights of Top1 cKO mice were comparable to WT mice at birth, but increased to a lesser extent at P7 and P15 (Fig. 1b). Top1 cKO were viable up to the second and third postnatal week (Fig. 1c). Top1 cKO mice also showed a severe motor deficit as assessed by the righting reflex assay at P7 and the geotaxis assay at P12 (Fig. 1d). No differences in body weight, viability, or motor function were observed in Top1 cHET compared to WT mice (Fig. 1b-d). These data indicate that deletion of Top1 in postmitotic excitatory cortical and hippocampal neurons did not overtly affect brain or body development up to birth, but impaired motor function and caused premature death within the first month of life.

**Top1 cKO mice show early-onset neurodegeneration.** To determine whether Top1 deletion affected postnatal brain size, we quantified brain weight in WT, Top1 cHET, and Top1 cKO mice.
and cortical layers (inset). Scale bar

Sections containing the cerebral cortex and hippocampus immunostained
were >0.05 in all comparisons. One-way ANOVA with Dunnett
comparison test. 

decrease in L2 lower cortical layers showed the most prominent effect (~30%
cKO mice relative to WT mice (Fig. 2c, Supplementary
1) and

To determine if the cortex and hippocampus were affected, we
immunostained P15 brain sections for NEUN (a pan-neuronal
marker), CUX1 (an upper layer cortical neuron marker), and
CTIP2 (enriched in lower layer cortical neurons and hippocampal
granule cells) (Fig. 2c). Cortical thickness was reduced by 40% in
Top1 cKO mice relative to WT mice (Fig. 2c, Supplementary
Fig. 2a). While all cortical layers were affected by Top1 deletion, lower
cortical layers showed the most prominent effect (~30% decrease in L2–4, ~70% decrease in L5, and ~40% decrease in L6) (Fig. 2c, Supplementary Fig. 2a). Hippocampal area was also drastically reduced (Supplementary Fig. 2b), with a stronger effect in CA1, CA2, and CA3 hippocampal regions (Fig. 2c). In contrast, the DG was not overly affected, consistent with no loss of TOP1 in this region (Supplementary Fig. 1). Top1 cHET cortical thickness was comparable to WT (Supplementary Fig. 2a).

Given the profound reduction in cortical thickness, we next
sought to understand the possible causes of this phenotype. To
determine if the loss in cortical thickness was due to a deficit in
cortical layering or to neurodegeneration, we compared Top1
cKO and cHET somatosensory cortex to that of WT during the
first 2 weeks of postnatal development (Fig. 3a, b). Cortical
neurogenesis and neuron migration begin at E11.5, when
Neurod3 is first expressed28, and terminate perinatally around
E17.5 (reviewed in ref. 29). At P0, the overall thickness and layer
organization of Top1 cKO and cHET cortex were comparable to
that of WT, indicating that neurogenesis and neuron migration
were not affected. However, Top1 cKO cortical thickness was affected at later timepoints. In particular, L5 and L6 were more vulnerable to Top1 loss, showing a significant decrease in thickness compared to WT at P7, and a collapse in thickness by P15. L4–2 were only significantly reduced at the P15
timepoint. Top1 cHET cortical development was comparable to
that of WT mice (Fig. 3a, b). These data suggest that homozygous
loss of Top1 caused rapid, early-onset neurodegeneration that
initially affected lower layer neurons and then extended to upper layers.

CTIP2/Bcl11b is a transcription factor that marks projection
neurons in L5 and L6 and is highly expressed in L5 corticospinal
motor neurons30. To evaluate the extent to which the decrease in
L5 thickness was due to neuronal loss over time, we quantified the
density and cell size of spinal projection neurons (CTIP2high). We
found that CTIP2high neuron density in Top1 cKO was reduced
relative to WT mice by P7, and these neurons were no longer
present by P15 (Fig. 3c, d). While CTIP2high neuron density
did not decline until P7, we observed a decrease in CTIP2high soma
size beginning at P2 (Fig. 3e). These data suggest that deletion of
Top1 affected CTIP2high L5 neurons as early as P2 and contributed to the demise of these neurons by P15. Since HET loss of Top1 did not affect any of the neurons or markers evaluated above, we did not examine Top1 cHET mice in
subsequent experiments.

Top1 cKO mice show neuronal apoptosis and neuroinflammation. We next performed immunostaining for cleaved-caspase 3 (cl-CASP3) and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), markers of intermediate and
late phases of apoptosis, respectively. Both markers were
increased in Top1 cKO cortex at P7, particularly in L5 and L6
where cell loss and layer thinning were most pronounced (Fig. 4a,
b, Supplementary Fig. 3a, b). Another early sign of apoptosis
is cell shrinkage, which was observed in L5 neurons starting at P2
(Fig. 3c, e). The cl-CASP3 signal was localized in the soma and
apical dendrite of L5 neurons (Fig. 4b), suggesting that neurons in
this region were undergoing apoptosis. Altogether, these data
indicate that the cortex develops normally in
Top1 cKO mice up to birth, but then shows signs of neurodegeneration by P2 that
initiates in lower layer excitatory neurons.

Neuroinflammation is seen in a variety of neurodegenerative
diseases (reviewed in ref. 31). To determine if neuron loss in
Top1 cKO mice was associated with astrogliaisis and/or microgliosis, we
immunostained P7 cortex for glial fibrillar acidic protein (GFAP) and ionized calcium-binding adaptor molecule 1 (IBA1), respectively. In contrast to WT mice, intense GFAP and IBA1 immunostaining was detected in Top1 cKO cortex in
expression is increased in many neurodegenerative diseases characterized by microglia activation, including AD, PD, and multiple sclerosis33. PET imaging of WT and Top1 cKO P15 mice injected with the radioisotope-labeled PBR ligand $^{18}$F-PBR1135 indicated a significant increase in the uptake of the ligand in the frontal lobe, cerebral cortex, and hippocampus of Top1 cKO brains relative to WT brains (Supplementary Fig. 4a, b). Increased $^{18}$F-PBR1135 binding was also detected in sections of the cerebral cortex from Top1 cKO mice using autoradiography (Supplementary Fig. 4a).

**Downregulation of long genes in Top1 cKO lower layer neurons.** To evaluate the extent to which Top1 deletion causes downregulation of long genes in vivo, we performed a small-scale single-cell RNA-seq experiment with cortical cells derived from two WT and two Top1 cKO P7 mice. We captured a total of 1,596 cells (683 WT, 913 cKO) and identified a median of ~2,300 transcripts per cell that corresponded to a median of ~1,750 genes per cell. Using unbiased clustering analyses, we detected three main cell clusters, which we annotated as excitatory neurons, inhibitory neurons, and glial cells based on the expression of marker genes (Fig. 5a, Supplementary Data 1). We then investigated transcriptional changes between WT and cKO excitatory neurons. As expected, Top1 expression levels were drastically decreased in excitatory neurons from cKO animals (~1.08 log fold change; adjusted [adj.] p value = $7.95 	imes 10^{-23}$), whereas levels in inhibitory neurons (~0.43 log$_2$ fold change; adj. p value = 0.99) and glial cells were similar between genotypes (~0.33 log$_2$ fold change; adj. p value = 0.37) (Fig. 5b). We identified 132 downregulated genes and only one upregulated gene in excitatory neurons (Supplementary Data 2). Consistent with our previous findings23,24, the average length of the downregulated genes in cKO excitatory neurons was 2.5-fold lower than the average gene length of all genes expressed in cortical neurons (151.4 kb vs. 59.3 kb) (Fig. 5c and Supplementary Data 2). No biased change in long gene expression was seen in the inhibitory neurons or glial cell clusters (Fig. 5c).

In the excitatory neuron cluster, many of the downregulated genes were involved in axon extension and morphology (Celi2, Rtn4, Hspa5, Gpmn6a, Zbbl8b18, Ephb4, Gap43, Hspa5, Nef1, Ank2, Nefn, and Gpmn6b) and in the induction of postsynaptic currents (Epha4, Snap25, Syt11, Nrxn1, Nrxn3, Cplx1, Snap91, and SncA) (Supplementary Data 2). These data were consistent with the loss of long projecting neurons and suggested that the function of excitatory circuits in Top1 cKO mice is affected. Genes involved in cholesterol biosynthesis and metabolic pathways (Scd2, Sgce, Hmgcs1, and Hmger) and genes relevant to AD (App, Pp3cb, Atp2a2, Calm2, and SncA) were also downregulated (Supplementary Data 2). Top1 cKO mice present a similar, but more severe, phenotype relative to mice depleted of mitogen-activated protein kinase 1/2 (MAPK1/2) in excitatory postmitotic neurons 36.

To evaluate neuroinflammation non-invasively, we performed in vivo imaging of peripheral benzodiazepine receptor (PBR) expression by positron emission tomography (PET) (Supplementary Fig. 4a, b). PBR is a mitochondrial protein found in the brain (mainly glial cells) and peripheral tissues32. At baseline, the brain has lower levels of PBR compared to peripheral tissues33. PBR

L5 and L6 at P7 (Fig. 4c, d), suggesting extensive neuroinflammation in Top1 cKO mice. To evaluate neuroinflammation non-invasively, we performed in vivo imaging of peripheral benzodiazepine receptor (PBR) expression by positron emission tomography (PET) (Supplementary Fig. 4a, b). PBR is a mitochondrial protein found in the brain (mainly glial cells) and peripheral tissues32. At baseline, the brain has lower levels of PBR compared to peripheral tissues33. PBR

WT cHET

CTIP2

high neurons in

0.0001. WT vs. cKO

p

(mainly glial cells) and peripheral tissues32. At baseline, the brain tary Fig. 4a, b). PBR is a mitochondrial protein found in the brain

expression by positron emission tomography (PET) (Supplemen-

in vivo imaging of peripheral benzodiazepine receptor (PBR)
fold change; −0.75; adj. p value <1.12 × 10⁻⁶ (Supplementary Data 2). To restrict our analysis to Neurod6⁺ excitatory neurons, we co-hybridized the sections with a probe to Neurod6 and used DAPI (4′,6-diamidino-2-phenylindole) to label all cells (Fig. 5d). We then used CellProfiler to quantify the number of Ptprd transcripts in Neurod6⁺ neurons (Fig. 5d–e; total number of Neurod6⁺ neurons identified: L2–4 WT 16,090, L2–4 cKO 18,401, L5 WT 13,929, L5 cKO 7,799, L6 WT 8,727, and L6 cKO 5,718). We observed a small decrease in Neurod6 transcripts in L5 cKO neurons compared to WT, consistent with Neurod6 being one of the downregulated genes in Top1 cKO mice, and no change in L2–4 and L6 neurons (Supplementary Fig. 5a). The percentage of Neurod6⁺ neurons in L2–4 was similar between WT and cKO cortical sections, while in L5 and L6 the percentage of Neurod6⁺ neurons was lower in cKO relative to WT (Supplementary Fig. 5b), consistent with loss of lower layer excitatory neurons at P7. While the number of Ptprd transcripts/Neurod6⁺ neuron was comparable in upper layers between WT and Top1 cKO mice, the number of Ptprd transcripts/Neurod6⁺ neuron was significantly reduced in L5 and L6 neurons of Top1 cKO mice (Fig. 5d–f), demonstrating reduced expression of this extremely long gene in lower, but not upper, layer neurons at P7.

Elevated mutation burden in Top1 cKO neurons. TOP1 inhibition or deletion in mitotic cells induces DNA damage and genomic instability37–40. To evaluate the extent to which Top1 deletion causes DNA damage in postmitotic neurons, we immunostained P0, P2, and P7 cortex for γH2AX, a marker of double-strand DNA (dsDNA) breaks. At P0 and P2 timepoints, levels of γH2AX were similar between WT and Top1 cKO mice (Supplementary Fig. 6a). At P7, roughly one-third of neurons presented γH2AX foci in Top1 cKO cortex. In contrast, only 3% of neurons showed γH2AX staining in P7 WT cortex (32.6 ± 5.42% neurons with γH2AX foci in Top1 cKO vs. 3.2 ± 2.6% neurons with γH2AX foci in WT; p value = 0.008, two-sided Student’s t test; data are the mean of percentages of neurons with γH2AX foci; n = 3 mice per genotype) (Fig. 6a). γH2AX accumulation in Top1 cKO mice extended to all cortical layers, although it was more pronounced in L6 (Fig. 6a–c, Supplementary Fig. 6b). Increased DNA damage in Top1 cKO cortex was further confirmed by immunostaining with phosphorylated p53-binding protein 1 (Supplementary Fig. 6c, d), another marker of dsDNA breaks11.

Extensive DNA damage, if not properly repaired, can increase somatic mutation burden. To evaluate copy number variations (CNVs) at the single-cell level, we isolated a small number of neurons (n = 39 WT; n = 43 cKO) by fluorescence-activated cell sorting (FACS) from P7 cerebral cortex of one WT and one Top1 cKO mouse (Supplementary Fig. 10). We identified an increase in the number of cKO neurons with CNVs compared to WT neurons (4/39 in WT; 14/43 in cKO) (Supplementary Fig. 6e). We repeated the experiment using a droplet-based whole-genome amplification (WGA) approach on larger groups of neurons from a different pair of mice42. A two-fold increase in CNV prevalence...
was detected in cKO neurons (24/123 in WT; 60/169 in cKO) (Fig. 6d, Supplementary Data 3), further supporting the previous results. These data suggest that TOP1 plays a key role in maintaining genomic integrity in postmitotic neurons.

**NAD\(^+\) supplementation reduces neuronal death in Top1 cKO mice.** PARP1 catalyzes the addition of poly-ADP ribose (PAR) to multiple substrates and consumes NAD\(^+\) in the process\(^{18-20}\). In DNA damage-induced neurodegenerative diseases, PARP1 is hyperactive and leads to increased PAR levels, energetic breakdown, and cell death\(^{17-20}\). Given the extensive DNA damage observed in neurons from Top1 cKO mice, we next evaluated the extent to which PARP1 levels and activity were changed via western blot analysis for PARP1 and its post-translational modification PAR, in WT and Top1 cKO P7 cortical lysates. We found an increase in both PARP1 and PAR levels in Top1 cKO cortical lysates relative to WT lysates (Fig. 7a, b, Supplementary Fig. 11), suggesting that deletion of Top1 causes an increase in PARP1 activity.

NAD\(^+\) supplementation was found to increase neuronal survival in models of neurodegeneration and to restore NAD\(^+\) levels in response to PARP1 hyperactivation\(^{18,43}\). Therefore, we hypothesized that increasing NAD\(^+\) levels may limit neurodegeneration in Top1 cKO mice. We first evaluated the extent to which two precursors of NAD\(^+\) synthesis, nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR), elevated NAD\(^+\) in the neonatal mouse brain. We found that only NR elevated NAD\(^+\) levels in the brain of P3 mice when injected intraperitoneally (i.p.) (Supplementary Fig. 7). To measure the amounts of NAD\(^+\) in WT and Top1 cKO mice and test whether daily injections of NR could maintain high levels of NAD\(^+\) over time, we injected i.p. WT and Top1 cKO mice with saline (control) or NR (200 mg/kg per day) beginning on P1 and quantified NAD\(^+\) levels in cortical extracts at P7 and P15.
Neurons NAD

Fig. 3a, b). Untreated WT mice showed an ~44% increase in breakdown and apoptosis at this age (Fig. 4a, b, Supplementary WT controls at P7 (94.8 ± 2.4 in P7 WT saline vs. 94.7 ± 6.2 in P7 WT Top1 cKO saline) (Table 1), consistent with the fact that only a small number of cortical neurons were undergoing energetic breakdown and apoptosis at this age (Fig. 4a, b, Supplementary Fig. 3a, b). Untreated WT mice showed an ~44% increase in NAD+ levels in the cortex from P7 to P15 (94.8 ± 2.4 in P7 WT saline vs. 136.7 ± 10.9 in P15 WT saline). In contrast, NAD+ levels did not increase from P7 to P15 in Top1 cKO cortical samples (94.7 ± 6.2 in P7 Top1 cKO saline vs. 92.8 ± 6.6 in P15 Top1 cKO saline) and showed an ~32% decrease relative to WT controls at P15 (136.7 ± 10.9 in P15 WT saline vs. 92.8 ± 6.6 in P15 Top1 cKO saline). Treatment with NR for 15 days restored NAD+ levels (136.7 ± 10.9 in P15 WT saline vs. 155.5 ± 22.9 in P15 Top1 cKO NR) (Table 1). These data indicated that Top1 cKO mice developed a deficit in cortical levels of NAD+ by P15 and suggested that cortical NAD+ levels could be restored by supplementation with NR.

We next evaluated the extent to which long-term NAD+ supplementation rescued motor defects and delayed premature death of Top1 cKO mice. NR treatment did not rescue the motor deficits of Top1 cKO mice (Fig. 8a), but NR treatment did increase the median survival of Top1 cKO mice by 5 days (18 days in saline injected vs. 23 days in NR treated), which corresponds to a 30% increase in lifespan (Fig. 8b).

To determine if NR treatment also reduced neuroinflammation and cell death in Top1 cKO mice, we immunostained NR-treated Top1 cKO P7 brain sections for cl-CASP3, TUNEL, GFAP, and IBA1. We found that cl-CASP3 activation and TUNEL staining were greatly reduced throughout the somatosensory cortex and hippocampus in NR-treated mice (Fig. 8c). Neuroinflammation was also strongly reduced, as evidenced by reduced IBA1 and GFAP staining (Fig. 8c). NR treatment also modestly increased hippocampal area, increased overall cortical thickness, and increased lower layer thickness in cKO mice at P15 (Fig. 8d, e). Moreover, we observed a 2- to 3-fold increase in the number of CTP2+ neurons in lower cortical layers and in the hippocampus of Top1 cKO mice treated with NR (Fig. 8d, e). These data suggest that neuron loss is only partially dependent on NAD+ depletion.

p53 loss partially rescues neurodegeneration in Top1 cKO mice. We asked whether partial or complete deletion of p53 could rescue neuron death in Top1 cKO mice. To answer this question, we crossed Neurod6Cre+/fl;Top1fl/fl mice with p53 KO mice to generate WT and Top1 cKO mice with p53 WT, HET, or KO alleles. Mice were assayed for motor tests at P7 and P12, brains were collected at P15 and cortical thickness was quantified by staining with CUX1 and CTIP2. p53 deletion failed to rescue motor deficits (Fig. 9a) and did not extend lifespan in a small cohort (n = 2) of Top1 cKO:p53 KO animals, which died in the third postnatal week. However, both homozygous and HET loss of p53 led to an ~1.5-fold increase in cortical thickness and an ~2.5-fold increase in hippocampal area in Top1 cKO mice (Fig. 9b, c), bringing these areas to ~60% of Top1 WT size. The rescue observed in Top1 cKO:p53 KO and Top1 cKO:p53 HET cortex was caused by an ~3-fold increase in L5 thickness and an

Fig. 6 Elevated DNA damage and more abundant CNVs in Top1 cKO mice. a P7 somatosensory cortex immunostained for γH2AX. Dashed lines delineate the cortical layering identified by NEUN (not shown). Scale bar = 300 μm. b Insets of P7 Top1 cKO cortical layers immunostained with NEUN and γH2AX, showing γH2AX foci. Scale bar = 20 μm. Images are representative of three independent experiments for a total of three mice per genotype. c Percentages of neurons with γH2AX foci in different layers of WT and cKO P7 cortex. Values are expressed as mean and error bars are ±SEM. Two-sided Student's t test. n = 3 mice per genotype. d Graph showing the overlap of all CNVs identified in WT and cKO neurons at the different genomic locations. Y-axis indicates the number of CNVs identified at the correspondent genomic location; above zero are duplications (dup), below zero are deletions (del). (Inset) CNV coverage, number of CNVs identified, and percentages of neurons with CNVs in WT and cKO mice.
Table 1 Quantification of NAD+ cortical levels in WT and cKO mice treated with saline or NR.

|               | Mean  | SEM  | P7 WT sal | P7 WT NR | P7 cKO sal | P7 cKO NR | P15 WT saline | P15 cKO saline | P15 WT NR |
|---------------|-------|------|-----------|----------|------------|------------|--------------|---------------|----------|
| P7 WT sal     | 94.8  | 2.4  | —         | —        | 159.4      | 146.3      | 136.7         | 136.7         | 136.7    |
| P7 cKO sal    | 94.7  | 6.2  | —         | —        | 0.336a     | —          | 92.8          | —             | 92.8     |
| P7 WT NR      | 159.4 | 7.2  | —         | —        | 0.007      | 0.007      | 0.007         | 0.086a        | —        |
| P7 cKO NR     | 146.3 | 3.8  | —         | —        | 0.108a     | —          | 92.8          | —             | 92.8     |
| P15 WT sal    | 136.7 | 10.9 | —         | —        | 0.007      | 0.007      | 0.007         | 0.086a        | 0.268a   |
| P15 cKO sal   | 92.8  | 6.6  | 0.314a    | 0.134a   | 0.314a     | 0.314a     | 0.007         | 0.007         | 0.008    |
| P15 WT NR     | 240.7 | 25.4 | —         | —        | 0.007      | 0.007      | 0.007         | 0.007         | —        |
| P15 cKO NR    | 155.5 | 22.9 | —         | —        | 0.308a     | 0.184a     | 0.007         | 0.007         | 0.008    |

Mean plus/minus SEM of NAD+ levels (pmol/mg) in cortical lysates of P7 and P15 WT and cKO mice treated with saline (sal) or NR beginning at P1. Adjusted p values for each comparison are shown (∗= non-significant p > 0.05; **adj. p < 0.001; ***adj. p < 0.0001). Three-way ANOVA with false discovery rate correction (two-stage step-up method of Benjamini, Krieger, and Yekutieli, Q = 0.05). For P7; n = 6 WT;saline mice, n = 5 WT;NR mice, n = 6 cKO;saline mice, n = 7 cKO;NR mice. For P15; n = 3 WT;saline mice, n = 4 WT;NR mice, n = 4 cKO;saline mice, n = 3 cKO;NR mice.

Fig. 8 NAD+ supplementation with nicotinamide riboside partially blocks neurodegeneration and increases lifespan of Top1 cKO mice. a P7 righting assay (left) and P12 geotaxis (right) performed on WT and Top1 cKO mice treated with saline or NR 200 mg/kg per day from P1 onward. For P7; n = 12 WT;saline mice, n = 12 WT;NR mice, n = 8 cKO;saline mice, n = 12 cKO;NR mice. For P12; n = 14 WT;saline mice, n = 14 WT;NR mice, n = 5 cKO;saline mice, n = 14 cKO;NR mice. Dashed lines represent cutoff time. ∗∗∗p < 0.05. One-way ANOVA with Dunnett’s multiple comparison test. b Kaplan-Meier survival curve of Top1 cKO mice treated with saline or 200 mg/kg per day NR from P1 onwards. Mantel-Cox test. WT = 11, cKO = 10 mice. c Top1 cKO mice were treated with saline or 200 mg/kg per day NR for 6 days starting from P1 and then brains were collected. Sections containing cortex (Ctx) and hippocampus (Hip) were immunostained for cl-CASP3, TUNEL, GFAP, and IBA1 (left panel), scale bar = 500 μm. The number of cells positive for each marker was quantified (right panel). ∗∗∗p < 0.05. Two-sided Student’s t test. For cl-CASP3 quantification n = 4 mice per condition, for TUNEL, GFAP, and IBA1 quantifications n = 4 cKO;saline mice, n = 3 cKO;NR mice. d CTIP2 staining of cerebral cortex and hippocampus of P15 Top1 cKO mice treated with saline or 200 mg/kg per day NR for 14 days. Scale bar = 300 μm. Dashed lines delineate cortical layering and hippocampus based on NEUN, CUX1, and CTIP2 staining. The solid line indicates the area used to quantify cortical thickness. Images are representative of at least three independent experiments for a total of three mice per genotype. e Hippocampal area and number of CTIP2+ cells per section of cortex and hippocampus. ∗∗∗p < 0.05. Two-sided Student’s t test. For thickness and area quantifications: n = 3 WT mice, n = 6 cKO;saline mice, n = 5 cKO;NR mice. For L5–6 CTIP2+ neurons quantification: n = 3 WT mice, n = 6 cKO;saline mice, n = 4 cKO;NR mice. For CA1 CTIP2+ neurons quantification: n = 3 WT mice, n = 3 cKO;saline mice, n = 4 cKO;NR mice. Two sections per mouse were used for all quantifications. Values are mean and error bars are +SEM, n.s. not significant.

We observed a complete rescue in the number of CTIP2+ neurons of the CA1 hippocampal region in Top1 cKO:p53 KO mice (Fig. 9b, c). To test whether the increase in cortical and hippocampal size in Top1 cKO:p53 KO mice was due to an increase in the number of TOP1+ neurons, we performed TOP1 and NEUN

~2-fold increase in L6 thickness, bringing L5 from ~18% to ~52% of Top1 WT L5 size and L6 from ~28% to 55% of Top1 WT L6 size (Fig. 9b, c). Both homozygous and HET loss of p53 in Top1 cKO mice led to an increase in the number of CTIP2+ neurons in lower cortical layers and the CA1 hippocampal region.
immunostaining of the cortex and hippocampus. While very few TOP1−/−NEUN+ neurons were observed in the lower cortical layers and CA1 hippocampal region of Top1 cKO:p53 WT mice, these areas were almost completely composed of TOP1−/−NEUN+ neurons in Top1 cKO:p53 KO (Supplementary Fig. 8). These data strongly suggest that neurodegeneration caused by Top1 loss is partially p53 dependent.

**Discussion**

Our data support a model whereby loss of TOP1 causes transcriptional stress in postmitotic neurons that leads to high levels of DNA damage, subsequent activation of DNA damage-induced cell death pathways and neurodegeneration (Supplementary Fig. 9). Interestingly, aberrant TOP1 activity has been shown to cause a similar phenotype in neurodegenerative genome instability syndromes. In these pathologies, the formation of R-loops, and genomic instability in mitotic cells has also been linked to transcriptional stress, accumulation of RNA:DNA hybrids (R-loops), and the formation of dsDNA breaks in postmitotic cells. TOP1 deficiency has also been linked to transcriptional stress, accumulation of R-loops, and genomic instability in mitotic cells. DNA damage caused by TOP1 depletion was attributed to the collision between transcriptional machinery and the DNA replication fork. Our data suggest that TOP1 loss induces DNA damage accumulation independent of DNA...
recombination in postmitotic neurons, possibly because of increased DNA torsional stress and R-loop formation during transcription. Further investigation will be required to understand the extent to which R-loops accumulate in Top1 cKO neurons. Altogether, these data indicate that Top1 maintains genomic integrity in the central nervous system.

Another interesting relationship between Top1 deletion and Top1cc expression is the downstream activation of multiple DNA damage-induced pathways, including p53 activation15 and increased PARP1 activity17. Consistent with other forms of neurodegeneration that feature DNA damage, Top1 cKO mice show increased PARP1 activity, reduced NAD+ levels, neuroinflammation, and neuronal death18–20. Our study suggests that Top1 cKO mice could be used to further understand the pathways involved in DNA damage-induced neurodegeneration in vivo, but on a greatly accelerated time scale.

We also found that treatment with NR restored NAD+ levels in the brain of Top1 cKO mice. This treatment increased lifespan by 30%, significantly reduced neuroinflammation, and reduced neuron death, including CTIP2+ neurons in the lower cortical layers and hippocampus. These results corroborate other studies indicating that NAD+ supplementation can extend lifespan, decrease neuroinflammation, and delay neuron death in neurodegeneration models, including in AD and DNA-repair-deficient mouse model.18,50–52 However, given that the cortical and hippocampal reduction were only modestly rescued by NR treatment, NAD+-independent pathways must also contribute to neurodegeneration in Top1 cKO mice.

In support of this possibility, genetic ablation of p53 led to a greater rescue in cortical thickness and hippocampal area compared to NR treatment and led to a near complete rescue in the number of CTIP2+ projecting neurons. The tumor suppressor p53 is the main driver of DNA damage-induced death in neurons and is a common downstream effector of multiple cellular stress sensors, including PARP120,53–55 However, neither p53 deletion nor NAD+ supplementation rescued the motor phenotypes and premature death observed in Top1 cKO mice. These data suggest that loss of Top1 could have additional effects that compromise neuron function, such as elevating somatic mutation burden (Fig. 6d), transcriptional downregulation of long synaptic genes23,24,26 (Fig. 5c–f), which attenuates synaptic activity24, impaired RNA splicing,56 and/or impaired ribosomal RNA biosynthesis.49,57 Thus, transcriptional decline and somatic mutations could incapacitate neurons well before cell loss is apparent.

Selective vulnerability of different neuronal populations to cell death has been described in many neurodegenerative diseases and it has often been attributed to intrinsic properties of the affected neurons.58 Our data indicate that among cortical neurons, lower layer cortical neurons are particularly sensitive to Top1 loss. Using single-cell RNA-seq and single-molecule in situ hybridization, we found that Top1 loss strongly impaired expression of long genes, with a bias for lower layer neurons. Many long genes encode proteins that play key roles in synapse formation and function. Downregulation of these genes is likely to compromise neuron physiology, as we previously found26 and health independent of DNA damage.

Alternatively, lower layer neurons could be more vulnerable because of their earlier birthdate and presumably earlier expression of Neurod6cre. Cortical layers are produced in an inside-out order, where lower layers are produced earlier than upper layers.29 Therefore, lower layer neurons have more time to accumulate transcriptional stress, DNA damage, and somatic mutations that affect neuronal functions and ultimately cause neurodegeneration. In support of this hypothesis, we observed that lower layer cortical neurons present higher levels of DNA damage and downregulation of long genes in Top1 cKO mice. However, counting this possibility, we found that L5 neurons are more vulnerable to Top1 deletion than L6 neurons, yet L5 neurons are born after L6 neurons.

The unique properties of L5 motor neurons, such as large soma size and the ability to establish extremely long distance axonal projections (requiring adhesion molecules that are encoded by long genes), are known to be more vulnerable to cell death.36,59 Projection neurons that maintain a large soma, lengthy projections, and higher transcriptional output of long genes, likely consume more energy than local projection neurons. L5 upper motor neurons are thus likely to be more sensitive to NAD+ depletion and energetic breakdown than other neuronal populations. Interestingly, ALS and other motor neuron neurodegenerative diseases have been associated to defects in R-loop resolution60, providing another possible explanation for why this neuronal population is more sensitive to Top1 loss. Ultimately, future studies will be needed to characterize the effect of Top1 deletion in other neuronal subtypes, including inhibitory neurons and neurons from other brain regions.

Methods
Mice. All animal procedures were approved by the University of North Carolina at Chapel Hill Animal Care and Use Committee. Mice were housed at temperatures of 18–23 °C with 40–60% humidity with food and water provided ad libitum and maintained on a 12-h light/dark cycle. Top1fl/fl conditional mice were previously described.14 Neurod6cre mice16 and p53+/− mice48 were obtained from Jackson Laboratory. Genomic DNA extracted from tail or ear samples was utilized for mouse genotyping by PCR using standard techniques. Primers for gene amplification are as follows (listed 5’–3’): Top1 geno2 GGACCGGGAAAGTCTAAGC and Top1 geno3 GGAGCCGGGAAAAAGTCTAAGC amplifying Top1 WT and KO and flxed alleles, CreF GTAGGACATGTCCAGGATGCC and CreR CTCTCA TCAGTACGTGAGAT amplifying the Cre allele; p53 WT-F CCCGAGTATCTGGAAGACAG and p53 WT-R ATAGGTGGCGGCTTCACTG amplifying p53 WT allele; p53 ex7 TATATCTACGAGCGGCGCT and p53 Neo TTCCTCGTGGTACCGGATAC amplifying p53 KO allele.

Motor assays. For therighting assay, P7 pups were placed on their backs on a sheet of paper and held in position for 3 s. Pups were then released and the total time needed to flip on their feet was recorded. For the negative geotaxis assay, P12 pups were held for 5 s on a 45° incline with the head pointing downward. Pups were then released and the total amount of time needed to face upward was recorded. For both assays, a cutoff maximum of 60 s was given for every trial. Pups were tested three times with a break of 30 s between each trial. The times recorded for the three trials were then averaged. Top1 cKO and cHET mice were compared with WT mice as littermates or age-matched males. Males and females mice were used for motor assessments. GraphPad Prism (version 7.00) was used for statistical analysis.

Tissue collection and immunostaining. At the indicated time points, mice were anesthetized and perfused first with phosphate-buffered saline (PBS) and then with 4% paraformaldehyde (PFA). Brains were dissected, post fixed in 4% PFA overnight at 4 °C, incubated in 30% sucrose at 4 °C, and embedded in M-1 embedding matrix (Thermo Scientific, 13113). Brains were cut on a cryostat and free-floating sections were collected and stored in a freezing solution consisting of PBS, ethylene glycol, and glycerol. Sections were rinsed in PBS, treated with citrate buffer for 30 min at 95 °C, rinsed again in PBS followed by rinses with Tris-buffered saline containing Triton-X-100 (TBS/TX, 0.05 M Tris, 2.7% sodium chloride, 0.3% Triton X-100, pH 7.6). Sections were blocked in 10% normal donkey serum in TBS/TX and then incubated in primary antibodies. After incubation in primary antibodies, sections were rinsed in TBS/TX and then with Tris-buffered saline containing Triton-X-100 (TBS/TX, 0.05 M Tris, 2.7% sodium chloride, 0.3% Triton X-100, pH 7.6). Sections were blocked in 10% normal donkey serum in TBS/TX (NDS/TBS/TX) for 1 h and then incubated overnight at room temperature in primary antibody cocktails diluted in NDS/TBS/TX. Primary antibodies used were rabbit anti-TOP1 (1:300; GeneTex, GXT63013, EPR5375), guinea-pig anti-NEUN (1:400; EMD-Millpore, ABN9029), rabbit anti-CUX1 (1:200; Santa Cruz Bio-technology, sc-13024), rat anti-CTIP2 (1:200; Abcam, ab18465), rabbit anti-cleaved-CASP3 (1:100; Cell Signaling Technology, 9664), rabbit anti-IBA1 (1:400; Wako, 019-19741), goat anti-ATF4 (1:750; Abcam, ab53554), rabbit anti-phospho-Histone H2A.X (1:50; Cell Signaling Technology, 2577), and phospho-388P1 (Ser1778) (1:50; Cell Signaling Technology, 2675).

After incubation in primary antibodies, sections were rinsed in TBS/TX and blocked with NDS/TBS/TX for 30 min. All secondary antibodies were diluted 1:200 in NDS/TBS/TX and applied for 1 h. The following secondary antibodies were used: donkey anti-rabbit Alexa 488 or Alexa 568 (Thermo Fisher Scientific, A21206 and A10042, respectively), donkey anti-goat IgG-Cy3 (Jackson ImmunoResearch Laboratories, 705-163-003), donkey anti-rat IgG-Cy3 (Jackson ImmunoResearch Laboratories, 712-165-153), and donkey anti-guinea-pig IgG Alexa 647 (Jackson ImmunoResearch Laboratories, 706-605-148). DAPI (1:4000; Fisher Scientific, A20438).
PET imaging with 18F-PBR111. PET imaging probe of PBR ligand, 18F-PBR111, was produced at the UNC BRIC Cyclotron and Radiochemistry Core facility according to a well-established radiosynthesis method. Radiochemical purity was >95% confirmed from the HPLC analysis. PET/MRI imaging was conducted in the UNC Small Animal Imaging facility using a small animal PET/MRI scanner (Siemens, ADAC, Inc., Spain) with a spatial resolution of 1.2 mm in the center field of view. Animals were anesthetized with isoflurane inhalation (1.5-2.5% isoflurane mixed with oxygen). A catheter was placed in the abdominal region for i.p. injection of radiotracer. WT and Top1 cKO P15 mice were placed on the imaging bed. 18F-PBR111 (~5 MBq in 50 μL) was administered to each animal through an i.p. catheter, followed by a 60-min dynamic scan. The computed tomography (CT) scan was conducted before the PET scan for attenuation correction and structure reference. Dynamic data were binned into six frames with 10 min/frame. Images were reconstructed using the 2D-OSEM (two-dimensional-ordered subset expectation maximization) algorithm with scatter, attenuation, and decay correction. Standardized uptake value was calculated by normalizing the signal to the injected activity and animal body weight. After PET/CT, brains were collected and autoradiography was conducted using a digital phosphor imaging system (Cyclone Plus storage phosphor system, PerkinElmer Inc.). GraphPad Prism (version 7.00) was used for statistical analysis.

Drop-seq procedure. P7 mice were decapitated and cortices were isolated, cut into pieces with a sterile razor blade, and incubated in papain diluted in standard artificial cerebrospinal fluid (aCSF) media (NaCl 124 mM, KCl 2.5 mM, NaH2PO4 1.2 mM, NaHCO3 24 mM, HEPES 5 mM, glucose 13 mM, MgSO4·7H2O 2 mM, CaCl2·2H2O 2 mM, pH 7.3) supplemented with (2R)-aminophosphonic acid (APV) and tetrodotoxin (TTX) at 37 °C for 30 min. Cortical pieces were washed and gently dissociated in aCSF media with APV and TTX. Cortical cells were then filtered, captured, and processed into single-cell transcriptomes using Drop-seq. Complementary DNA libraries were sequenced on two lanes of a HiSeq2500 on Rapid Run mode. FASTQ files were processed using the Drop-seq Toolkit 1.2 where possible and aligned to the mouse genome (mm10) using STAR. Barcoded bead synthesis errors were corrected using a publicly available command line script.

Single-cell RNA-seq bioinformatics. Cells expressing fewer than 300 different genes or whose mitochondrial transcripts exceeded 10% were excluded. Genes expressed in fewer than three discrete brain regions were removed. To be considered optimized near neighbors (>67 nearest neighbors), CellRanger Single-cell bioinformatics were correctly classified as "hotspots" for positive-CNVs, and any CNVs located entirely within the boundaries of a hotspot were excluded. To further safeguard against
false positives, CNVs under 5 Mb in length were also excluded. Bin level copy number estimates for all single cells were downloaded from Ginkgo and plotted with filtered CNV results in R (version 3.4.1) using ggplot2 (version 2.2.1) to generate CNV profiles.

Western blotting. P7 cortices were dissected from both Top1 cKO and WT control mice and lysed in RIPA buffer (0.05 M Tris-HCl, pH 8, 0.15 M NaCl, 0.5% deoxycholic acid, 1% NP-40, and 0.1% sodium dodecyl sulfate (SDS)), Halt Protease Inhibitor Cocktail (Thermo Fisher, 87785), and Halt Phosphatase Inhibitor Cocktail (Thermo Fisher, 78420). Lysates were sonicated and cleared by centrifugation. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Fisher, 23227). Equal amounts of protein were denaturing in reduced sample buffer, separated by SDS-PAGE (polyacrylamide gel electrophoresis) gels, and blotted to nitrocellulose membranes (Bio-Rad). Blots were blocked with Odyssey Blocking Buffer (Fisher, 927-40000) for 1 h at room temperature, and then incubated overnight at 4 °C with primary antibodies. The primary antibodies used were rabbit anti-PARP1 (1:1000, Cell Signaling, 9332) rabbit anti-PAR-pADPR (1:1000, R&D Systems, 4336-APC-050), and mouse anti-GAPDH (1:1000, Thermo Fisher, MA5-15738). After washing with TBS containing 0.5% Tween 20 (TBS-T), membranes were incubated with goat anti-mouse IRDye 680 (1:10,000 Licor Odyssey, 925-68070) and IRDye 800CW donkey anti-rabbit (1:10,000 Licor Odyssey, 926-32213) diluted in Odyssey Blocking Buffer 1 h at room temperature. Blots were washed with TBS-T and detection was performed with the Odyssey CLx Imaging System (LI-COR) using the Image Studio Software (Odyssey).

NAD+ quantifications. Cortices were dissected from WT and Top1 cKO mice and flash-frozen in pieces ranging from 15 to 30 mg in weight. Cortical samples were collected 1 h after the last i.p. injection with saline, NR, or NMN. All cortical samples were thawed and processes for NAD+ quantification at the same time using EnzyChrom NAD+/NADH Assay Kit (BioAssay Systems, E2ND-100) following the manufacturer’s instructions. NAD+ quantification was collected using Synergy 2 microplate reader (Biotek) and using the Gen5 (version 2.0) software. The resulting NAD+ concentration was multiplied to the final volume of reagents for each sample and divided by the total amount of starting tissue to calculate NAD+ pmol/mg of tissue. GraphPad Prism (version 7.00) was used for statistical analysis.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Single-cell genomic data are available from the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/PRJA548496). Single-cell RNA-sequencing data are available from the NCBI Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146672). All source data are provided in a Source Data file.

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Author contributions
M.J.Z., G.F., and A.M.M. designed the study, G.F. performed all mouse experiments, single-molecule in situ hybridization, image quantification, and data analysis, B.T.-B. performed immunohistochemistry, J.K.N performed single-cell RNA-sequencing and analysis, H.M. provided assistance with experiments, H.Y. and Z.L. performed PET imaging and [18F]PBR111 synthesis, W.D.C. and M.J.M. performed CNV data acquisition, W.D.C., M.J.M., and J.M.S. performed CNV analysis, G.F. and M.J.Z. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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