Robust elimination of genome-damaged cells safeguards against brain somatic aneuploidy following Knl1 deletion

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The brain is a genomic mosaic shaped by cellular responses to genome damage. Here, we manipulate somatic genome stability by conditional Knl1 deletion from embryonic mouse brain. KNL1 mutations cause microcephaly and KNL1 mediates the spindle assembly checkpoint, a safeguard against chromosome missegregation and aneuploidy. We find that following Knl1 deletion, segregation errors in mitotic neural progenitor cells give rise to DNA damage on the missegregated chromosomes. This triggers rapid p53 activation and robust apoptotic and microglial phagocytic responses that extensively eliminate cells with somatic genome damage, thus causing microcephaly. By leaving only karyotypically normal progenitors to continue dividing, these mechanisms provide a second safeguard against brain somatic aneuploidy. Without Knl1 or p53-dependent safeguards, genome-damaged cells are not cleared, alleviating microcephaly, but paradoxically leading to total pre-weaning lethality. Thus, mitotic genome damage activates robust responses to eliminate somatic mutant cells, which if left unpurged, can impact brain and organismal fitness.

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maintenance of genome stability across divisions is essential to proliferating cells. In embryonic cerebral cortex, neurons and glia are generated by neural progenitor cells (NPCs) via successive rounds of cell divisions. Somatic mutations inevitably arise during DNA synthesis and chromosome segregation, and their impact is alleviated by DNA repair, cell cycle checkpoints, and elimination of cells with genome damage. Left uncorrected or unpurged, these mutations lead to somatic mosaicism. The adult human brain harbors multiple types of somatic mosaic mutations, including aneuploidy. Emerging evidence suggests that somatic mutations can contribute to brain disorders. However, the mechanisms that curtail the propagation of brain somatic mutations, and the consequence of their impairment, remain incompletely understood.

Here, we generated a conditional allele of Knn1 to manipulate genome stability in embryonic mouse brain, and leveraged this model to study the consequences of somatic genome instability in vivo. Knn1 (formerly CASC5 or BLINKIN) is a kinetochore component required for the spindle assembly checkpoint (SAC), which safeguards correct chromosomal segregation during mitosis. In the presence of chromosomes unattached to microtubules, KNN1 functions as a scaffold for the assembly of the mitotic checkpoint complex, a potent inhibitor of anaphase. Upon secure attachment of all chromosomes, SAC is deactivated, and anaphase proceeds. In the absence of the Knn1−/−BUB3-BUB1 pathway, the ROD-ZWILCH-ZW10 pathway can activate SAC in response to unattached kinetochores, but SAC is deactivated prematurely, resulting in segregation errors. Disruption of KNN1-BUB3-BUB1 components thus leads to numerical (whole chromosome) aneuploidy, and anaphase, and can contribute to brain disorders. However, the mechanisms that curtail the propagation of brain somatic mutations, and the consequence of their impairment, remain incompletely understood.

We find in mice that conditional deletion of Knn1 from mitotic cortical NPCs leads to frequent chromosome segregation errors. The resulting missegregated chromosomes carry DNA damage in the form of double strand breaks (DSBs). Likely independently of aneuploidy, this DNA damage triggers rapid p53-dependent apoptotic and downstream microglial phagocytic responses that extensively eliminate the cells with chromosome missegregation. By leaving only karyotypically normal NPCs to continue dividing, these p53-dependent mechanisms provide a second safeguard against brain somatic aneuploidy in addition to the SAC. Applied en masse following Knn1 deletion, however, they cause massive cell loss and severe microcephaly. In the absence of both Knn1 and p53 safeguards against aneuploidy, genome-damaged cells are not eliminated from the brain. Paradoxically, this accumulation of somatic mutant cells, despite partially alleviating microcephaly, results in complete pre-weaning lethality. Our work thus unravels robust cellular processes in embryonic brain for eliminating cells with severe genome damage, which if left unpurged, can affect brain function and organismal fitness.

**Results**

**Knn1 deletion led to cortical NPC loss and microcephaly.** Consistent with Knn1 function in the SAC, publicly available single-cell RNA-seq data from embryonic cortex showed Knn1 expression in proliferating NPCs in the germinatal zones, the ventricular zone (VZ) and subventricular zone (SVZ), but not in post-mitotic neurons, and bulk RNA-seq showed forebrain Knn1 expression throughout neurogenesis (Supplementary Fig. 1a). Constitutive deletion of Knn1 in mice led to lethality on embryonic day E6 (MGI Ref. ID: J:175597). We recovered a conditional-ready lacZ reporter Knn1 allele (Knn1tm1at(EUCOMM)Hmgu, Supplementary Fig. 1b) from frozen spermatozoa. LacZ reporter expression was confirmed in E15.5 cortex (Supplementary Fig. 1c), and Knn1 conditional allele (Knn1fl/fl) was generated by crossing with Flp recombinease mice to remove the lacZ cassette.

To conditionally delete Knn1, we used hGFAP-Cre, a transgenic line that mediates Cre recombination in cortical NPCs starting at E12.5. Knn1fl/fl/hGFAP-Cre (cKO) mice were viable at birth. On postnatal day P4, compared to control (ctrl), cKO mice exhibited a 40% decrease in cortical area (ctrl, 21 ± 1.0 mm²; cKO, 13 ± 0.8 mm², mean ± s.e.m., p = 1.3E−3, two-tailed unpaired t-test) and a 32% decrease in cortical plate thickness (ctrl, 947 ± 9.1 µm; cKO, 643 ± 12.1 µm, p = 2.05E−5), consistent with microcephaly in patients with Knn1 mutations. Knn1 cKO mice were smaller in size and exhibited subviability, with ~75% survival at weaning (P21) and ~50% survival at P150 (n = 11 animals; Fig. 1c).

To investigate neuronal subtypes in cKO brain, we analyzed cortical layer markers. At P4, TBR1-expressing layer (L1/6) and BCL11B(CTIP2)-expressing L5 neurons were not significantly altered in number or lamination (Fig. 1d). However, POU3F2-expressing L2–5 neurons were reduced by 55% (ctrl, 334 ± 31.2; cKO, 151 ± 12.8 per 200 µm column, p = 0.017, two-tailed unpaired t-test) and CUX1-expressing L2–4 neurons were reduced by 85% (ctrl, 206 ± 11.5; cKO, 30 ± 3.2 per 200 µm column, p = 8.8E−6). Agenesis of the corpus callosum was observed in cKO (Supplementary Fig. 1d). Thus, layer cell fates were specified following Knn1 deletion, but upper layer (L2–4) neurons were selectively reduced, whereas deep layer (L5–6) neurons were not significantly affected.

In cortical neurogenesis, NPCs give rise first to deep layer neurons, then upper layer neurons. The loss of late-born upper layer neurons is consistent with a progressive decrease in NPCs. We therefore analyzed NPCs at E13.5, E15.5, and E16.5. SOX2-expressing apical progenitors (APs) and EOMES(TBR2)-expressing intermediate progenitors (IPs) showed stage-dependent reductions in number, each exhibiting a significant loss by E15.5 (Fig. 1e) and a >50% reduction by E16.5 (EOMES: ctrl, 65 ± 0.9; cKO, 32 ± 3.3 per 200 µm column, p = 7.4E−3, SOX2, ctrl, 138 ± 16; cKO, 51 ± 2.1, p = 0.031, two-tailed unpaired t-test). Thus, deletion of Knn1 led to progressive loss of NPCs. In E16.5 cKO cortex, some SOX2+ NPCs were positioned outside VZ and SVZ, suggesting a disorganization of embryonic cortical layering.

In addition to hGFAP-Cre, we conditionally deleted Knn1 using Emx1-Cre, which mediates Cre recombination in cortical NPCs starting at E10.5. Earlier Knn1 deletion by Emx1-Cre led to microcephaly more severe than hGFAP-Cre (Supplementary Fig. 1e) and a loss of both deep and upper layer neurons (Supplementary Fig. 1f). This increased phenotypic severity is consistent with a progressive loss of NPCs following Knn1 deletion and a requirement for Knn1 by NPCs during both early and late neurogenesis. Together, these results suggest that Knn1 deletion impaired NPC proliferation, survival, or maintenance.
controls (Fig. 2a and Supplementary Fig. 2a). Consistently, DAPI DNA staining revealed an abundance of pyknotic nuclei, a condensation of chromatin and hallmark of apoptosis rarely found in controls (Fig. 2b and Supplementary Fig. 2b). In cKO, pyknotic nuclei showed a stereotyped clustering (arrowheads; Fig. 2b).

We next analyzed the progression of apoptosis in cKO. At E13.5, within one day of hGFAP-Cre onset, CC3 was already increased in cortical wall, which at this age was mostly composed of NPCs, suggesting that apoptosis rapidly followed Knl1 deletion from NPCs (Fig. 2c). At E15.5, near the peak of neurogenesis, CC3 was abundantly present in the germinai zones and intermediate zone (IZ), but was largely absent from CP, where RBFOX3(NeuN)-positive neurons had settled after migration (Fig. 2d). By E17.5, near the end of neurogenesis, CC3 staining was only marginally increased (Fig. 2e). To assess the timing of apoptosis, we tracked NPC using a dual pulse of thymidine analogs 5-ethyl-2'-deoxyuridine (EdU) and 5-chloro-2'-deoxyuridine (GluU) (Fig. 2f, g), which are incorporated into DNA in S-phase. CldU was given at E16.5, 4 h prior to analysis to label cells that recently entered S-phase. In cKO, EdU was given at E15.5, to track cells that entered S-phase 24 h prior to analysis. In
Fig. 2 Robust and rapid apoptosis following Knl1 deletion. a Cleaved Caspase 3 (CC3) immunostaining showed extensive apoptosis in E15.5 cKO cortex (scale bar: 100 μm). b DAPI staining revealed an abundance of pyknotic nuclei in E15.5 cKO cortex (scale bar: 50 μm). Many pyknotic nuclei exhibited a stereotyped clustering (arrowheads). c CC3 immunostaining showed that apoptosis was present in E13.5 cKO cortex, one day after the onset of hGFAP-Cre-mediated Knl1 deletion at E12.5. d At E15.5, near the peak of neurogenesis, CC3 staining (green) was extensive in cortical VZ, SVZ, and intermediate zone (IZ), but largely absent from RBFOX3+ (NeuN+) cortical plate (CP) neurons (red) in cKO. e By E17.5, CC3 staining showed only a moderate increase in cKO compared to ctrl (scale bar: 50 μm). f, g Analysis of E16.5 cortex following a 4-h pulse of CldU (red) and a 24-h pulse of EdU (blue, scale bar: 100 μm) revealed extensive apoptosis in EdU+ cells (open arrowheads) in ctrl or cKO, but revealed extensive apoptosis in EdU+ cells (solid arrowheads) in cKO. EdU+ migrating neurons (yellow arrows) were present in the IZ of ctrl but largely absent from cKO (inset scale bar: 20 μm). h Quantification of CC3 colocalization with CldU or EdU (mean, two-tailed unpaired t-test, n = 3 animals). LGE lateral ganglionic eminence, h hours.

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This suggested that in cKO, a significantly increased proportion of cells was unable to progress normally through M-phase, which is in agreement with a recent in vitro study of a patient KNL1 mutation33. Consistent with incompletion or delayed completion of mitosis, cKO cells largely failed to become post-mitotic neurons 24 h after S-phase. In control, many cells labeled with a 24 pulse of EdU were present in the IZ (yellow arrows, Fig. 2g), a region containing newly post-mitotic neurons migrating to the CP. In cKO, however, EdU-labeled cells largely failed to enter the IZ (Fig. 2g and Supplementary Fig. 2e). Together, these data revealed that cKO NPCs largely failed to complete mitosis or...
Knl1 deletion led to DNA damage and p53 activation in NPCs.

Apoptosis is controlled by multiple regulatory mechanisms, including transcriptional regulation. To gain insights into potential transcriptional mechanisms underpinning apoptosis in Knl1 cKO, we performed transcriptome analysis. We reasoned that although apoptosis activates mRNA decay, the initial transcriptional events that trigger apoptosis, and potential population changes in the transcriptome, can be detectable. We generated unique molecular identifier (UMI) RNA-seq libraries by Click-seq of cKO and littermate control cortices at E15.5, the peak of apoptosis in cKO. In Click-seq, UMI tagging of individual cDNA molecules enabled de-duplication of PCR-amplified reads. Analysis of ERCC spike-in RNA standards revealed excellent quantification within a dynamic range that covered essentially all analyzed genes (Supplementary Fig. 3a). Analysis of differential gene expression using edgeR revealed a total of 47 differentially expressed genes (68 transcripts including gene models) in cKO with a false discovery rate (FDR) of <0.05, (Fig. 3a, b and Supplementary Table 1). Strikingly, of the 19 upregulated genes, 8 are known to be transcriptionally activated by the tumor suppressor p53, bound at their genomic locus by p53, or both. Among the top three upregulated genes, Ano3, Eda2r, and Pvt1, and downregulation of Eomes in cKO compared to ctrl (mean, two-tailed unpaired t-test, ctrl: n = 7, cKO: n = 4 animals). Immunostaining of E15.5 cortex revealed significant increases in the number of cells labeled by p53 or DNA damage marker γH2AX in cKO compared to ctrl (mean, two-tailed unpaired t-test, n = 3 animals, scale bar: 100 μm). p53 was localized to nuclei (solid arrowheads) and present in pyknotic cells (open +). Double immunostaining revealed p53 activation in SOX2+ NPCs in E15.5 cKO VZ (arrowheads). The vast majority of cells (89 ± 1.5%) with p53 activation were SOX2+ NPCs (mean, cKO: n = 4 animals), coloc. colocalization.

Fig. 3 DNA damage and activation of p53 and p53 target genes in Knl1 cKO brain. a Unique molecular identifier (UMI) RNA-seq volcano plot comparing cKO (n = 4) to ctrl (n = 7 animals) E15.5 cortex. P-value was calculated with likelihood ratio tests and false discovery rate (FDR) was calculated using the Benjamini–Hochberg procedure. Differentially expressed genes (FDR < 0.05) are indicated in red. b All 19 significantly upregulated genes, p53 target genes (pink), microglial genes (blue), and interneuronal genes (green) are indicated. Droplet digital RT-PCR (ddRT-PCR) validated upregulation of p53 target genes Ano3, Eda2r, and Pvl1, and downregulation of Eomes in cKO compared to ctrl (mean, two-tailed unpaired t-test, ctrl: n = 7, cKO: n = 4 animals). d Immunostaining of E15.5 cortex revealed significant increases in the number of cells labeled by p53 or DNA damage marker γH2AX in cKO compared to ctrl (mean, two-tailed unpaired t-test, n = 3 animals, scale bar: 100 μm). p53 was localized to nuclei (solid arrowheads) and present in pyknotic cells (open +). e Double immunostaining revealed p53 activation in SOX2+ NPCs in E15.5 cKO VZ (arrowheads). The vast majority of cells (89 ± 1.5%) with p53 activation were SOX2+ NPCs (mean, cKO: n = 4 animals), coloc. colocalization.
cell type-dependent apoptosis in Knl1 cKO and supported p53-activated transcription as a pathway towards apoptosis.

In the absence of DNA damage, p53 is targeted by ubiquitin ligase MDM2 for degradation. Following DNA damage, MDM2 is phosphorylated by ATM, which abrogates MDM2 degradation of p53. We immunostained for stabilized p53, and found a widespread loss of p5341. We immunostained for stabilized p53, and found a substantial decrease in p53 levels in cKO (Fig. 3d). The vast majority of p53+ cells were SOX2+ (89 ± 1.5%, Supplementary Fig. 3c), suggesting that DNA damage and p53 activation occurred in NPCs. By co-staining p53 with SOX2 (APs), SOX2+ neurons, or pKAP1+ cells were preferentially located in VZ and SVZ, and largely absent from CP, suggesting that DNA damage and p53 response occurred in NPCs. To directly identify cells with disrupted SAC, we co-stained p53 with SOX2 (APs), SOX2 (IPs), or RBFOX3 (neurons) in E13.5 cKO (Supplementary Fig. 3d). The vast majority of p53+ cells were SOX2+ (89 ± 1.5%, Fig. 3e), suggesting that DNA damage and p53 activation occurred in NPCs following Knl1 deletion. Supporting this, γH2AX and p53 were widespread in cKO VZ by E13.5 (Supplementary Fig. 3e), when the majority of cortical cells were NPCs and undergoing massive apoptosis at E15.5. Together, these analyses suggested that DNA damage, acquired in mitotic NPCs, elicited p53-dependent response following Knl1 deletion.

Missegreted chromosomes with DNA damage in Knl1 cKO NPCs. Disruption of SAC components causes chromosome missegregation and numerical aneuploidy. Aneuploidy, however, can be tolerated by neural cells and does not inhibit neuronal development. The doubling of missegreted chromosome in the cleavage furrow that are prone to DNA damage can give rise to lagging chromosomes at the cleavage furrow. In this context, we found an abundance of anaphase cells, only a minority of which exhibited potential chromosome missegregation. In striking contrast, in control E15.5 VZ, we found an increase in VZ cells labeled by phospho-(p)KAP1 (TRIM28) (Supplementary Fig. 3c), an ATM substrate and marker of heterochromatic DSBs. Notably, p53+, γH2AX+, or pKAP1+ cells were preferentially located in VZ and SVZ, and largely absent from CP, suggesting that DNA damage and p53 response occurred in NPCs. To directly identify cells with disrupted SAC, we co-stained p53 with SOX2 (APs), SOX2 (IPs), or RBFOX3 (neurons) in E13.5 cKO (Supplementary Fig. 3d). The vast majority of p53+ cells were SOX2+ (89 ± 1.5%, Supplementary Fig. 3e), suggesting that DNA damage and p53 activation occurred in NPCs following Knl1 deletion. Supporting this, γH2AX and p53 were widespread in cKO VZ by E13.5 (Supplementary Fig. 3e), when the majority of cortical cells were NPCs and undergoing massive apoptosis at E15.5. Together, these analyses suggested that DNA damage, acquired in mitotic NPCs, elicited p53-dependent response following Knl1 deletion.

Attenuated apoptosis following Knl1/Trp53 co-deletion. Although co-deletion of Trp53 abolished the p53-dependent transcriptional response, chromosome missegregation from SAC disruption should persist in dKO. Indeed, we found significant increases in chromosome missegregation and DNA breaks in dKO (Fig. 6a–c and Supplementary Fig. 6a). Next, we investigated whether apoptosis could be elicited in the absence of p53 and/or Knn1. We found an increase in CC3 immunostaining in E15.5 dKO compared to control (Fig. 6d), but that increase was modest compared to the p53-intact cKO. Pyknosis
was also present (Fig. 6e, f, ctrl, 4 ± 0.6; cKO, 327 ± 6.9; dKO, 18 ± 2.7 per 200 µm column). But compared to cKO, pyknosis was not increased to the same extent (dKO 18-fold lower than cKO, \( p = 1.2 \times 10^{-13} \), ANOVA with post-hoc t-test), suggesting that although apoptosis was present, it was less efficient compared to p53-dependent mechanisms. This was supported by reduced clearance of γH2AX+ cells (Fig. 6g). Compared to cKO, γH2AX+ cells were 1.7-fold more numerous in dKO (cKO, 42 ± 2.6, dKO, 71 ± 4.3 per 200 µm column, \( p = 3.7 \times 10^{-4} \)). Cells labeled by heterochromatic DSB marker pKAP1 were also less efficiently cleared (Supplementary Fig. 6d). Together, these results showed that cells with genome damage as a result of Knl1 deletion underwent p53-dependent apoptosis in cKO. In the absence of p53 in dKO, apoptosis occurred at significantly lower levels and was less efficient at clearing cells with genome damage.

Robust phagocytosis of apoptotic cells by microglia in Knl1 cKO. In Knl1 cKO, we found an abundance of pyknotic nuclei compared to controls in the embryonic brain that were cleared by early postnatal ages (Fig. 7a), implicating a robust mechanism for their elimination. In vivo, post-apoptotic cells are eliminated via phagocytosis, by macrophages in peripheral tissues. Our RNA-seq of cKO showed significant increases in Lcp2 and Wdfy4, two genes selectively expressed in microglia (Fig. 3b and Supplementary Table 1)\(^{26,49}\). Microglia are resident phagocytes present...
in embryonic brain as early as E10.5. To analyze microglia, we immunostained for ADGRE1 (F4/80), a marker of macrophagic cells. At E15.5, we found a striking change in microglial morphology (Fig. 7b). Confocal z-stack analysis revealed that microglia acquired a ballooned morphology. Each ballooned microglia contained dozens of phagocytosed pyknotic nuclei (Fig. 7c), which explained their stereotyped clustering (Fig. 2b). Some of the pyknotic nuclei were CC3+, confirming that they were apoptotic cells (Fig. 7c). In cKO, microglial size was increased by 2.5-fold (Fig. 7d, ctrl, 189.8 ± 21.29; cKO, 481.1 ± 35.17 µm²; dKO, 325.4 ± 19.19 µm², p = 9.2E–3; number: cKO, 12; dKO, 27 ± 2.3 per 400 µm column, p = 0.060, ANOVA with post-hoc t-test), and consistent with significantly lower levels of apoptosis in dKO (Fig. 6d–f). Thus, our analyses of Knl1/Trp3 dKO showed that chromosomal damage can lead to apoptotic and microglial phagocytic responses via a p53-independent mechanism. However, in the absence of p53, these responses were attenuated and significantly less efficient at clearing genome-damaged cells, thus partially alleviating microcephaly associated with Knl1 deletion.

To determine whether the microglial response was associated with p53, we examined E15.5 dKO. We found significant increases in both number and size of microglia compared to control (Fig. 7c–e). These increases, however, were attenuated compared to those in cKO (Fig. 7d, e; ctrl: cKO, 481.1 ± 35.17 µm²; dKO, 325.4 ± 19.19 µm², p = 9.2E–3; number: cKO, 12; dKO, 27 ± 2.3 per 400 µm column, p = 0.060, ANOVA with post-hoc t-test) and consistent with significantly lower levels of apoptosis in dKO (Fig. 6d–f). Thus, our analyses of Knl1/Trp3 dKO showed that chromosomal damage can lead to apoptotic and microglial phagocytic responses via a p53-independent mechanism. However, in the absence of p53, these responses were attenuated and significantly less efficient at clearing genome-damaged cells, thus partially alleviating microcephaly associated with Knl1 deletion.

Unpurged somatic mutants and total pre-weaning lethality in dKO. To assess the efficiency with which p53-dependent mechanisms cleared genome-damaged cells following Knl1 deletion, we karyotyped NPCs from E14.5 cortex. Remarkably, mitotic NPCs in cKO had normal karyotypes comparable to control (chromosome number, p = 0.71, Fisher’s exact test, Fig. 8a, b), suggesting that cells with DNA damage from a previous round of mitosis were largely cleared by p53-dependent
responses prior to the next cell cycle. The remaining mitotic cells, therefore, were karyotypically normal. Qualitatively, we observed that the number of metaphase spreads were lower in cKO compared to control, which was consistent with loss of NPCs in cKO. To determine the genomic consequences of Knl1 deletion in the absence of p53, we karyotyped NPCs from dKO. Strikingly, aneuploidy was found in the majority of dKO NPCs in the absence of p53, we karyotyped NPCs from dKO. Consistent with this, microcephaly as a result of severe genome-damaged cells, if present in substantial numbers triggered rapid p53-dependent apoptotic and downstream microglia phagocytic responses that efficiently eliminated cells with chromosome missegregation from embryonic brain, causing severe microcephaly (Fig. 8d). However, because genome-damaged cells were cleared, the remaining cells were largely euploid, and cKO organismal survival at weaning was at ~75%. Following co-deletion of Trp53 with Knl1, genome-damaged cells were not efficiently cleared and somatic mutant cells remained in the brain. Despite partial alleviation of microcephaly, the dKO showed total pre-weaning lethality. This paradox suggested that severely genome-damaged cells, if present in substantial numbers in the brain, can impact organismal fitness.

**Discussion**

Genomic mosaicism in the brain is the combined outcome of cumulative developmental and post-developmental somatic mutations, and cellular responses that correct or purge these mutations. In this study, we manipulated somatic genome...
stability during embryonic brain development and gained insights into the robust mechanisms that eliminate cells with somatic genome damage from the brain, and the consequences of leaving somatic mutant cells unpurged.

Mammalian cells lack an aneuploidy checkpoint, and thus rely on the SAC to ensure correct chromosome segregation during mitosis as a safeguard against aneuploidy. By conditional deletion of Knl1 from NPCs, we found that disruption of the SAC did not inevitably lead to brain somatic aneuploidy. In mitotic NPCs, DNA damage on missegregated chromosomes was a potent trigger for rapid p53-dependent apoptotic and microglial mechanisms that efficiently cleared cells with segregation errors, thus leaving only karyotypically normal NPCs to continue dividing. Protection against brain somatic aneuploidy is therefore multilayered: (1) SAC delays anaphase onset until secure microtubule attachment on all chromosomes and (2) in the event of SAC disruption in NPCs, DNA damage on the resulting missegregated chromosomes triggers p53-dependent mechanisms to remove these cells. In the normal brain, these mechanisms rapidly eliminate the small proportion of NPCs with such genome damage, thereby maintaining genome stability. Applied en masse, as in Knl1 deletion, apoptotic elimination of these cells would lead to massive cell loss and microcephaly. In normal adult brain, the frequency of numerical aneuploidy remains controversial, with rates ranging from 0% to 47% in human brain and 0% to 57% in mouse brain. Brain somatic aneuploidy arises from segregation errors in NPCs. Our finding that the resulting missegregated chromosomes carry DNA damage that triggers elimination of damaged cells is thus consistent with brain-derived somatic aneuploidy being rare in normal brain.

Knl1 is expressed in mitotic cells and functions in the SAC, a mitotic checkpoint. This role of KNL1 and the segregation errors in cKO NPCs convergently support mitosis as the point of primary deficit that ultimately elicits robust apoptotic responses following Knl1 deletion. This is consistent with our result that a majority of cells became apoptotic within 24 h of S-phase (Fig. 2). Furthermore, a human KNL1 mutation causes mitotic defects and cell death in hESC-derived neural progenitors, providing support that the mitotic function of KNL1 in NPCs is conserved. We have not excluded the possibility that apoptosis can also occur post-mitosis after initial mitotic segregation errors. The minority of cells that were not rapidly eliminated within this time frame may undergo apoptosis by other mechanisms, including cell cycle delay. In addition, although microglia phagocytosis is efficient, following massive apoptosis in cKO, clearance of post-apoptotic cells may be delayed, thus explaining transient accumulation of cleaved caspase staining outside the germinal zones (Fig. 2).

Collectively, the DNA damage on missegregated chromosomes, the activation of p53 in NPCs, and the p53-DSB transcriptomic signature converge on DNA DSBs as the key trigger for rapid p53...
activation following \textit{Knl1} deletion. The precise mechanisms that generate DSBs following missegregation remain unknown and would require technical advances from the field to resolve. There are several possibilities. Lagging chromosomes trapped in the cleavage furrow can acquire DSBs during cytokinesis\textsuperscript{44}. However, physical cutting by the furrow is unlikely, as chromosome bridges would trigger the abscission checkpoint\textsuperscript{53}. Micronuclei, formed when lagging chromosomes are excluded from either daughter nuclei, can also carry DNA damage\textsuperscript{43,54}. It has been proposed that the micronuclear envelope is liable to rupture, which can expose DNA to cytoplasmic nucleases\textsuperscript{42}, thus contributing to chromosomal pulverization and chromothripsis\textsuperscript{43,54}. Consistent with this, we found no evidence of persistent micronuclei in postnatal CKO brain, suggesting that they had been eliminated by apoptosis following p53 activation. Furthermore, following SAC disruption, anaphase can proceed prematurely in the presence of under-replicated or insufficiently decatenated DNA, ultimately leading to ultrafine bridges\textsuperscript{39} that can contribute to DNA damage if not resolved during mitosis.

Although aneuploidy has been associated with p53 upregulation, whether aneuploidy itself can be the trigger for rapid p53 activation is debated\textsuperscript{14,56}. Two recent studies have shown that numerical aneuploidy does not inevitably cause p53 activation\textsuperscript{42,57}. However, p53 does respond to structural aneuploidies generated by segregation errors and chromosomal breaks. Consistent with these emerging data, \textit{Knl1} deletion in vivo led to DNA damage on missegregated chromosomes and rapid apoptosis, suggesting that mitotic DNA damage, not aneuploidy itself, triggered the rapid p53-dependent responses. Further supporting this, in \textit{Bub1B\textsubscript{HI}/HI} hypomorphic mutant mice, widespread aneuploidy present in \(\sim 40\%\) of brain cells\textsuperscript{58} did not cause massive apoptosis or developmental microcephaly\textsuperscript{39}. However, we have not excluded the possibility that in the longer term, aneuploidy can contribute to apoptosis.

To investigate the consequence of disrupting both the SAC and p53-dependent safeguards against brain somatic aneuploidy, we co-deleted \textit{Trp53} with \textit{Knl1}. Following \textit{Trp53} co-deletion, segregation errors and DNA damage persisted, but the robust transcriptional, apoptotic, and microglial phagocytic responses were largely abrogated. Without p53, NPCs with chromosome mis-segregation were not eliminated and widespread mosaic aneuploidy was present, thus confirming p53-dependent mechanisms as a second safeguard against brain somatic aneuploidy. Without massive elimination of somatic mutant cells, \textit{Trp53} co-deletion partially alleviated microcephaly resulting from \textit{Knl1} deletion, but paradoxically led to complete pre-weaning lethality. The precise cause of DKO lethality, however, is unknown. At the cellular level, aneuploidy, unrepaired DNA damage, or both could contribute. At the organismal level, DKO pups were able to breathe and suckle, and we have not been able to ascertain potential causes of pre-weaning death. Our results, however, do strongly support the possibility that somatic mutant cells derived from divisions in the absence of both SAC and p53-dependent safeguards, if present in substantial numbers, can affect brain and organismal fitness.

Altered SAC function has only recently emerged as a mechanism of microcephaly\textsuperscript{19,22}. We found that SAC disruption in \textit{Knl1} CKO led to microcephaly, but likely not as a direct result of aneuploidy. Instead, SAC disruption caused frequent segregation errors in NPCs. DNA damage on the resulting missegregated chromosomes triggered robust removal of damaged cells, resulting in large-scale cell loss and microcephaly. Interestingly, centrosomal deficit can cause microcephaly and activate p53 without
increasing γH2AX, suggesting that centrosomal dysfunction does not necessarily cause DBS, unlike Knl1 deletion. These results thus highlight mechanistic differences between SAC disruption and centrosomal dysfunction in microcephaly.

Microglia have emerged as key players in CNS development, homeostasis, and disease. In Knl1 cKO, microglia underwent morphologic activation, increased in number, and exhibited a remarkable capacity for eliminating apoptotic cells with genome damage. Our work thus implicated a novel role for microglia in maintaining genome stability during embryonic development. Interestingly, recent work suggested that microglia can induce apoptosis of brain cells. Thus, in vivo, microglia may accelerate apoptosis in addition to clearing apoptotic debris, playing more than one role in curtailing the propagation of cells with genome damage in the brain.

**Methods**

**Mouse lines.** A conditional–ready lacZ reporter Knl1 allele (Knl1tm1a(EUCOMM)Hqnp, EUCOMM (HEP00665, 2_E05)) was obtained from EUCOMM and recovered from cryopreserved spermatozoa at the University of Michigan Transgenic Core. The Knl1fl/fl conditional allele was generated by crossing with a Flpo recombinase transgene to remove the neomycin resistance gene cassette from the germline via FRT site recombination. Neomycin cassette-deletion was confirmed by PCR. The Knl1fl/fl mice were used to generate conditional mutants and genotyped by PCR. The mice used in this study were: p53tm1a (The Jackson Laboratory, Stock No. 008462), ROSA26fl/+ (The Jackson Laboratory, Stock No. 012930), IGFAP-Cre (The Jackson Laboratory, Stock No. 004600), and Emil1-Cre (The Jackson Laboratory, Stock No. 005628). All experiments were carried out in compliance with ethical regulations for animal research. Our study protocol was reviewed and approved by the University of Michigan Institutional Animal Care & Use Committee.

**Primary culture of mouse NPCs.** Mouse NPCs were isolated from Knl1 cKO, Knl1flTrp53 dKO, and littermate control E15.5 cortices, enzymatically dissociated with Papain/Dispase/DNase1 (Worthington Biochemical), and cultured in neurobasal medium ( Gibco) supplemented with bFGF ( Gibco), B27 ( Gibco), Glutamax (Invitrogen), and penicillin/streptomycin ( Invitrogen) on glass coverslips precoated with poly-L-ornithine and laminin.

**Immunohistochemistry and confocal imaging.** Embryonic and neonatal brains were dissected and fixed by immersion in 4% PFA overnight at 4 °C. Mouse brains were sectioned at 70 µm with a Vibratome VT1000S (Leica). For immunostaining, brain sections were incubated in blocking solution containing 5% normal donkey serum (Jackson Immuno Research Laboratories), 1% BSA, 0.1% glycine, 0.1% L-lysine, and 0.3% TritonX-100 in PBS for 1 h. Sections were then incubated overnight at 4 °C in primary antibodies diluted in blocking solution, washed, and incubated with the appropriate fluorescent secondary antibodies and DAPI or processed for immunohistochemistry. Stained sections were mounted with VECTASHIELD Antifade Mounting Medium (Vector Laboratories) and analyzed. Images were acquired with the Olympus confocal microscope FV1000. ImageJ and Photoshop were used for image processing. Primary antibodies: rabbit anti-SOX2 (Millipore,1:500), rat anti-SOX2 (Thermo Fisher Scientific, 1:500), rat anti-EOMES (TBB2) (Thermo Fisher Scientific, 1:500), chicken anti-RBFOX3 (NEUN) (Millipore, 1:500), rabbit anti-cleaved Caspase-3 (CC3) (Cell Signaling Technology, 1:500), mouse anti-VIM (phospho-Ser55) (Enzo Life Sciences, 1:500), rat anti-BCL11B (Abcam, 1:500), rabbit anti-CUX1 (Santa Cruz Biotechnology, 1:250), rat anti-Ki67 (Abcam,1:250), rabbit anti-p53 (Leica Microsystems, 1:250), rabbit anti-histone H2A.X (phospho-Ser139) (Cell Signaling Technology, 1:250), rabbit anti-IBA1 (Alkf) (Wako, 1:500), rabbit anti-ADGRE1 (F4/80) (Abcam, 1:500), goat anti-POU3F2 (BRN2) (Santa Cruz Biotechnology, 1:250), rabbit anti-TBR1 (Abcam, 1:500), rabbit anti-phospho KAP1(S824) (Bethyl, 1:500), mouse anti-SATB2 (Abcam, 1:500), rat anti-CldU (Accurate Chemical and Scientific Corporation, 1:250), chicken anti-GFP (Abcam,1:500), rabbit anti-GFP (Thermo Fisher Scientific, 1:500). Secondary antibodies: Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Labs, 1:250), Alex Fluo 488 AffiniPure Donkey Anti-Rat IgG (H+L) (Jackson ImmunoResearch Labs, 1:250), Alexa Fluor 488 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Labs, 1:250), Alexa Fluor 488 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Labs, 1:250), Alexa Fluor 488 AffiniPure Donkey Anti-Rat IgG (H+L) (Jackson ImmunoResearch Labs, 1:250), Alexa Fluor 488 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Labs, 1:250), Alexa Fluor 488 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Labs, 1:250).

**RNA purification and droplet digital PCR (ddPCR).** Total RNA was isolated from freshly microdissected embryonic 15.5 cortices using Trizol and RNA Clean & Concentrator Kit. 600 ng of RNA was used as input for cDNA synthesis using SuperScript III (Invitrogen). Diluted cDNA was used for expression level analysis using ddPCR (QX200 Droplet PCR System, Bio-Rad). Probe sets (Prime-Time qPCR Probe Assays) were purchased from Integrated DNA Technologies. For ddPCR, the reaction mixture was prepared with template cDNA, 2XddPCR Supermix (No UTP) (Bio-Rad), target probes with HEX fluorescence, and control probe against house-keeping gene Sptz2 with FAM fluorescence. Oil droplets were generated with the QX200 droplet generator (Bio-Rad) and transferred to the C1000 Touch Thermal cycler (Bio-Rad) for thermocycling. Following PCR amplification, the droplets were analyzed in the QX200 droplet reader (Bio-Rad), which analyzed each droplet individually using a two-color detection system.

**Primers.** Primers and probes for ddRT-PCR: 

**Prt1:**

- primer1 GCCACCTGCAAATCTCTGT
- primer2 CACTGAAAACAAAGACCGAAAC
- probe /56-FAM/TCCAGAGTGTZeni/CAGGAGAAGATGACA/3IABkFQ/Eomes:
- primer1 CAGAAGAAAACCTGTTCCCTCAT
- primer2 GTCCTCAGTGCTCAGTTGA
- probe /56-FAM/AGCCGCTGTCAGTTGACCTCA
- primer1 CGCGACCACACCCTCCGAGA
- probe /56-FAM/GAGACGACTCA/3IABkFQ:
- Genotyping primers:
  - Knl1/fl_200 F TCAAGATTHTTGTAGATGCCC
  - Knl1/fl_200 R GAACGGATTTGGATGTTGGAGAG
  - probe /56-FAM/GGAGCAACA/3IABkFQ:
  - Genotyping primers:
    - Knl1_fl_200_F TCAAGATTHTTGTAGATGCCC
    - Knl1_fl_200_R GAACGGATTTGGATGTTGGAGAG

CluD, IdU, and EdU labeling.**

**Methods**

**CluD, EdU, and IdU labeling.** Timed-pregnancies were obtained by detection of a vaginal plug (E0.5). Timed-pregnant mice were pulse-intraperitoneally with thymidine analogues CluD, IdU, or EdU at 0.05 mg/g of body weight. Mice were analyzed for incorporation of CluD or IdU using specific antibodies. Incorporation of EdU was analyzed by Click reaction using fluorescent azide.

**Metaphase chromosome spread.** Mouse NPCs were isolated from Knl1 cko, Knl1flTrp53 dko, and littermate control E14–15 embryonic cortices and cultured in Neurobasal Medium (Gibco) supplemented with bFGF (Gibco), B27 (Gibco), Glutamax (Invitrogen), and Penicillin/Streptomycin (Invitrogen). Cells were cultured in less than 36 h, and treated with colcemid (10 ng/ml) and MG132 (10 µm) for 3 h. Cells were then subjected to hypotonic swelling (by incubating in 0.56% KCl) for 15 min and fixed in Carnoy's fixative (75% methanol, 25% acetic acid). Chromosome spreads were prepared on clean, dry slides.

**Comet assay.** Single cell gel electrophoresis comet assay was performed using the SCGE assay Kit (Enzo Life Sciences). E14.5 embryonic cortices were dissected from Knl1 cko, Knl1flTrp53 dko, and littermate control, dissociated into single cells, and resuspended in pre-chilled PBS. Cells were mixed with low melting point agarose at a volume ratio of 1:50 and transferred in 100 pl aliquots onto pre-warmed slides. Slides were immersed in pre-chilled lysis solution for at least 1 h and placed in pre-chilled alkaline solution for 1 h. Electrophoresis was performed at 22 V in the TBE buffer for 30 min. Comets were stained with CY3GREEN dye for 30 min and imaged.

**Anaphase analysis.** NPCs were cultured on coverslips and pulsed with EdU for 12 h. Cells were washed with PBS and fixed with 4% PFA for 30 min at room temperature and washed three times with PBS for about 20 min. Cells were then subjected to Click reaction using fluorescent azide and immunostained with anti-IBA1 primary antibody as described above. DNA was counterstained with DAPI pulsed in PBS for 5 min. Coverslips were mounted with VECTASHIELD Antifade Mounting Medium (Vector Laboratories).
ClickSeq. RNA-seq libraries were generated using ClickSeq36. Total RNA was extracted using TriZol (Invitrogen), treated with DNase1, and cleaned and concentrated using the RNA Clean and Concentrator Kit (Zymo Research). Ribosomal RNA was removed using NEBNext rRNA Depletion Kit (NEB). ERCC RNA spike-in standards (Thermo Fisher Scientific) were used to assess library quality. Reverse transcription was carried out with SuperScript II (Invitrogen) using 1:30 5 mM AaDNTP-DNTP and 5′ Genomic Adapter-6N RT primer (GTGACTGGAGTTCA′ uridylated intermediates degraded by DIS3L2.

Image data analysis. Image data quantification was performed using ImageJ (Fiji) with age-matched and spatially matched brain sections from mutants and control cortices.

Statistical analysis. Knl1 mutant and control group comparison statistical analysis were performed using GraphPad Prism 7 (GraphPad Software) using unpaired two-tailed Student’s t-test. A p-value of <0.05 was considered statistically significant. ERCC quantification performed by Salmon using ERCC Mix 1 sequences34. The mean and 95% confidence interval were calculated by aggregating all measurements of ERCC sequences of equal concentration across all samples. Gene lists associated with p53 activation and microglia were matched by gene symbol. Knl1 EKO and dKO intersectional comparison was performed using a paired two-tailed Student’s t-test.

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

Data availability

The RNA-seq data that support the findings of this study have been deposited to NCBI GEO with the accession number GSE114956. The source data underlying Figs. 1–8 and Supplementary Figs. 1–6 are provided in a Source Data file.

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