Rad50 Is Dispensable for the Maintenance and Viability of Postmitotic Tissues

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The majority of spontaneous chromosome breakage occurs during the process of DNA replication. Homologous recombination is the primary mechanism of repair of such damage, which probably accounts for the fact that it is essential for genome integrity and viability in mammalian cells. The Mre11 complex plays diverse roles in the maintenance of genomic integrity, influencing homologous recombination, checkpoint activation, and telomere maintenance. The complex is essential for cellular viability, but given its myriad influences on genomic integrity, the mechanistic basis for the nonviability of Mre11 complex-deficient cells has not been defined. In this study we generated mice carrying a conditional allele of Rad50 and examined the effects of Rad50 deficiency in proliferative and nonproliferative settings. Depletion of Rad50 in cultured cells caused extensive DNA damage and death within 3 to 5 days of Rad50 deletion. This was not associated with gross telomere dysfunction, suggesting that the telomeric functions of the Mre11 complex are not required for viability. Rad50 was also dispensable for the viability of quiescent liver and postmitotic Purkinje cells of the cerebellum. These findings support the idea that the essential functions of the Mre11 complex are associated with DNA replication and further suggest that homologous recombination is not essential in nondividing cells.

The Mre11 complex regulates both DNA damage checkpoint function and repair. Its checkpoint functions appear to be primarily related to its role as a DNA double-strand break (DSB) sensor which binds DNA damage and activates ATM (ataxia-telangiectasia [AT] mutated). The ATM kinase transduces the damage signal via phosphorylating mediators of the damage response (30, 42), which promotes cell cycle arrest, DNA repair, and apoptosis. Mre11 complex functions are compromised in the human chromosome instability syndromes Nijmegen breakage syndrome and AT-like disorder, which are caused by hypomorphic mutations in Nbs1 and Mre11. Cells derived from patients and from mouse models of these diseases exhibit spontaneous DNA damage, ionizing radiation (IR) sensitivity, and checkpoint defects (25, 27, 48, 52, 57).

The complex’s primary role in DNA repair is in recombinational DSB repair, and this role likely underlies its essential nature. In Saccharomyces cerevisiae, the complex governs homologous recombination (HR) and nonhomologous end joining (NHEJ) (19), whereas in vertebrate systems it primarily functions in HR (51, 61, 62). In fact, studies of Nbs1-deficient cells suggest that the Mre11 complex may inhibit NHEJ in mammals (62). Data from several species also implicate the Mre11 nuclease in the metabolism of topoisomerase adducts (40, 43, 49). This highly conserved function could also explain why the Mre11 complex is essential.

The Mre11 complex’s function at telomeres may also be required for viability. Telomeres protect the ends of linear chromosomes from being recognized as DSBs and thereby activating the DNA damage response (DDR) (9). In S. cerevisiae the Mre11 complex influences telomere length maintenance (5, 28), whereas in mammals the complex interacts with the telomere binding protein Trf2 and localizes to telomeres (63). Loss of Trf2 results in telomere uncapping, causing activation of the DDR, telomere fusions, and senescence (7). Given the association of Mre11 with Trf2, it is conceivable that acute Mre11 complex deficiency in the mouse would phenocopy Trf2 loss and similarly lead to cell death as a result of telomere uncapping.

Conclusions regarding the essential nature of HR in general (33, 47, 53) and the Mre11 complex specifically (10, 17, 45, 59, 62) have been derived from the analysis of proliferating cells in vitro or in vivo. The coincidence of DNA replication and the formation of spontaneous DSBs prompted us to test whether the Mre11 complex and, by extension, HR would be essential in quiescent or postmitotic tissues in which the frequency of spontaneous DSBs is significantly reduced. To examine this issue, we generated mice containing a conditional Rad50 allele in which the Rad50 gene could be inactivated in quiescent and postmitotic cells.

Our results indicate that Rad50 is not required for homeostasis or viability of quiescent hepatocytes of the adult liver; nor does it appear to be required for maintenance of postmitotic Purkinje cells of the cerebellum. In contrast, Rad50 was required for viability of proliferating tissue culture and bone marrow cells. Rad50-deficient hepatocytes that were induced to divide via hepatectomy were able to achieve limited proliferation; nor does it appear to be required for maintenance of postmitotic Potter cells of the cerebellum. In contrast, Rad50 was required for viability of proliferating tissue culture and bone marrow cells. Rad50-deficient hepatocytes that were induced to divide via hepatectomy were able to achieve limited proliferation and telomere maintenance. The complex is essential for cellular viability, but given its myriad influences on genomic integrity, the mechanistic basis for the nonviability of Mre11 complex-deficient cells has not been defined. In this study we generated mice carrying a conditional allele of Rad50 and examined the effects of Rad50 deficiency in proliferative and nonproliferative settings. Depletion of Rad50 in cultured cells caused extensive DNA damage and death within 3 to 5 days of Rad50 deletion. This was not associated with gross telomere dysfunction, suggesting that the telomeric functions of the Mre11 complex are not required for viability. Rad50 was also dispensable for the viability of quiescent liver and postmitotic Purkinje cells of the cerebellum. These findings support the idea that the essential functions of the Mre11 complex are associated with DNA replication and further suggest that homologous recombination is not essential in nondividing cells.
Mre11 complex and, by extension, HR may be dispensable in the replication-associate functions of the Mre11 complex account for its essential nature.

MATERIALS AND METHODS

Mice. Mice were housed in ventilated rack caging in a pathogen-free facility. The Institutional Animal Care and Use Committee of Memorial Sloan-Kettering Cancer Center approved animal use protocols. Mice were maintained on a mixed C57BL/6 and 129Sv background. For mice carrying an inducible Rad50 allele (Rad50ind), genotypes were determined using the primers TGTGATCGATGCC AAGGTAATGCGTCT (sense), and TCAGAGAAGCTTGGTGTGATCA ATTCT (antisense). Rad50ind genotypes were determined using CGCTGTTAA CAGACTAGTCGCC (sense) and the antisense primer from the Rad50ind PCR above. All other genotype strategies were described previously (3, 29, 37).

Rad50ind targeting. Details of the Rad50ind targeting construct will be provided upon request. Targeting and Southern blot analyses of embryonic stem cell clones were carried out using previously described methods (37).

Ear fibroblast derivation. Mice were anesthetized with isoflurane, and ear tissues were collected using sterile scissors. Ear fragments were rinsed twice each in 70% ethanol and phosphate-buffered saline (PBS) supplemented with 100 mg/ml kanamycin. Tissue was transferred into 0.3 ml of protease solution (4 mg/ml each of collagenase D and dispase in Dulbecco’s modified Eagle’s medium [DMEM]; filter sterilized), cut into pieces, and incubated at 37°C for 45 min. DMEM (1.5 ml) containing 10% fetal bovine serum (Gemini), 1X glutamine, and 5X antibiotic-antimycotic solution was added, and samples were incubated at 37°C overnight. Cells were dissociated by pipetting, passed through a 40-μm pore-size cell strainer, and plated in DMEM as above except using 1X antibiotic-antimycotic solution. Cells were passaged on reaching confluence and immortalized via transfection with a plasmid expressing simian virus 40 (SV40) large T-antigen. Transformed cells were grown in DMEM plus 10% fetal calf serum (FCS; HyClone).

Rad50 knockdown in cultured cells. Adenovirus was commercially obtained (University of Iowa Gene Transfer Vector Core), and cells were infected in suspension at 2.5 × 105 cells/ml at a multiplicity of infection of 10 under low-serum conditions (2% FCS in DMEM) with 5 μg/ml polybrene for 2 h at 37°C on a rotating shaker.

Lentiviral production, concentration, and determination of titers were carried out using established methods (12, 36). For infections using a lentivirus-Cre-green fluorescent protein vector, cells were infected in suspension at 1 × 106 cells/ml at a multiplicity of infection of 10 in DMEM plus 10% FCS with 5 μg/ml polybrene. Tubes were spun at 1,900 rpm (600 × g) for 90 min, with occasional stops to manually resuspend cells. After viral infection, cells were plated and grown in DMEM containing 10% FCS (HyClone).

Cellular assays. Western blotting was carried out on 40 μg of protein lysates prepared by subjecting cells to three freeze-thaw cycles in NETN buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40 plus protease inhibitors). Binding and washing steps were done in 5% milk and PBS-Tween 200 buffer. Rabbit anti-Rad50 polyclonal (1:5,000; custom Petriti lab antibody m84-7) and antianti mouse monoclonal (1:1,000; AC-40; Sigma) primary antibodies and species-specific secondary antibodies (Pierce) were used. For horseradish peroxidase signal was detected with ECL Plus reagent (Amersham). m84-7) and antiactin mouse monoclonal (1:1,000; AC-40; Sigma) primary antibodies. Slides were washed two times for 15 min in wash I (0 mM Tris-Cl, pH 7.2, 0.1% bovine serum albumin, with DAPI added to the second wash), dehydrated in ethanol as above, and dried; coverslips were mounted. A total of >1,000 metaphase chromosomes from a minimum of 15 spreads were analyzed.

For immunofluorescence-FISH, cells were seeded on multwell slides, fixed for 15 min in 2% PFA, washed in PBS, and blocked for 30 min (1 mg/ml bovine serum albumin, 1% goat serum, 0.1% Triton X-100, 1 mM EDTA). Slides were incubated at room temperature for 3 h with 53BP1 antibody (1:1,000 diluted in blocking solution; Novus), washed, incubated with secondary antibody for 2 h, fixed for 5 min in 2% PFA, and washed in PBS. Slides were dehydrated and denatured for 5 min each under conditions as described for telomere FISH. Slides were hybridized overnight at room temperature using a TelC-fluorescein isothiocyanate probe (1:1,000; Applied Biosystems). Slides were washed two times for 15 min in FISH wash (70% formamide, 2 h in a dark, humid chamber; slides were washed two times in PBS, with DAPI added to the second wash. For IR-treated samples >1,000 53BP1 foci from at least 35 cells were analyzed for colocalization with telomeres; for Cre-treated TsH2ind (Trf2-deficient) and Rad50ind (Rad50-deficient) samples, >200 foci from at least 18 cells were analyzed; for untreated samples and Rad50ind (Rad50-proficient) Cre-treated samples, 53BP1 focus-positive cells were rare, but >65 foci from at least 11 cells were analyzed.

Partial hepatectomy (PH) and liver regeneration analyses. Ms-Cre-mediated Rad50 deletion was induced by injecting 6- to 12-week-old mice twice with 400 mg of poly(I)-poly(C) ([poly-I-poly(C)] Sigma) intraperitoneally at 48-h intervals. PHs were performed after a wait of >28 days for bone marrow recovery. Mice were anesthetized with isoflurane, an incision was made above the abdomen, the large left and two median lobes of the liver were extruded, and lobes were ligated with silk suture and excised. Incisions were cleaned and stitched, and analgesic was applied. Studies conducted on mice at 2, 3, 4, and 6 weeks post-PH were performed in triplicate; studies at days 1 and 6 post-PH were performed in duplicate. For proliferative indices, bromodeoxyuridine (BrdU) injections were administered at 24-h intervals (50 mg/g of body weight) up to 3 days post-PH. Livers were used for calculation of regeneration kinetics, genotyping, and histological preparations.

Histological sample preparation and staining. Tissue samples for histological analyses were fixed overnight at 4°C in 4% PFA, stained at 4°C in 70% ethanol, and then processed for paraffin embedding. Sections (8 μm) were prepared, and were dehydrated and stained at the Memorial Sloan-Kettering molecular cytogenetics core for γH2AX, phospho-H3, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay, and Rad50. p16 and p19 staining was carried out by the Memorial Sloan-Kettering pathology core. For γH2AX and TUNEL analyses, >200 cells per sample were scored. For phospho-H3 analyses >250 cells per sample were scored for determining mitotic index and >45 cells per sample were scored for phospho-H3. Phospho-H3-positive nuclei with chromatin beginning to condense were classified as prophase; those with condensed unaligned chromosomes were scored as prometaphase; aligned, unsegregated chromosomes were scored as metaphase; condensed chromosomes undergoing segregation were scored as anaphase; and condensed sister nuclei were scored as telophase.

Behavioral analyses. To assess gait abnormalities in P52Cre-cm experiments, mice were conditioned to walk down a corridor, and gait was recorded by dipping front paws in nontoxic red paint and hind paws in blue paint. Measurements from three to four runs per mouse were averaged. At 4 months, two controls and five mutants were analyzed; at 16 months three controls and four mutants were analyzed. To assess balance, studies were conducted as described previously with two controls and four mutant mice at 16 months (22).

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RESULTS

Rad50 gene targeting and deletion in cultured cells. We generated mice carrying an inducible allele of Rad50 (Fig. 1A and B) to investigate the consequences of Rad50 deficiency in vivo. These mice were bred with the Rad50lox/lox strain (37) to produce Rad50ind mice. A PCR strategy was devised to determine the status of the inducible allele (Fig. 1A), and in conjunction with the Rad50lox-specific PCR (37), final genotypes were determined.

SV40-immortalized ear fibroblast cultures were derived and used to assess the effects of Rad50 deletion in vitro. Adenovirus- or lentivirus-mediated delivery of Cre resulted in deletion of the Rad50ind allele and production of the Rad50− allele (Fig. 1A and C). Time course analysis indicated that Cre-mediated recombination was complete at 2 days postinfection (data not shown). At 4 days after lentivirus-Cre infection, Rad50 protein was undetectable (Fig. 2A), and the levels of the remaining complex members were diminished (data not shown). Rad50 depletion was faster in adenovirus-infected cultures, and a moderate increase in phenotypic severity was noted, most likely due to Cre-independent effects of adenovirus. Because of this, adenovirus experiments were terminated at 4 days whereas lentivirus experiments could be carried to 6 days. Because of this, adenovirus experiments were terminated at 4 days whereas lentivirus experiments could be carried to 6 days postinfection.

Following Rad50 deletion, cells were examined for indices of spontaneous DNA damage. Four days after adenovirus-Cre infection, 61% of Rad50lox− cells contained two or more γ-H2AX foci, whereas 18% of Rad50lox/lox control cells exhibited this staining pattern ($\chi^2 = 39; P = 3.4 \times 10^{-11}$). Analysis of metaphase spreads indicated that 42% of Rad50lox− cells contained two or more aberrations 6 days after lentivirus-Cre infection versus 5% in controls (Fig. 2B) ($\chi^2 = 16; P = 0.0013$). At this time point, cells primarily exhibited chromatid breaks (55.8% versus 10% in controls), but fragments, chromosome breaks, fusions, and tri-/quadra-radial exchange structures were also frequently observed (Table 1 and Fig. 3A; see also Fig. S1A in the supplemental material). Endoreduplicated cells containing four sister chromatids were occasionally seen, as previously noted in Nbs1-deficient B cells (45). Nuclear aberrations were also observed in Rad50-deficient cells including micronuclei, telophase bridges, and fragmented nuclei (see Fig. S1B in the supplemental material). These aberrations were likely the by-product of cell division in the presence ofacentric or dicentric chromosomes.

Consistent with the DNA damage observed, Rad50-deficient cells lost viability rapidly. Fluorescence-activated cell sorter analysis at 4 days postinfection revealed a sevenfold increase in the percentage of sub-G1 events ($P < 0.0013$). Expression of a dominant negative Trf2. Southern blot analysis of telomeric DNA from Rad50lox/lox cells at 2 and 4 days postdeletion did not reveal the presence of aberrantly large telomeric DNA fragments (see Fig. S1C in the supplemental material), as would be expected to arise from telomere fusions and which are seen upon expression of a dominant negative Trf2 (Trf2MMAM) (55). Southern blots also did not reveal precipitous telomere shortening, and despite an increase in the frequency of chromosome fu-
sions, telomere sequence was rarely observed at fusion sites in metaphase spreads examined by telomere FISH (Fig. 3A). Additionally, in contrast to Trf2-deficient cells that exhibit telomere dysfunction-induced DNA damage foci (TIFs) (50), no increase in TIFs was observed in Rad50-deficient cells (Fig. 3B to F). These data indicate that Mre11 complex deficiency does not result in telomere uncapping and argue against telomere dysfunction as the underlying basis of lethality in Rad50<sup>−/−</sup> cells.

**Rad50 deletion in vivo.** To query the role of the Mre11 complex in proliferative and postmitotic settings in vivo, the Mx-Cre transgene was crossed into Rad50<sup>+/ind</sup> mice. In Mx-Cre-containing mice, injection with double-stranded pl-pC induces Cre expression in both proliferative and nonproliferative tissues, including bone marrow, thymus, and liver (10, 29). Cre expression in the liver enabled analysis of Rad50 deficiency under both quiescent and proliferating conditions since quiescent hepatocytes can be stimulated to proliferate by resection of the liver mass.

The floxed Rad50 allele was inactivated in adult Mx-Cre transgenic mice, and bone marrow samples were analyzed. Genotype PCR results indicated that deletion of the Rad50<sup>ind</sup> allele occurred within 6 days of pl-pC administration (data not shown). Since all mice were alive at 4 weeks and showed no signs of anemia, we hypothesized that deletion did not occur in 100% of hematopoietic stem cells, permitting repopulation of the bone marrow with hematopoietic cells that had escaped deletion. We confirmed this at 7 weeks after pl-pC injection, when loss of the Rad50-inducible allele was detected in liver and bone marrow of Rad50<sup>+/-</sup> controls as well as in the liver of Rad50<sup>−/−</sup> animals. At this time, the Rad50<sup>ind</sup> (i.e., functional) allele was still present in bone marrow from Rad50<sup>−/−</sup> animals (Fig. 4A). These data indicate that the viable bone marrow cells detected were those that had escaped Rad50 deletion. This demonstrates that Rad50 is required for the viability of bone marrow cells, consistent with observations made in Nbs1-deficient mice (10).

To assess whether Rad50 deficiency was lethal in quiescent cells, we examined the livers of Rad50<sup>−/−</sup> Mx-Cre mice after administration of pl-pC. Hepatocytes of the adult liver are largely quiescent unless liver injury or resection induces regeneration. Rad50 deletion in the liver (Fig. 4A and 5A) resulted in a slight increase in levels of spontaneous DNA damage as assessed by γ-H2AX labeling (Fig. 5C). Nevertheless, liver function, as inferred from serum levels of liver enzymes, was unaffected up to 36 weeks postdeletion (see Fig. S2A in the supplemental material). Additionally, survival up to 40 weeks postdeletion was not significantly compromised, and no liver-associated pathology was observed. These results indicate that, in contrast to hematopoietic cells, quiescent Rad50-deficient hepatocytes are viable and functional.

We examined a second tissue in which DNA replication is rare, terminally differentiated neurons. The Pcp2-Cre mouse line (3) was used to test the requirement for Rad50 in maintenance of postmitotic Purkinje cells. In this strain, Cre expres-

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sion is activated postnatally under the control of the Purkinje cell-specific Pcp2 promoter. Although loss of Rad50 in Pcp2-Cre mice was evident in postmitotic Purkinje cells of adults (Fig. 4B), no alteration in cerebellar size or architecture was noted, and no increase in spontaneous \( H_{2}AX \) foci or pyknotic nuclei was observed in Purkinje cells of the mutants. Furthermore, mice lacking Rad50 did not show signs of ataxia or balance abnormalities up to 16 months postdeletion (\( P = 0.037; P = 0.85 \)), and only minor gait abnormalities were observed (Fig. 4C) (for stride length at 4 months, Wilcoxon rank sum \( W = 11,591 \) and \( P \) [two-sided] = 0.32; for stride length at 16 months, \( W = 14,206 \) and \( P \) [two-sided] = 0.16). These data support the interpretation that the Mre11 complex is dispensable for the viability of nondividing cells.

**Rad50 deletion in proliferating hepatocytes.** Having established that Rad50 was dispensable in quiescent hepatocytes and postmitotic Purkinje cells, we induced proliferation in Rad50-deficient liver to determine whether proliferating hepatocytes would be similarly robust. The \( Rad50^{\text{ind}} \) locus was deleted in Mx-Cre transgenic mice at least 4 weeks prior to regeneration studies to allow for bone marrow recovery. Mutant and control mice then underwent PH to remove two-thirds of the liver to induce regeneration. In contrast to the bone marrow, which relies upon the stem cell pool for repopulation, the proliferation of hepatocytes accounts for liver repopulation; hence, approximately two cell divisions per hepatocyte are required for full recovery of the liver mass (14). Mean survival after this procedure is approximately 5 days when regeneration is impaired (for examples, see references 4 and 60).

Livers from \( Rad50^{\text{ind}} \) mice lacked detectable signal from the inducible locus when assayed by genotype PCR, both before and after regeneration (Fig. 4A). Furthermore, immunohistochemical staining of kidney and liver sections indicated that Rad50 was present in kidney cell nuclei of all mice, whereas Rad50-deficient hepatocytes exhibited only background levels of staining in comparison to controls (Fig. 5A). These data indicate that regeneration occurred in the absence of Rad50. Rad50\(^{-} \) liver regeneration (as assessed by calcu-
lation of liver weight recovery) did not significantly differ from controls (Fig. 5B) \((\chi^2 = 0.58; P = 0.90)\). Furthermore, liver function (see Fig. S2B in the supplemental material) was uncompromised at 2 and 16 weeks post-PH, and survival up to 36 weeks was comparable to controls.

Although liver function appeared normal, staining with \(\gamma\text{-H2AX}\) antibody revealed a marked increase in the frequency of hepatocytes exhibiting DNA damage foci in \(Rad50^{+/}\) livers following regeneration (Fig. 5C) \((\chi^2 = 30.8; P = 1 \times 10^{-5})\). Damage appeared within 24 h of surgery and peaked at 3 days post-PH when 79% of hepatocytes exhibited two or more foci. Damage persisted at essentially the same level up to 8 days. In contrast, hepatocytes from control livers peaked at 20% \(\gamma\text{-H2AX}\) positive and declined to 1% by 8 days post-PH.

Given the evidence for DNA damage in repopulating hepatocytes, we assessed DNA damage checkpoint activation in that setting. Hepatectomized mice were injected with BrdU immediately after surgery and at 24-h intervals thereafter, a procedure that labels a subset of replicating cells. The cumulative percentage of BrdU-positive hepatocytes at 3 days post-PH, the time frame in which the bulk of regeneration occurs (14), was significantly higher in controls than in mutant livers sections \((38\% \pm 7.7\% \text{ versus } 22\% \pm 7.7\%, \text{respectively}; \chi^2 = 51; P = 8.8 \times 10^{-12})\), suggesting that DNA damage accumulating during repopulation impaired the proliferation of Rad50-deficient hepatocytes. This interpretation predicts that the mitotic index of \(Rad50^{+/}\) hepatocytes at time points during repopulation would be reduced relative to controls.

To assess cell division, liver sections were labeled with the mitotic marker phospho-histone H3. Mitotic cells were observed beginning 2 days posthepatectomy. In control livers, 15% (standard deviation, \(\pm 11\%\)) of hepatocytes had the speckled phospho-H3 pattern of cells at the G2/M border (21) and 4% \((\pm 3\%\)) were present between prophase and telophase of mitosis. The remaining cells were either in G0 or interphase (Fig. 6A; see Fig. S2C in the supplemental material for examples of G2/M and G2/interphase nuclei). In Rad50-deficient livers 27% \((\pm 23\%\)) appeared to be at the G2/M boundary and less than 1% \((\pm 0.6\%\)) were in mitosis \((\chi^2 = 79; P = 6.14 \times 10^{-16})\). At 3 days post-PH, mitotic indices had decreased in all mice, but the pattern was similar to that observed at day 2: G2/M levels were approximately threefold higher in mutants, but mitotic cells were 20-fold less frequent than in controls \((\chi^2 = 158; P = 5.6 \times 10^{-18})\). At 4 days post-PH, mitotic ratios were less than 0.1% in all mice. These results show that mitotic progression is impaired in Rad50-deficient hepatocytes. A similar increase in cells at the G2/M boundary was observed in Nbs1-deficient B cells (45).

To determine whether Rad50-deficient cells that enter mitosis ultimately divide, mitotic cells were classified according to their mitotic progress through prophase, prometaphase, metaphase, anaphase, and telophase. These cells were identified on the basis of their morphological and staining patterns as described in Materials and Methods (Fig. 6B). There was a significant difference between mutant and control distributions at 2 days post-PH (Fig. 6B) \((\chi^2 = 24; P = 7.01 \times 10^{-5})\). This difference was due to a reduction in anaphase \((7.1\% \text{ in controls versus } 3.2\% \text{ in mutants})\) and telophase cells \((13.9\% \text{ in controls versus } 4.7\% \text{ in mutants})\). At 3 days post-PH, the distributions did not significantly differ \((\chi^2 = 1.2; P = 0.87)\), and anaphase and telophase frequencies were comparable \((6\% \text{ and } 14\%, \text{respectively, in controls versus } 5\% \text{ and } 13\% \text{ in mutants})\). These data indicate that despite high levels of damage observed in Rad50-deficient cells, a subset of cells are able to enter mitosis and divide either by resolving damage before mitotic entry or by dividing in the presence of damage.
The reduction in proliferation and impaired mitotic progression in Rad50/− mice was associated with a decrease in liver cellularity. A reduction in the average number of cells per field (59 in controls versus 41 in mutants at 8 days post-PH; \( W = 489 \) and \( P \) [two-sided] = 9.92 × 10⁻⁵) and an increase in the average nuclear area per hepatocyte (3,251 pixels in controls versus 4,113 pixels in mutants; \( W = 26,076 \) and \( P \) [two-sided] = 3.8 × 10⁻⁸) suggest that the cellularity of Rad50-deficient livers is reduced and, further, that Rad50/− hepatocytes may undergo endoreduplication.

Since γ-H2AX foci persisted several days after regeneration, we analyzed hepatocytes for markers of apoptosis and senescence. A TUNEL assay of liver sections indicated that there was no increase in apoptotic cells from either mutant or control mice up to 12 days post-PH. Similarly, markers of senescence such as p16 and p19 were absent from repopulating hepatocytes. These data suggest that, as in other proliferating cells examined, Rad50 deficiency has a profound effect on genome integrity in proliferating hepatocytes. The apparent

FIG. 5. Liver regeneration is normal in Rad50-deficient cells, but hepatocytes exhibit indices of DNA damage. (A) Kidney and liver sections from control and Rad50\(^{-/}\) Mx-Cre− mice were immunohistochemically stained with Rad50 antibody. Examples from mice sacrificed 4 weeks after pl-pC injection and 6 days post-PH are shown. (B) Percent liver weight recovery was calculated, and results were graphed for control and mutant mice up to 8 days post-PH. (C) Immunohistochemical staining of liver sections prepared from control and mutant mice were used to determine the percent γ-H2AX-positive hepatocytes before and after PH. Examples of γ-H2AX-labeled sections from Rad50\(^{+/−}\) and Rad50\(^{-/−}\) livers at 2 days post-PH are shown. quiesc, quiescent.

FIG. 6. Rad50-deficient hepatocytes divide at a reduced rate. The mitotic index at 2 days post-PH was determined by immunofluorescent labeling with phospho-H3 and DAPI staining of sections from control and Rad50\(^{-/}\) livers (A). (B) Phospho-H3-positive cells at 2 days post-PH were classified according to the indicated stages, and the percentages in each class were graphed for control and mutant mice. Representative images from the various stages are shown. Red, phospho-H3 (Ph-H3); pseudo-green, DAPI.
persistence of DNA damage notwithstanding, liver function did not appear to be grossly impaired.

**DISCUSSION**

**DNA repair in postmitotic tissues.** In terminally differentiated tissues, the rates of cellular turnover can range from days to months or even decades. In the case of murine Purkinje cells, proliferation ceases at day 14 of embryogenesis (20), and the cells persist over the organism’s life span without dividing. In these tissues, DNA repair associated with chromosome breakage, the most common source of spontaneous DSBs, will be commensurately rare. Spontaneous chromosome breakage has also been attributed to oxidative damage induced by reactive oxygen species and free radical by-products of oxidative metabolism. Nondividing cells, predominantly in G1, lack a sister chromatid and rely upon NHEJ for DSB repair. This may account for the observation that DNA ligase IV-deficient mice exhibit widespread apoptosis in postmitotic neurons (31) and that disruption of HR in the developing nervous system primarily affects proliferating neural precursors (41). These data predict that Mre11 complex-dependent homologous recombination, which promotes the sister chromatid as a repair template (6, 26), may not be required for maintenance of postmitotic cells. To test this hypothesis, we analyzed the effects of Rad50 deletion in postmitotic tissues of the mouse.

As expected from previous studies in proliferating cells (45), Rad50 deficiency led to profound chromosome instability and death within 4 to 6 days. In contrast, that condition was well tolerated in quiescent hepatocytes and postmitotic Purkinje cells, and no overt pathology was observed. These findings support the possibility that the Mre11 complex and, by extension, Mre11 complex-dependent HR are dispensable in nonproliferative tissues.

Implicitly, these data also suggest that DSBs that arise in nondividing cells—some of which likely result from oxidative damage—are not repaired in an Mre11 complex-dependent manner. An alternative possibility is that such DSBs are sufficiently rare that they do not deleteriously affect postmitotic tissue homeostasis. The phenotypic outcomes of NHEJ-deficient mice, in which postmitotic neurons are severely affected (2, 15, 16, 18, 41), argue against the latter possibility. A parsimonious interpretation is that the Mre11 complex does not play a significant role in mammalian NHEJ, and so Rad50 deficiency does not affect the repair of such lesions.

The data also suggest that mitigation of other consequences of oxidative damage such as base modifications is not heavily dependent on the Mre11 complex. It is clear from a number of studies that certain endogenous DNA lesions such as abasic sites can trap topoisomerase I-DNA covalent complexes (43). Recent studies suggest that the Mre11 complex may contribute to the resolution of such intermediates (11, 35, 40, 54) although TDP1 (13, 35) may play a more significant role in this regard. Furthermore, the most common form of oxidative DNA damage in mammalian cells, 7,8-dihydro-8-oxoguanine, stabilizes the noncovalently DNA-bound form of topoisomerase I (32, 44) and thus is not likely to be removed by either TDP1 or the Mre11 complex.

**Essential functions of the Mre11 complex.** The Mre11 complex’s effects on chromosome metabolism are diverse; however, several lines of evidence suggest that its functions are acutely required during DNA replication. The complex associates with chromatin during S phase in undamaged cells and forms foci that colocalize with PCNA (38, 39). Treatment of cells with agents that cause replication fork stalling or collapse results in additional recruitment of the Mre11 complex (34, 39, 56). Recruitment of the Mre11 complex in either circumstance may reflect a role in stabilization, processing, or repair of damaged replication forks.

Furthermore, the major recombinational repair function of the complex is the promotion of DNA damage-induced sister chromatid recombination (6, 26). A primary determinant of this function is the zinc hook domain of Rad50, in which two cysteine residues from each of two protomers coordinate a zinc atom to establish a dimerization interface. This mode of interaction underlies molecular bridging by the Mre11 complex, which we have proposed enforces the physical proximity of participating DSBs (39) in the DSB repair reaction (8, 24, 58).

The chromosomal outcomes observed in Rad50<sup>H9004/H11002</sup> fibroblasts, Mre11-deficient DT40 cells (61), and Nbs1-deficient B cells (45) support the interpretation that the essential function of the Mre11 complex in vertebrates is the metabolism of DNA repair-associated damage. The effects of Rad50 depletion on hepatocyte repopulation are also consistent with the view that proliferation is severely impaired by Rad50 deficiency and suggest that the consequences of Rad50 loss are manifest within the first passage through S phase.

On the other hand, the data do not support the possibility that the complex’s role at the telomere underlies its essential nature as depletion of Rad50 did not result in acute telomere dysfunction. No telomere fusions—which would indicate telomere uncapping—were observed in metaphase cells upon Rad50 deletion, and telomere dysfunction-induced foci (50) were not evident in interphase nuclei (Fig. 3B to F).

The deletion of Rad50 in postmitotic Purkinje cells did not phenocopy the mild ataxia seen in Atm<sup>H11022/H11022</sup> mice (1), even at relatively advanced ages (>12 months) (Fig. 4C and data not shown). This raises the possibility that events during neuronal development may contribute to Purkinje cell loss in AT and AT-like disorder patients. This interpretation is consistent with the report of ataxia in Nestin-Cre mice that lose Nbs1 during development of the nervous system (17) as well as studies implicating ATM (23, 46) and HR (41) during proliferation of precursors rather than maintenance of postmitotic Purkinje cells and neurons.

It remains an open question as to whether other essential members of the DDR pathway are dispensable for the maintenance of postmitotic or quiescent cells. Resolution of this issue is required to fully understand the role of the DDR in vivo.

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