Neurogenesis requires TopBP1 to prevent catastrophic replicative DNA damage in early progenitors

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The rapid proliferation of progenitors during neurogenesis requires a stringent genomic maintenance program to ensure transmission of genetic fidelity. However, the essential factors that govern neural progenitor genome integrity are unknown. Here, we report that conditional inactivation of mouse TopBP1, a protein linked to DNA replication, and a key activator of the DNA damage response kinase ATR (ataxia telangiectasia and rad3-related) is critical for maintenance of early-born neural progenitors. During cortical development TopBP1 prevented replication-associated DNA damage in Emx1-progenitors which otherwise resulted in profound tissue ablation. Notably, disrupted neurogenesis in TopBP1-depleted tissues was substantially rescued by inactivation of p53 but not of ATM. Our data establish that TopBP1 is essential for preventing replication-associated DNA strand breaks, but is not essential per se for DNA replication. Thus, TopBP1 is crucial for maintaining genome integrity in the early progenitors that drive neurogenesis.

Maintaining genome stability is critical for mammalian development and is achieved by the functional integration of DNA replication and DNA damage signaling. This is particularly important during neural development, where defective DNA damage signaling can result in a wide range of human diseases that are characterized by neurodegeneration, neurodevelopmental disease or brain tumors. However, little is known about specific factors that modulate and maintain stem and progenitor genome integrity in vivo during neurogenesis.

Topoisomerase II binding protein 1 (TopBP1) is important for DNA replication initiation and cell cycle checkpoint activation. Mammalian TopBP1 contains eight BRCA1 C-terminal (BRCT) domains that facilitate protein-protein interactions between key replication and checkpoint factors. TopBP1 has been linked to the prereplication complex because, together with factors such as Treslin (Ticrr) and GEMC1, it is required for loading essential replication initiation components including Cdc45. DNA damage arises during replication via frequent stalled replication forks or when DNA breaks occur by free radicals generated during cellular metabolism. When replication forks stall, TopBP1 is important for initiating a cellular checkpoint. In this capacity, a key function seems to be activation of the ATR kinase to facilitate establishment of a checkpoint by phosphorylation of Chk1. The TopBP1-ATR-Chk1 signaling axis is key to DNA damage resolu-

The rapid proliferation of progenitors during neurogenesis requires a stringent genomic maintenance program to ensure transmission of genetic fidelity. However, the essential factors that govern neural progenitor genome integrity are unknown. Here, we report that conditional inactivation of mouse TopBP1, a protein linked to DNA replication, and a key activator of the DNA damage response kinase ATR (ataxia telangiectasia and rad3-related) is critical for maintenance of early-born neural progenitors. During cortical development TopBP1 prevented replication-associated DNA damage in Emx1-progenitors which otherwise resulted in profound tissue ablation. Notably, disrupted neurogenesis in TopBP1-depleted tissues was substantially rescued by inactivation of p53 but not of ATM. Our data establish that TopBP1 is essential for preventing replication-associated DNA strand breaks, but is not essential per se for DNA replication. Thus, TopBP1 is crucial for maintaining genome integrity in the early progenitors that drive neurogenesis.

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Although in vitro data indicate that TopBP1 is critical for DNA replication, the physiological settings requiring this function are unknown. Here, we show that there is a broad requirement for TopBP1 throughout neurogenesis, where it functions to prevent DNA damage accumulation in progenitor cells. Notably, unlike DNA damage signaling after ATR inactivation, DNA damage signaling after TopBP1 loss is p53 dependent. Finally, our data show that TopBP1 is not essential per se for replication; rather, the critical biological function of this protein during neurogenesis is most likely the activation of cellular checkpoints after replication-associated DNA damage.

RESULTS
Neural inactivation of TopBP1
To understand the biological function of TopBP1, we determined its role in the nervous system, as this tissue is often primarily affected in human syndromes associated with DNA repair deficiency. We generated a Topbp1 allele in which loxP sites flank exons 3–6, which results in an out-of-frame mutation when Cre recombinase is used to drive gene deletion. We used Nestin-cre (Nes-cre) to generate Topbp1Nes-cre mice, in which TopBP1 was inactivated throughout the nervous system. In Topbp1Nes-cre mice, TopBP1 was efficiently inactivated in neural tissues, whereas in non-neural tissues, such as the thymus and spleen, normal protein amounts were present. Topbp1Nes-cre mice were viable and were similar in size to wild-type (WT) littermates at birth, although they became runted within a week and generally did not survive beyond 2 weeks of age.

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Figure 1 Topbp1 deletion in the nervous system. (a) TopBP1 contains eight BRCA1 C-terminal (BRCT) domains. These encompass an amino-terminal Treslin (Tcim) interacting domain, a C-terminal ATR-activating region, a BACH1-binding region and an ATM phosphorylation (ATM-phos) site (*) in BRCT domain 5. Bottom: a conditional Topbp1 allele has loxP sites flanking exons 3 and 6 and generates an out-of-frame mutation after Cre-mediated recombination. (b) TopBP1 protein is absent in P0 Topbp1Nes-cre brain tissues but is present at normal levels in the thymus and spleen, whereas Nbs1 and actin levels are similar in all mutant tissues. Cereb, cerebellum. (c) Topbp1Nes-cre neonates (*) are initially similar to littermate controls but become runted by P4. (d) The Topbp1Nes-cre cerebellum lacks granule neurons, resulting in a disorganized Purkinje cell placement as shown at P0 using calbindin staining (purple) and at P12 using the neuronal differentiation marker β-tubulin III (TuJ1). (e) Quantitative real-time PCR shows that Topbp1 deletion occurs by E12.5 in the cortex but not the liver. Error bars, s.e.m. (f) Western blot analysis of E14.5 Topbp1Nes-cre tissues shows that TopBP1 is deleted in the cortex but not the liver; p53pSer18 expression is also elevated in Topbp1Nes-cre tissue. Nbs1 and Ponceau staining are loading and blotting controls, respectively. (g) Apoptosis (arrows) occurs in proliferating cells in the developing cortex at E13.5. VZ, ventricular zone; H&E, hematoxylin and eosin; P1, propidium iodide.

Topbp1Nes-cre mice were characterized by neurodevelopmental abnormalities (Fig. 1d and Supplementary Fig. 1). We found a general reduction in cellularity in the Topbp1Nes-cre brain and a pronounced defect in cerebellar development. Analysis of the postnatal day (P) 0 cerebellum showed disorganized Purkinje cells and an absence of an external granule layer, which resulted from apoptosis in the rhombic lip during development (Fig. 1d and see below). Elsewhere, less notable effects were observed, although the hippocampal region was also markedly affected (Supplementary Fig. 1). Additionally, developmental gliogenesis was similar in Topbp1Nes-cre tissue to that in controls (Supplementary Fig. 2).

TopBP1 loss affects cortical development

To further determine the function of TopBP1 during neural development, we focused on cortical development, which commences from embryonic day (E) 10.5 (refs. 34,35). We initially determined Topbp1 gene deletion levels and protein loss in the Topbp1Nes-cre developing cortex. Quantitative analysis of isolated neopallium using real-time PCR indicated that Topbp1 deletion was maximal between E12.5 and E13.5 (Fig. 1e), and western blot analysis confirmed that TopBP1 protein was absent at E14.5 (Fig. 1f). Of note, TopBP1 loss led to phosphorylation of p53, consistent with DNA damage–induced activation of p53 (Fig. 1f). TopBP1 deletion resulted in widespread apoptosis by E13.5 as shown by cells positive for terminal deoxynucleotidyl transferase dUTP nick-end-labeling (TUNEL) in the ventricular zone of the developing cortex (Fig. 1g). These proliferating areas also accumulated DNA damage as revealed by immunostaining for phosphorylated histone H2AX (γH2AX), which occurred early and was widespread between E11.5 and E12.5, and preceded apoptosis (Supplementary Fig. 3a,b). Analysis of cell types undergoing apoptosis indicated that cells positive for the radial glial progenitor marker BLBP were largely intact, although they contained γH2AX (data not shown), indicating that TopBP1 loss was associated with apoptosis of newborn progenitors, as abundant overlap between cells positive for TUNEL and for proliferating cell nuclear antigen (PCNA) occurred at E16.5 (Fig. 2a). Moreover, apical progenitors identified by immunostaining for Ser10-phosphorylated histone H3 (H3pSer10) were intact and largely unperturbed in Topbp1Nes-cre tissue at E13.5 (Fig. 2b).

Apoptosis in the Topbp1Nes-cre neocortex resulted in an overall reduction in cortical size (Supplementary Fig. 1b). The cortex comprises six layers derived from inside-out patterning, with early cortical progenitors fated to produce deep-layer neurons (layers VI–V)34,35. To determine how TopBP1 deletion during progenitor proliferation and differentiation affected layering in the Topbp1Nes-cre cortex, we used layer-specific markers. Analysis at P0 and P10 using Tbr1 and Ctip2 showed that the deep layers (layers VI–V) formed to a large extent in the Topbp1Nes-cre cortex (Fig. 2c). In contrast, upper cortical layers, identified by Cux1, Brn2 and Satb2 immunostaining, were reduced in size, suggesting persistent DNA damage in longer-cycling progenitors eventually compromised upper cortical layers (Fig. 2c).

Expansion of Emx1 cortical progenitors requires TopBP1

Our data indicate that despite efficient deletion of Topbp1 during neurogenesis substantial cortical development occurs in the Topbp1Nes-cre brain. Given the in vitro studies identifying a crucial role for TopBP1 function
during replication\textsuperscript{9,17}, the large amount of cell proliferation associated with neurogenesis in the Topbp1\textsuperscript{Nes-cre} tissue was unexpected.

To understand these findings, we considered whether the timing of gene deletion influences the outcome of TopBP1 inactivation. We assessed this using Emx1\textsuperscript{-cre} to drive TopBP1 deletion in early cortical progenitors. Emx1\textsuperscript{-cre} expresses in the dorsal telencephalon earlier than Nes\textsuperscript{-cre} (~E10 rather than E11)\textsuperscript{37,38}. Emx1\textsuperscript{-cre} expression occurs in cells that give rise to the hippocampus and parts of the motor, somatosensory and visual cortices, but it does not express in the ventral telencephalon\textsuperscript{37,38}. In contrast to the Topbp1\textsuperscript{Nes-cre} phenotype, there was a loss of all cortical structures derived from Emx1\textsuperscript{-expressing} progenitors in Topbp1\textsuperscript{Emx1-cre} mice (Fig. 3a and Supplementary Fig. 4a).

To ascertain the cause of this phenotype, we examined the embryonic neocortex at E13.5, when Emx1\textsuperscript{-expressing} progenitors are rapidly expanding\textsuperscript{37}. In Topbp1\textsuperscript{Emx1-cre} tissue, but not control tissue, we found abundant apoptosis indicated by immunostaining for activated caspase-3, by TUNEL and by resultant reduced cellularity after hematoxylin and eosin staining (Fig. 3b and Supplementary Fig. 4b). Cell loss was also evident by reduced 5-bromodeoxyuridine (BrdU) incorporation and fewer histone H3pSer10-positive G2/M phase cells at the ventricular (apical) surface (Fig. 3c). This extensive cell loss is sufficient to account for the absence of the cortices at birth. We also found that TopBP1 inactivation led to widespread γH2AX immunostaining by E11.5 that colocalized with proliferating PCNA-positive cells in the dorsal telencephalon (Supplementary Fig. 4c). Whereas the dorsal telencephalon was affected in the Topbp1\textsuperscript{Emx1-cre} brain, other regions, including the ventral telencephalon and the cerebellum, showed no developmental abnormalities, underscoring the specificity of Emx1\textsuperscript{-expression} expression (Supplementary Fig. 4d).

**Emx1 progenitors proliferate after Lig4 or Xrcc1 loss**

To further gauge the essential requirement for TopBP1 in the cortical progenitors, we determined the comparative importance of the key DNA repair factors DNA ligase IV (Lig4) and Xrcc1. These factors are central for DNA strand break repair by means of nonhomologous end-joining and base excision repair, respectively; germline deletion of either is embryonic lethal, and deletion throughout the nervous system using Nes\textsuperscript{-cre} results in microcephaly and DNA damage accumulation\textsuperscript{39,40}.

We compared cortical development in Lig4\textsuperscript{Emx1-cre} and Xrcc1\textsuperscript{Emx1-cre} to that in Topbp1\textsuperscript{Emx1-cre} mice. Loss of Lig4 or Xrcc1 resulted in cortices with milder developmental perturbations, although a marked effect

**Figure 2** Apoptosis and cortical layering disruption in the Topbp1\textsuperscript{Nes-cre} brain. (a) Apoptosis in the Topbp1\textsuperscript{Nes-cre} E16.5 cortex is associated with PCNA\textsuperscript{+} proliferating cells (arrowheads) but not BLBP\textsuperscript{+} radial glia progenitors. (b) Apical progenitors (arrows) identified using histone H3pSer10 immunostaining in E13.5 Topbp1\textsuperscript{Nes-cre} and control neocortex. (c) Analysis of cortical development in P0 and P10. Topbp1\textsuperscript{Nes-cre} and WT tissue using Tbr1 and Ctip2 to mark layers VI–III, Cux1 to identify layers IV–II and Brn2/Satb2 to identify layers V–II reveals disrupted layer development in the Topbp1\textsuperscript{Nes-cre} cortex. VZ, ventricular zone. Roman numerals indicate cortical layers.

**Figure 3** Dorsal telencephalon progenitors are lost in Topbp1\textsuperscript{Emx1-cre} mice. (a) Cortices and other structures developing from the dorsal telencephalon are missing in the Topbp1\textsuperscript{Emx1-cre} brain (red dotted outlines), shown by Nissl staining at P5. Arrowheads, lateral ventricle; CC, corpus callosum; hippo, hippocampus; RMS, rostral migratory stream. (b) Abundant apoptosis is observed in the E13.5 Topbp1\textsuperscript{Emx1-cre} neocortical tissue shown using TUNEL and activated caspase-3 staining. Differences in the numbers of apoptotic cells between genotypes were significant (Student’s t-test). Active caspase-3-positive cells in 1 mm\textsuperscript{2} (± s.e.m.; P < 0.05): control, 0; Topbp1\textsuperscript{Emx1-cre}, 734 ± 30.6. TUNEL-positive cells in 1 mm\textsuperscript{2} (± s.e.m.; P < 0.0001): control, 7.0 ± 0.81. Topbp1\textsuperscript{Emx1-cre}, 988 ± 27.4. H&E, hematoxylin and eosin; P1, propidium iodide. (c) Fewer proliferating cells indicated by histone H3pSer10 (arrows) or BrdU incorporation staining are seen in the E13.5 Topbp1\textsuperscript{Emx1-cre} neocortical tissue. Differences in the numbers of proliferating cells between genotypes were significant (t-test): BrdU-positive cells in 0.27 mm\textsuperscript{2} (± s.e.m.; P < 0.0001): control, 438 ± 26.5; Topbp1\textsuperscript{Emx1-cre}, 206 ± 10.3. H3pSer10-positive cells in 1 mm\textsuperscript{2} (± s.e.m.; P < 0.0001): control, 91 ± 4.59; Topbp1\textsuperscript{Emx1-cre}, 27.7 ± 1.71. Widespread DNA damage in Topbp1\textsuperscript{Emx1-cre} mice is shown by γH2AX and PCNA immunostaining (right) in regions indicated in white boxes in center panels. DAPI (blue) stains nuclei.
on the hippocampus was apparent (Fig. 4a). Accordingly, cortical layering was relatively intact in both the Lig4<sup>Emx1-cre</sup> and Xrcc1<sup>Emx1-cre</sup> mice, with each of the six layers present (Fig. 4b). Nonetheless, substantial DNA damage accumulated, as γH2AX was abundant in Lig4<sup>Emx1-cre</sup> tissue, consistent with Lig4 gene deletion throughout the dorsal cortex leading to persistent unrepaired DNA (Fig. 4c). These data indicate that despite the essential neural repair functions of Lig4 and Xrcc1, TopBP1 fulfills a distinct role in maintaining genome stability of progenitors.

**Progenitor loss after TopBP1 inactivation requires p53**

In the nervous system, DNA damage–induced apoptosis is often dependent on p53 or ATM<sup>41</sup>. To determine whether this signaling pathway contributes to the Topbp1-deleted phenotype, we generated compound Topbp1 mutants with coincident deletion of either Trp53 (encoding p53) or Atm.

Loss of p53 resulted in an obvious amelioration of the Topbp1<sup>Nes-cre</sup> cortical defects that were particularly noticeable in the case of hippocampal development, although this rescue was only partial (Fig. 5a and Supplementary Fig. 5a). Notably, widespread DNA damage was observed in the rescued Topbp1<sup>Nes-cre;Trp53<sup>−/−</sup></sup> dentate gyrus, indicating that chronic genome instability was a feature of this tissue (Fig. 5a and Supplementary Fig. 6a,b). Consistent with this developmental

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**Figure 4** Analysis of cortical development in Lig4<sup>Emx1-cre</sup> and Xrcc1<sup>Emx1-cre</sup> brain. (a) Nissl staining identifies the Lig4<sup>Emx1-cre</sup> and Xrcc1<sup>Emx1-cre</sup> cortex (Ctx) and hippocampus (arrows; dentate gyrus (DG) and CA1 region) and shows the relative levels of defective development after Lig4 or Xrcc1 loss. Relative gene deletion (± s.e.m.) was determined in genomic DNA isolated from the cortex using real-time PCR. For Xrcc1: WT, 1.22 ± 0.033; Xrcc1<sup>Emx1-cre</sup> 0.46 ± 0.006. For Lig4: WT, 0.86 ± 0.038; Lig4<sup>Emx1-cre</sup>, 0.19 ± 0.004. Tbr2 marks the DG. (b) Cortical markers were used to assess development in the Lig4<sup>Emx1-cre</sup> and Xrcc1<sup>Emx1-cre</sup> cortex. Roman numerals indicate cortical layers. (c) DNA damage (γH2AX) accumulates in the Lig4<sup>Emx1-cre</sup> cortex.

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**Figure 5** Defective neurogenesis after Topbp1 inactivation requires p53 but not ATM signaling. (a) The hippocampal defects in Topbp1<sup>Nes-cre</sup> brain involve p53 signaling, as Topbp1<sup>Nes-cre;Trp53<sup>−/−</sup></sup> mice show partial rescue of hippocampal dentate gyrus (DG) development as shown using calbindin immunostaining to assess histology at P5. In contrast to p53 loss, ATM loss did not rescue defective Topbp1<sup>Nes-cre</sup> hippocampal development. Despite recovered development, TopBP1 loss nonetheless resulted in persistent DNA damage as demonstrated by γH2AX foci (arrows). (b) Analysis of apoptosis using TUNEL or proliferation using histone H3pSer10 indicates that p53 but not ATM signaling is involved in the Topbp1<sup>Nes-cre</sup> phenotype. *P < 0.01, **P < 0.05; error bars, s.e.m. (c) Cortices that fail to develop in Topbp1<sup>Nes-cre</sup> mice are rescued by coincident loss of p53 (red dotted outlines). However, the hippocampal formation is not rescued in the Topbp1<sup>Nes-cre;Trp53<sup>−/−</sup></sup> brain. (d) Although substantial cortical rescue occurs in Topbp1<sup>Nes-cre;Trp53<sup>−/−</sup></sup> mice, cortical layering shows disorganization as indicated using Cux1 or Tbr1 and Clup2 co-staining. (e) DNA damage is present throughout the P5 Topbp1<sup>Nes-cre;Trp53<sup>−/−</sup></sup> cortices (arrows), and γH2AX foci are frequently observed in PCNA-positive proliferating cells (arrows).
rescue, apoptosis resulting from TopBP1 loss was also abrogated in Topbp1Nes-cre;Trp53−/− tissue, and cell proliferation recovered as determined by histone H3 phosphorylation (Fig. 5b). In contrast to Trp53 loss, inactivation of Atm did not influence the Topbp1-null phenotype (Fig. 5a,b). The rescue of the Topbp1Nes-cre phenotype by p53 loss also occurred in other Topbp1Nes-cre brain regions. The E13.5 Topbp1Nes-cre embryonic cerebellum showed apoptosis in proliferating cells of the ventricular zone, which was blocked after loss of p53, but not Atm (Supplementary Fig. 7a). Quantification of both proliferation and apoptosis showed that inactivation of p53 blocked apoptosis and restored proliferation to WT levels in the Topbp1Nes-cre;Trp53−/− cerebellum and ganglionic eminence, whereas Atm loss worsened the phenotype (Supplementary Fig. 7b).

Although rescue of the Topbp1Nes-cre phenotype by p53 loss was extensive, it only extended the lifespan of the Topbp1Nes-cre mice by around 10 d (Supplementary Fig. 5b). This likely reflects the profound functional consequence of persistent DNA damage after TopBP1 loss in replicating cells throughout the brain. These data indicate that DNA damage–associated apoptosis after TopBP1 loss is p53 dependent and that cells can proliferate in the absence of TopBP1, but at the expense of DNA damage accumulation.

Given the degree of development in Topbp1Nes-cre;Trp53−/− neural tissue, we also determined how p53 inactivation affects tissue loss in cortical progenitors (Fig. 5d). This rescue resulted in establishment of cortical layering, albeit less organized than in WT tissue (Fig. 5d). Most notably, the upper cortical layers were more affected, probably as a result of chronic DNA damage during neurogenesis. Recovered development in Topbp1Emx1-cre;Trp53−/− tissue showed perturbation of homeostasis, as these regions were marked by gliosis (Supplementary Fig. 6c,d). One exception to the rescue in the Topbp1Emx1-cre;Trp53−/− tissue was the hippocampus, as this structure was absent from the Topbp1Nes-cre;Trp53−/− brain (Fig. 5c). This situation contrasts the partially recovered hippocampal development in the Topbp1Nes-cre;Trp53−/− brain (Fig. 5a).

The different hippocampal phenotypes between the Topbp1Emx1-cre;Trp53−/− and Topbp1Nes-cre;Trp53−/− likely reflect the enhanced sensitivity of early hippocampal progenitors toward genotoxic stress in the absence of TopBP1. The Topbp1Emx1-cre;Trp53−/− cortex was associated with γH2AX-immunopositive cells that colocalized with PCNA immunopositive cells, indicating that Topbp1-null cells had survived and were replication competent (Fig. 5e).

DNA strand breaks accumulate in TopBP1-null progenitors

Despite the rescue of developmental defects by p53 deficiency after TopBP1 inactivation, DNA damage was associated with TopBP1 loss and was frequently colocalized with proliferating cells (Fig. 5e and Supplementary Figs. 3 and 6a). Although this reinforces the notion that TopBP1 is critical for maintaining genome stability but is dispensable for DNA replication, it raises the question of the physiological nature of the DNA damage. As γH2AX is only a surrogate marker for DNA damage, we directly addressed DNA integrity in cortical progenitors in situ using the alkaline comet assay (ACA) to measure DNA strand breaks. We isolated progenitors from E13.5 neocortex from Topbp1Nes-cre;Trp53−/− and control embryos and then immediately subjected these to ACA. In this way we were able to directly assess DNA integrity in vivo at a specific developmental stage (Fig. 6a). To determine the amount of strand breakage, we included an irradiated series of acutely isolated WT cerebellar granule neuron precursors as a comparison to the cortical progenitors. We found that all individual cortical isolates from TopBP1 mutant tissue had markedly increased DNA strand breaks. The levels of DNA damage were similar to those produced by ~2 Gy of radiation in granule neuron precursors (Fig. 6a), which is estimated to be about 80 double-strand breaks per nucleus42, although the damage measured by the ACA also includes single-strand breaks. Notably, the levels of in situ DNA breaks were similar between cortical progenitors from Topbp1Nes-cre and Topbp1Emx1-cre progenitors, indicating that both Cre drivers are effective at TopBP1 deletion despite the more profound defects of cortical development in Topbp1Emx1-cre tissue.
Because TopBP1 deletion by either Nes-cre or Emx1-cre resulted in similar amounts of DNA damage in progenitors at E13.5, we determined the relative sensitivity of progenitor populations to DNA damage at different early embryonic developmental times. Using very low doses (0.2 Gy) of ionizing radiation that generate ~8 breaks per G1 genome, we quantified apoptosis by determining the ratio of TUNEL+ cells to total cells in a defined area in the ventricular zone of E11.5, E12.5 and E14.5 embryos. We found that there was a significantly enhanced susceptibility to DNA damage–induced apoptosis in early embryos compared with later-stage embryos, indicating a lower threshold for apoptosis in earlier progenitors compared with those generated later (Fig. 6b).

Collectively, these data indicate that TopBP1 deletion results in the accumulation and persistence of DNA strand breaks in cortical progenitors, and also that the relative levels of DNA damage are similar in Topbp1Emx1-cre and Topbp1Nes-cre progenitors. Thus the difference in phenotype after deletion via the different Cre drivers is not due to different numbers of DNA strand breaks, but rather the enhanced susceptibility of earlier-born cortical progenitors to DNA damage after TopBP1 loss.

Defective DNA damage signaling in TopBP1 deficient cells

As TopBP1 loss resulted in elevated DNA damage, we determined the competency of TopBP1 deficient cells to respond to and repair DNA damage. We initially attempted to use Topbp1lox/loxP mouse embryonic fibroblasts (MEFs) and retroviral transduction with murine stem cell virus (MSCV)-cre to delete Topbp1. However, retroviral introduction of Cre caused widespread apoptosis of the Topbp1lox/loxP cells after 48 h, whereas Topbp1+loxP cells used as a control were unaffected by MSCV-cre. This likely reflects hypersensitivity of the Topbp1-deleted cells to DNA breaks associated with retroviral integration.

Therefore, we established Topbp1creTM cells, in which TopBP1 can be inactivated after tamoxifen administration (CreTM is a tamoxifen-inducible version of Cre that uses the actin promoter and CMV enhancer). We found that efficient TopBP1 deletion occurred in Topbp1creTM MEFs after 4-hydroxytamoxifen (4-OHT) administration and that loss of TopBP1 resulted in a modest upregulation of p53, probably from replication-associated DNA damage (Fig. 7a). After 4-OHT-mediated deletion (48 h treatment) Topbp1creTM MEFs ceased proliferation, although they remained viable. Using these, we found that loss of TopBP1 did not alter Chk2 activation or p53Ser18 phosphorylation after DNA damage by ionizing radiation. However, consistent with ATR activation requiring TopBP1, Chk1Ser317 phosphorylation was reduced after ionizing radiation (IR) (n = 50 cells); R0 and R240, recovery after 0 and 240 min, respectively. (f) DNA repair assessed after IR (10 Gy) and time in camptothecin (CPT; 14 µM) using the ACA. Tdp1−/− primary astrocytes were established from null mice. R60, recovery after 60 min. Error bars, s.e.m.

DNA repair is near normal in TopBP1 deficient cells

We also determined DNA repair competency in TopBP1-inactivated cells using primary Topbp1Nes-cre astrocytes challenged with ionizing radiation or camptothecin. Although we were unable to establish Topbp1Nes-cre astrocytes, probably as a result of DNA damage induced cell cycle arrest, we were able to generate Topbp1Nes-cre;Trp53+−/− astrocytes (that presumably overcame DNA damage-induced p53-dependent cell cycle arrest). However, compared with WT cultures, Topbp1Nes-cre;Trp53+−/− astrocyte cultures only reached ~30% of the confluency of control astrocyte cultures and were characterized by chronic DNA damage accumulation indicated by widespread γH2AX foci that was not observed in controls (Fig. 7c). This DNA damage accumulation was associated with replication abnormalities, as BrdU and 5-ethyl-2′-deoxyuridine (EdU) incorporation in TopBP1 astrocytes showed a marked reduction compared with controls (Fig. 7d and Supplementary Fig. 8).

**Figure 7** Analysis of DNA damage responses in TopBP1-depleted cells. (a) Radiation-induced DNA damage response was investigated using tamoxifen (4-OHT)-induced deletion of Topbp1 in MEFs. WT are Topbp1+/+, creTM, and KO are Topbp1lox/loxP;creTM. TopBP1, Chk2, p53(P(Ser18)), Chk1(P(Ser317)) and actin indicate the result of probing with the respective antibodies. Activation of Chk2 is indicated by a mobility shift (arrow). Ponceau staining and actin immunostaining show protein loading. Asterisk indicates decreased Chk1(P-ser317) immunostaining after TopBP1 inactivation. (b) Cerebellar extracts (Cereb) or astrocytes from WT or Topbp1Nes-cre mice were immunoblotted as described above for MEFs, and also with antibodies recognizing total Chk1 and glial fibrillary acidic protein (GFAP); longer exposure revealed clear GFAP staining in the cerebellum (not shown). The trace TopBP1 signal in replication-defective null astrocytes is from meningeal fibroblast contamination. Radiation was 4 Gy with 2 h recovery. (c) Topbp1Nes-cre;Trp53+−/− astrocytes show elevated endogenous DNA damage indicated by γH2AX immunostaining (arrows). Astrocyte purity is shown by GFAP immunostaining. (d) DNA repair in control and Topbp1Nes-cre;Trp53+−/− astrocytes determined using EdU pulselabeling after BrdU prelabeling. TopBP1 deficiency compromised DNA replication as shown by a reduction of EdU uptake; *P < 0.001. (e) DNA repair in Topbp1Nes-cre;Trp53+−/− astrocytes determined by quantifying removal of γH2AX foci after ionizing radiation (IR) (n = 50 cells); R0 and R240, recovery after 0 and 240 min, respectively. (f) DNA repair assessed after IR (10 Gy) and time in camptothecin (CPT; 14 µM) using the ACA. Tdp1−/− primary astrocytes were established from null mice. R60, recovery after 60 min. Error bars, s.e.m.
Despite a normal ionizing radiation–induced DNA damage response, the high endogenous levels of γH2AX indicate extensive DNA damage, most likely strand breaks associated with compromised replication. Therefore, we assessed DNA strand break repair after ionizing radiation, by measuring resolution of the resultant γH2AX foci in Topbp1Nes-cre;Trp53−/− cells. We found that ionizing radiation–induced γH2AX foci were resolved in Topbp1Nes-cre;Trp53−/− astrocytes, suggesting that this type of exogenously induced DNA damage could be repaired effectively after TopBP1 loss (Fig. 7e). Additionally, we measured DNA repair after genotoxic stress using the ACA. We found that although Topbp1Nes-cre cells had a higher basal DNA damage, these cells repaired DNA breaks at a rate similar to that of control cells after either ionizing radiation or camptothecin. As a comparison to Topbp1Nes-cre genotype, we included DNA repair–deficient tyrosyl-DNA phosphodiesterase 1 null (Tdp1−/−) cells and confirmed that these were defective after both genotoxic agents (Fig. 7f). Together, these data indicate that TopBP1 is not essential for repair of DNA strand breaks induced by agents that require base excision repair and nonhomologous end-joining/homologous recombination pathways. Thus, DNA strand break pathways are competent in TopBP1-null tissue, and the persistence of DNA breaks likely reflects excessive DNA damage accumulation and perturbed steady-state repair.

DISCUSSION

Coordinated integration of DNA replication and damage signaling is necessary for unperturbed mammalian development. While our understanding of the biochemistry of DNA replication and repair has rapidly advanced during the last few years, it nonetheless remains a challenge to apply this knowledge to physiological systems. The nervous system is particularly relevant in this regard, as it is often a primary tissue affected in human diseases associated with defects in the response to DNA damage5. In the present study, we show that the ATR activator TopBP1 is critical for the prevention of DNA damage accumulation and apoptosis in early-born neural progenitors, thereby maintaining homeostasis at a critical stage of nervous system development (Supplementary Fig. 9).

We found that TopBP1 inactivation by Emx1-cre in dorsal telencephalon progenitors during the very early stages of cortical neurogenesis (from ~E10) resulted in cortical ablation. In contrast, TopBP1 inactivation at later stages by Nes-cre resulted in less substantial defects in cortical development. The main reason for these different outcomes is the timing of Cre-mediated deletion: Emx1-cre targets specific progenitors at least 1 d earlier than Nes-cre. Although Emx1-cre targets earlier-born cortical progenitors, we found similar levels of DNA strand breaks in dorsal telencephalon progenitors at E13.5 after TopBP1 inactivation by either Cre driver. This raises the likelihood that there is a different threshold of tolerance to DNA damage in the early-born progenitors, perhaps reflecting an increased need for vigilance to genome integrity in stem or early-progenitor populations. This assertion is supported by our finding of a greater sensitivity of cortical progenitors to DNA damage at E11 than at E14. Indeed, although they cycle rapidly, early-born cortical progenitors have an extended S-phase that would allow enhanced genome surveillance and integrity. When these cells switch to neuron production, S-phase shortens, suggesting that genome maintenance is an important feature of uncommitted progenitors14. Also, as cortical expansion occurs, G1 lengths in progenitors undergoing differentiation, highlighting the dynamic events associated with cell cycle regulation during neurogenesis36,44,45.

Altered transcriptional programming in cortical progenitors may also influence the outcome of genotoxic stress. For example, by the time they are targeted using Nes-cre, the Emx1-1-expressing progenitor cells in the dorsal telencephalon have already undergone substantial expansion and cell fate determination by Lhx2 to commit to cortical genesis38. Therefore, progression from stem or uncommitted progenitor can result in altered cell cycle dynamics, intrinsic replication differences and differential sensitivity to DNA damage. In contrast to TopBP1, inactivation of the key DNA repair factors Xrcc1 and DNA ligase IV by Emx1-cre did not fully disrupt cortical genesis. This is also the case for Brca1 inactivation by Emx1-cre, although in that case early-born but not late-born progenitors are lost by apoptosis46. Taken together, one interpretation of our data is that the outcome of DNA damage is regulated differently as progenitors mature, and that TopBP1 contributes specifically to genome stability in early-born progenitors. Alternatively, and despite equal levels of DNA strand breaks in both Topbp1Emx1-cre and Topbp1Nes-cre progenitors at E13.5, the greater cell loss in Topbp1Emx1-cre brains may reflect the more protracted period of proliferation before Nes-cre expression.

The consequences of TopBP1 loss for newborn progenitors in the cerebellum were similar to those in the cortex. Topbp1Nes-cre mice showed pronounced cerebellar agenesis, as the external granule layer failed to expand from the rhombic lip. The cerebellar ventricular zone progenitors were also markedly affected, and the resultant lack of development lead to a cerebellum consisting of a disorganized collection of cells, such as Purkinje cells that arise before Nes-cre activation.

Although our data highlight the importance of TopBP1 for preventing replication-associated DNA damage, most prominently they show that it is not required per se for replication. This is because neural development defects in Topbp1Nes-cre or Topbp1Emx1-cre can be substantially rescued by concomitant p53 loss, leading to continued tissue development. These data contrast with other studies that show TopBP1 to be essential for the assembly of DNA replication pre-initiation complexes, a function tightly linked to factors such as Treslin (Ticrr) and GEMC1 (refs. 6,9,10). The in vivo nature of our study maintains the physiological parameters that support development and cell proliferation, unlike other studies that use cell-free extracts or in vitro cell culture systems. However, consistent with those other studies, we found severely compromised proliferation of TopBP1-null cells in culture, and coincident inactivation of p53 was required to extend their viability. Nonetheless, Topbp1Nes-cre;Trp53−/− astrocytes can only undergo a few rounds of division, during which they accumulate abundant DNA damage.

Although a key function of TopBP1 is activation of ATR, the more severe neurogenesis defects after TopBP1 loss than after ATR inactivation47 argue for a broader role in genome maintenance. Furthermore, TopBP1 loss activated a DNA damage response resulting in p53-mediated apoptosis. This contrasts the different outcomes of ATR and p53 inactivation53, suggesting loss of TopBP1 in vivo results in DNA lesions and/or DNA damage signaling that are qualitatively different from those in ATR-deficient tissues. Although ATM can also be important in modulating p53-dependent DNA damage-induced apoptosis in the nervous system48, ATM loss did not rescue defects in Topbp1Nes-cre mice. This is reminiscent of other situations in the nervous system wherein DNA damage leads to ATM-dependent apoptosis in immature postmitotic neural cells but not in proliferative cells49,50.

Taken together, our data are compatible with a model in which TopBP1 maintains replication fidelity by means of checkpoint activation, rather than having an essential role in replication preinitiation complex assembly. In its absence, replication proceeds, but DNA fidelity is compromised through the accumulation of stalled replication forks and other DNA damage during S-phase. This damage would normally signal S-phase and other factors such as the BACH1 helicase50 for checkpoint activation and homologous recombination repair17.
Thus, our study reveals the indispensable requirement for TopBP1 to prevent DNA damage accumulation in early-born progenitors, where maintenance of genome integrity is of paramount importance for neurogenesis.

METHODS

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

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

Y.L.S. and H.R.R. performed all experiments characterizing the Topbp1 mutant mouse and contributed to writing the manuscript. S.M.D. contributed the analysis of the L4q and Xrcc1 conditional mutant mice. J.Z. generated western blot data. P.J.M. was project leader and produced the final version of the manuscript.

COMPETING FINANCIAL INTERESTS

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Western blot analysis. Western blot analysis was done using astrocyte cell lines (genotypes as indicated in the main text and figures) at passage 1 or 2, or tissues (cortex, cerebellum, liver, thymus and spleen) from both P4/P5 control mice (Topbp1loxP/Nes-cre) and conditional knockout mice (Topbp1Nes-cre). Protein extracts were prepared by using lysis buffer (50 mM Tris-HCl, 200 mM NaCl, 0.2% NP-40, 1% Tween-20, 1 mM NaF, 1 mM sodium vanadate, 50 mM β-glycerophosphate, 2 mM PMSF, and protease inhibitor cocktail (Roche)) and quantified using the Bradford assay (Bio-Rad). Proteins (50 µg per lane) were separated through a 4–12% Bis-Tris SDS polyacrylamide gel (Invitrogen) and transferred onto nitrocellulose membrane (Bio-Rad). Blots were sequentially immunostained with mouse anti-TopBP1 antibody (rabbit, 1:1,000, Millipore/Chemicon) followed by horseradish peroxidase–conjugated secondary antibody (1:1,000, GE Healthcare) and detected using ECL Plus chemiluminescence (GE Healthcare). The blots were also probed with anti-Nbs1 (rabbit, 1:500, Cell Signaling), anti-Chk1 (mouse, 1:1,000, Millipore), anti-p53ser15 (rabbit, 1:1,000 Bio-labs), anti-Chk1 (rabbit, 1:500, Cell Signaling) and anti-p-Chk1ser317 (rabbit, 1:800, Bethyl) and processed as described above. Anti-actin (goat, 1:500, Santa Cruz Biotech) western blots or Ponceau-stained transfer membrane were used as protein-loading controls.

Quantitative real-time PCR. To confirm deletion of the Topbp1 gene using cre lines (both Emx1-cre and Nes-cre), genomic DNA was isolated at several different time points. For Emx1-cre, the dorsal portion of the cerebral cortex was separated from the rest of brain. Similarly, the entire cerebral cortex was removed from the embryonic brain for Nes-cre. Quantitative real-time PCR using SYBR Green was performed according to the manufacturer’s guideline (iQ5 multicolor real-time PCR detection system, Bio-Rad). The following primer sets were used to measure the quantity of exon 4 (targeted exon) and exon 11 of the Topbp1 gene. The deletion efficiency was calculated as a ratio between exon 4 and exon 11. Each group contained 2 to 4 embryos. The primers are as follows: Exon 4 forward: 5′-GGGCCACAGCAGAAATGGTGCTGACATGC-3′; Exon 4 reverse: 5′-TGATACAGGATGCTTGCAAGATGATGA-3′. In situ cell counts. To determine cell cycle indices and apoptosis in Topbp1Nes-cre tissue during brain development, E13.5 embryos were subjected to quantitative analysis. Three embryos for each genotype were analyzed. Immunopositive signals for BrdU, PCNA, H3Pser10, TUNEL or active caspase-3 were measured within 1 or 0.27 mm² from at least three representative sections of the neocortical cortex, cerebellum and ganglionic eminence per embryo. Similar quantification methods were applied to ionizing radiation–induced apoptosis (TUNEL) in embryos collected 6 h after 0.2 Gy ionizing radiation at E11.5, E12.5 and E14.5. In this case, the ratio of TUNEL-positive signals to propidium iodide–positive neurons was calculated using ImageJ (1.46b, NIH), and analyzed using Prism v4 software (GraphPad).

Isolation of primary astrocytes and embryonic fibroblasts. Primary astrocytes were prepared from P1–P2 mouse brains. Cortices were dissociated by passage through a 5-ml pipette and cells were suspended in Dulbecco’s modified Eagle’s medium and Ham’s nutrient mixture F-12 (1:1 DMEM/F12, Gibco-BRL) supplemented with 10% FBS (v/v), 100 U/ml penicillin, 100 µg/ml streptomycin and 20 ng/ml epidermal growth factor (Millipore). Primary astrocytes were established in Primaera T-25 tissue culture flasks (Falcon) at 37 °C in a humidified 5% CO2-regulated (5%) incubator. Mouse embryonic fibroblasts (MEFs) were isolated from E13.5 embryos and grown at 37 °C in DMEM/10% FCS supplemented with β-mercaptoethanol in a humidified CO2-regulated (5%) incubator.

For DNA replication analysis, astrocytes were cultured in the presence of 10 µM BrdU (Sigma). Five days later cells were trypsinized, resuspended in medium containing BrdU and seeded onto 1 mm glass coverslips (渔器) at ~20% confluency. Twenty hours later, the BrdU–enriched medium was removed and cells were incubated with prewarmed medium alone (0 min) or medium
containing 10 µm EdU (Invitrogen) for 15, 30 or 60 min. Cells were then washed, fixed in 4% PBS-buffered paraformaldehyde and permeabilized with 0.5% PBS-buffered Triton X-100. Labeling of EdU was performed using the Click-It EdU Alexa 488-azide detection kit (Invitrogen) using the manufacturer’s suggested protocol. Cells were then treated with 2 N HCl for 30 min at 37 °C and then immunostained with Alexa 555–conjugated anti-BrdU (Invitrogen, 1:500, clone MoBU-1). Cells were counterstained with DAPI (Vector Laboratories). Cells counts were analyzed using a Student’s t-test.

**Comet assays.** Quiescent primary astrocytes were treated with either 14 µM camptothecin for 60 min at 37 °C or γ-irradiation (20 Gy, 137Cs). Following treatments, cells were incubated for various times in drug-free medium at 37 °C to assess recovery from damage. Cells were trypsinized and suspended in prechilled PBS, mixed with an equal volume of 1.2% low-melting-point agarose (Invitrogen), maintained at 42 °C and immediately layered onto frosted glass slides (Fisher) precoated with 0.6% agarose and maintained in the dark at 4 °C for all subsequent steps. Slides were immersed in prechilled lysis buffer (2.5 M NaCl, 10 mM Tris-HCl, 100 mM EDTA, 1% (v/v) Triton X-100, 3% (v/v) DMSO, pH 10) for 1 h, washed with prechilled distilled water (twice for 10 min each) and placed in prechilled 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-HCl (pH 7.0). Comets were stained with SYBR Green (1:10,000 in PBS, Sigma) for 10 min. Experiments were performed in triplicate and 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 200× magnification.

For in vivo comet analysis, tissue from E13.5 embryonal dorsal telencephalon was triturated into a single-cell suspension in prechilled PBS-buffered 2% glucose. Cells were embedded into LMP agarose and underwent alkaline comet analysis as outlined above. Samples were prepared and analyzed from every embryo collected and genotyping was done post-experimentally, ensuring a blinded analysis of strand break levels. Cerebellar granule neurons were prepared from P6 WT mice via a Percoll gradient as previously described43; neurons were irradiated on ice at the specified dose rate and immediately embedded in agarose for alkaline comet analysis. At least 300 comet tail moments were measured from each sample.

**Statistical analysis.** Cell counts were determined using a Student’s t-test and were calculated using Prism V4 software (GraphPad); P < 0.05 was considered significant. Box plots representing comet data were calculated using Deltagraph 6 (Redrock Software) from tail moment data generated using the Comet Assay IV system (Perceptive Instruments).