Mammalian RNase H2 removes ribonucleotides from DNA to maintain genome integrity

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Ribonuclease H (RNases H) are endonucleases which cleave the RNA moiety of RNA/DNA hybrids. Their function in mammalian cells is incompletely understood. RNase H2 mutations cause Aicardi-Goutières syndrome, an inflammatory condition clinically overlapping with lupus erythematosus. We show that RNase H2 is essential in mouse embryonic development. RNase H2–deficient cells proliferated slower than control cells and accumulated in G2/M phase due to chronic activation of a DNA damage response associated with an increased frequency of single-strand breaks, increased histone H2AX phosphorylation, and induction of p53 target genes, most prominently the cyclin-dependent kinase inhibitor 1 encoding cell cycle inhibitor p21. RNase H2–deficient cells featured an increased genomic ribonucleotide load, suggesting that unrepaired ribonucleotides trigger the DNA damage response in these cells. Collectively, we show that RNase H2 is essential to remove ribonucleotides from the mammalian genome to prevent DNA damage.
shows clinical overlap with systemic lupus erythematosus (SLE) and, like SLE, is characterized by an uncontrolled type I IFN response. AGS is also caused by mutations in TREX1 (three prime repair exonuclease 1; Crow et al., 2006a) and SAMHD1 (Rice et al., 2009) encoding an intracellular 3′-5′ exonuclease and a deoxynucleoside triphosphate triphosphohydrolase, respectively. Mutations of Trex1 are associated with human lupus (Lee-Kirsch et al., 2007), and Trex1 deficiency results in type I IFN–dependent multi-organ inflammation in mice (Morita et al., 2004; Stetson et al., 2008; Gall et al., 2012). This led to a concept of autoimmunity caused by defective degradation of intracellular nucleic acids and their sensing by innate receptors. In this study, we describe that loss of RNase H2 results in increased numbers of ribonucleotides in genomic DNA, spontaneous DNA breaks, and activation of a DNA damage response in mouse cells.

RESULTS AND DISCUSSION

Early embryonic lethality in Rnaseh2c<sup>−/−</sup> mice

All three subunits of yeast and human RNase H2 are required for enzymatic activity in vitro (Cerritelli and Crouch, 2009) and mutations in any of the three human subunits give rise to AGS. Biallelic null mutations have not been described for any of the three Rnaseh2 genes and presumably result in early lethality. We generated Rnaseh2c<sup>−/−</sup> mice carrying a deletion of the entire coding region (Fig. 1), likely resulting in complete loss of RNase H2 activity. Homozygous embryos were small at embryonic day 9.5 (E9.5) and were not detected at later time points (not depicted), whereas heterozygous neonates were obtained in expected numbers (Fig. 1). Embryos with presumably complete abrogation of RNase H2 activity were also generated by crossing a conditional Rnaseh2b<sup>−/−</sup> mouse line (see H2, topoisomerase I (topo I) processes misincorporated ribonucleotides and is responsible for part of the increased mutation rate (Kim et al., 2011).

Mutations in RNase H2 cause Aicardi–Goutières syndrome (AGS), a pediatric inflammatory disorder resembling intrauterine viral infection (Crow et al., 2006b). The condition
Materials and methods) to a general Cre-deleter strain (Lallemand et al., 1998) and displayed lethality similar to Rnaseh2c−/− embryos (unpublished data). Thus, RNase H2 activity is essential in mouse embryonic development.

Perinatal lethality in mice with reduced RNase H2B expression

In addition to Rnaseh2c−/− mice, a mouse line carrying a gene trap cassette inserted into Rnaseh2b intron 4/5 (Rnaseh2b knockout first [KOF] mice) was analyzed. The gene trap cassette is expected to be spliced to exon 4 and terminate translation (Testa et al., 2004). Mice homozygous for the KOF allele were underrepresented at birth (Fig. 2), were significantly smaller compared with littermate controls, and died perinatally (Fig. 2). Already at E14.5, homozygous embryos were small but present at Mendelian ratios (Fig. 2). Histological analysis revealed proportionally small organs. Many tissues were edematous and displayed extended vessels, as well as bleedings, suggesting defects in vessel wall architecture (Fig. 2). FACS analysis of E18.5 liver and thymus revealed a massive reduction of cellularity (unpublished data). Because the gene trap cassette may allow normal mRNA splicing to some degree, we analyzed E14.5 fetal liver (not depicted) or fibroblasts for residual normal transcripts and found them reduced to a small percentage compared with control levels (Fig. 2). Correspondingly, RNase H2 activity was reduced to 6% of normal values in extracts from E14.5 fibroblasts (Fig. 2) and liver (not depicted). Fibroblasts from two additional Rnaseh2bKOF/KOF and control embryos yielded similar results (unpublished data). Thus, the Rnaseh2bKOF mutation represents a hypomorphic allele with low residual enzyme activity.

RNase H2 deficiency impairs proliferation and triggers a DNA damage response

Rnaseh2bKOF/KOF E14.5 fibroblasts proliferated slower than control cultures (Fig. 3) and this defect became more pronounced with increasing passage numbers. Reduced proliferation of these cultures was not a result of enhanced apoptosis because fractions of propidium iodide+ and annexin V+ cells were not increased (unpublished data). DNA content analysis revealed mildly increased numbers of cells in S and G2/M phase in (asynchronous) 1-wk-old mutant versus control cultures (Fig. 3) and this defect became more pronounced with increasing passage numbers. Reduced proliferation of these cultures was not a result of enhanced apoptosis because fractions of propidium iodide+ and annexin V+ cells were not increased (unpublished data). DNA content analysis revealed mildly increased numbers of cells in S and G2/M phase in (asynchronous) 1-wk-old mutant versus control cultures (Fig. 3). The fraction of mutant cells in G2/M markedly increased with the duration of the culture. In E18.5 fibroblasts, the accumulation of cells in G2/M was striking already after 1 wk of culture, and 4-wk-old E18.5 fibroblast cultures contained 70–80% of cells in G2/M (Fig. 3). This finding suggested that the problem caused by RNase H2 deficiency exacerbates with increasing numbers of divisions.

To test the proliferative capacity of mutant hematopoietic cells, we transferred E14.5 Rnaseh2bKOF/KOF liver cells into lethally irradiated recipients. Transfer of a standard radioprotective...
contaminating non–B cells. Thus, most half of the residual RNase H2 activity was accounted for by from 96.5–97.7% (unpublished data), indicating that about 316 impairs cell cycle progression but does not preclude substantial proliferative expansion of hematopoietic cells. We generated mice by Flpe-mediated deletion of the gene trap cassette of the Rnaseh2b KOF allele to create conditional Rnaseh2b knockout mice. Rnaseh2b FLOX mice were normal in spleen mice (Rickert et al., 1997) for B cell–specific gene inactivation. (Cre-mediated deletion of exon5 introduces a frame shift.) B cell numbers of Rnaseh2b FLOX/CD19-Cre+ mice were depleted to 5.6 ± 2.1% (n = 2). Post-sort analysis determined the purity of the sorted B cells to range from 96.5–97.7% (unpublished data), indicating that about half of the residual Rnase H2 activity was accounted for by contaminating non–B cells. Thus, most Rnaseh2b FLOX/FLOX CD19-Cre+ B cells were completely devoid of Rnase H2 activity. CD19-Cre–driven recombination commences in B cell development and is ongoing at the pre–B cell stage (Rickert et al., 1997). Our findings indicate that complete deficiency for Rnase H2 in precursor B cells does not preclude a limited number of divisions required to fill up peripheral B cell compartments. Proliferation of LPS-stimulated Rnaseh2b FLOX/FLOX B cell cultures was reduced to 80% compared with controls (Fig. 3) with a moderate accumulation of mutant B cells in G2/M (Fig. 3). Collectively, RNase H2 deficiency impairs proliferation but does not preclude considerable proliferative expansion in vivo.

Microarray–based gene expression profiling of Rnaseh2b KOF/KOF versus WT E14.5 liver cells revealed a striking up-regulation of p53–inducible genes in Rnaseh2b KOF/KOF versus control E14.5 liver cells (GEO database accession no. GSE36687). The top seven genes up-regulated in mutant versus control included four p53 target genes, with Cdkn1a, encoding cyclin–dependent kinase inhibitor 1 (p21), being the most prominent up-regulated gene (Fig. 4). Enhanced transcription of these genes was validated by quantitative (q) RT–PCR (Fig. 4).

A large number of genes involved in mitosis showed reduced transcript levels in the mutant samples (unpublished data). Our results suggested that Rnase H2–deficient cells feature cell cycle arrest mediated via p53–induced p21 expression. To detect DNA damage that triggered p53 activation, we stained E18.5 thymocytes and liver cells for phosphorylated histone H2AX (γ–H2AX), which is induced in chromatin flanking double–strand breaks, and found significantly increased numbers of γ–H2AX foci per nucleus in mutant cells (Fig. 4). In addition to enhanced H2AX phosphorylation, we detected increased numbers of spontaneous single–strand breaks in DNA from embryos with complete Rnase H2 deficiency (see next section). Collectively, our data indicate that Rnase H2–deficient cells spontaneously activate a DNA damage response.

Similar to our findings in Rnase H2–deficient cells, compromised proliferation, spontaneous DNA damage, and chronic checkpoint activation were also reported for a Trex1−/− mouse embryonic fibroblast line and fibroblasts from AGS patients with Trex1 mutations by Yang et al. (2007). Trex1−/− cells featured p21 expression, a defective G2/M
cell cycle checkpoint, and a block in G1/S transition (Yang et al., 2007). These findings, however, were not reproduced by Stetson et al. (2008), who did not find impaired proliferation or evidence of a DNA damage response in primary embryonic fibroblasts from *Trex1*Δ/Δ mice. In our primary RNase H2-deficient cells, the G2/M checkpoint seemed to be intact and may be responsible for the observed p21-mediated arrest. Although p21 is well known to block G1/S transition, p21 was also shown to arrest cells in G2 by inhibition of CDK1 (Bunz et al., 1998).

**RNase H2 deficiency results in an increased genomic ribonucleotide load**

DNA from RNase H2-deficient yeast displays enhanced sensitivity to alkaline hydrolysis, indicating an increased genomic ribonucleotide content (Nick McElhinny et al., 2010). To test whether RNase H2 deficiency in mammalian cells is associated with increased numbers of ribonucleotides in DNA, we used bacterial RNase HII to introduce nicks into chromosomal DNA at positions of ribonucleotides. DNA was purified using standard procedures and contains few double-strand breaks as a result of shear stress, resulting in large fragments in the range of several hundred kilobases. Nicks were detected by DNA polymerase I-dependent nick translation incorporating label into the DNA, which was then run on an agarose gel and visualized by autoradiography. Assuming equal mean length of the single strand polymerized de novo by pol I in mutant versus WT DNA and loading of equal amounts of DNA per lane (determined photometrically for each sample), incorporation of label correlates with numbers of nicks present in the genomic DNA. We found massive RNase HII-dependent incorporation of label into DNA from RNase H2-deficient (*mh201Δ; Nick McElhinny et al., 2010; but not WT (Fig. 5) yeast, confirming that the increased sensitivity of mutant yeast DNA to alkaline hydrolysis (Nick McElhinny et al., 2010) was caused by the presence of increased numbers of ribonucleotides. Likewise, DNA from E9.5 *Rnaseh2bΔ/Δ* (Fig. 5) and *Rnaseh2cΔ/Δ* (Fig. 5) embryos displayed RNase HII-dependent incorporation of label, indicating increased numbers of ribonucleotides. Similar results were obtained for two additional *Rnaseh2bΔ/Δ* and four additional *Rnaseh2cΔ/Δ* embryos (unpublished data). Importantly, for all 11 mutant embryos tested, the assay consistently revealed increased incorporation of label compared with DNA from control embryos also in those samples without pretreatment with bacterial RNase HII (Fig. 5 and not depicted), demonstrating that DNA from RNase H2-deficient cells contains higher numbers of spontaneous single-strand breaks as compared with control DNA. The increased genomic ribonucleotide load in RNase H2-deficient mouse cells demonstrates that, like the yeast enzyme, mammalian RNase H2 also functions to remove ribonucleotides from genomic DNA. Our assay did not discriminate whether the accumulating ribonucleotides in RNase H2-deficient DNA represented single nucleotides misincorporated during replication, incompletely removed Okazaki primers, or R-loop structures. Because RNase H1—which cannot cleave at single ribonucleotides but requires at least four subsequent ribonucleotides (Cerritelli and Crouch, 2009)—did not compensate for the loss of RNase H2, it appears likely that the accumulating ribonucleotides in RNase H2-deficient mouse cells are single nucleotides misincorporated by DNA polymerases.

Our data strongly suggest that defective ribonucleotide repair triggers the spontaneous damage response of mutant cells. Breaks in RNase H2-deficient DNA may in part reflect lower chemical stability of the ribonucleotide-containing genome. In addition, topoisomerase-mediated cleavage of ribonucleotide-containing DNA could explain the higher frequency of strand breaks in RNase H2-deficient cells. Topo I was reported to cleave double-stranded DNA substrates containing single ribonucleotides resulting in a single-strand break with one 2',3’-cyclic phosphate end (Sekiguchi and Shuman, 1997; Kim et al., 2011). The damage response could also...
be a result of collisions of widespread topo I cleavage complexes with replication forks resulting in replication-mediated double-strand breaks (Furuta et al., 2003) or to compromised resolution of RNA/DNA hybrids arising from spontaneous errors of RNA biogenesis. Such R-loop structures represent substrates for RNase H activity in yeast and their persistence was demonstrated to predispose to double-strand break formation (Walba et al., 2011; Stirling et al., 2012).

The relevance of the spontaneous damage response we found in RNase H2–deficient mouse cells for the pathogenesis of AGS is unclear. Genome instability and increased incidence of neoplastic disease were not reported in RNase H2–associated AGS. Embryonic lethality, spontaneous DNA damage response, and increased genomic ribonucleotide load were also described in another study of RNase H2 mutant mice (Reijns et al., 2012).

**Lethal phenotype of RNase H2–deficient embryos is independent of type I IFN**

Deficiency for Trex1 causes AGS and/or Lupus, both of which are inflammatory conditions characterized by a chronic type I IFN response (Crow, 2011). Trex1−/− mice develop type I IFN and lymphocyte-dependent autoimmune multi-organ inflammation (Morita et al., 2004; Stetson et al., 2008; Gall et al., 2012). The IFN response initiated in utero before E14 (Gall et al., 2012). Similar to Trex1 mutations, defects in RNase H2 also cause AGS. We therefore expected spontaneous IFN-mediated pathology in our RNase H2–deficient mice. However, crossing the Rnaseh2bKOF strain to type I IFN receptor-deficient (IFNAR1−/−) mice (Müller et al., 1994) did not result in a rescue of perinatal lethality (unpublished data). Both Rnaseh2bKOF/KOF IFNAR1−/− E17.5 embryos obtained so far featured similar growth retardation as Rnaseh2bKOF/KOF animals with intact type I IFN system and living Rnaseh2bKOF/KOF IFNAR1−/− neonates were not found. In accordance with this finding, gene expression profiling of Rnaseh2bKOF/KOF E14.5 liver cells (Fig. 4) did not reveal a clear signature of up-regulated IFN-responsive genes (unpublished data). Few IFN-inducible genes were found up-regulated; however, several of these are also responsive to p53 and may not reflect type I IFN effects in our mice. Inflammatory changes were not found upon detailed histological analysis of serial sections of whole mount E14.5, E18.5, or stillborn Rnaseh2bKOF/KOF embryos (unpublished data). A cross to RAG1−/− mice did not rescue the pathology of Rnaseh2bKOF/KOF animals (unpublished data), indicating that lymphocytes have no role in the lethal phenotype. We speculate that IFN-mediated autoimmune pathology might commence at later stages of ontogeny in mouse models of less severe or conditional RNase H2 deficiency which bypass early lethality. A milder reduction of RNase H2 activity in AGS patients compared with our mouse models may explain that a DNA damage–related phenotype dominates in the latter but not in RNase H2–associated AGS. Whether a low-level chronic DNA damage response contributes to the pathogenesis of AGS and related human disorders remains an open question.

**MATERIALS AND METHODS**

**Mice.** Rnaseh2b−/− mice were generated by homologous recombination in JMB8.65 (C57BL/6) cells. For targeted deletion of all four exons of the Rnaseh2b gene, upstream and downstream homology regions (1,800 and 900 bp, respectively) were amplified from C57BL/6 genomic DNA (primers: 5′ HR up, 5′-GGACTTCTAGATGTCATTTGGCAGCCCTCTGGAGGAGA-3′; 5′ HR down, 5′-CGTACCGGTAGGATTGAAGGTGGAGCCCGCCTTTGAG-3′). PCR products were digested with ApaLI and cloned into the vector pBluescript (using restriction sites contained in the primers) to flank a PGK-gb2-neomycin resistance cassette, which was then inserted into the multiple cloning site. For negative selection against random integration of the targeting vector, a diptheria toxin cassette was inserted downstream of the 3′ homology region. The linearized vector was transfected into embryonic stem (ES) cells and recombinant clones were detected by Southern blot analysis of ApaLI-digested DNA. The probe was amplified from BAC RP23-156A18 (BACPAC Resources Center; primers: H2c probe up, 5′-CTGAAACGAGATGGTGAGTGGACCATGAGGAC-3′; and H2c probe down, 5′-GTCTCTAATAGGCCAGAGAAATTCCTCTTG-3′) and labeled fragments of 9.3 and 5.7 kb in WT and targeted locus, respectively. Positive clones were injected into albino C57BL/6 foster mothers. Chimeric animals were crossed to C57BL/6 mice and offspring was genotyped by PCR (primers: H2c wt forward, 5′-CACAGATCCCCTTCACCACCTTGGA-3′; H2c neo forward, 5′-CTCGTTGCCGAATATCATGGTGAGGAGA-3′; and H2c reverse, 5′-CCGGAAAGACACCCAAAGGAGGAA-3′) to detect germline transmission of the mutation. Heterozygous Rnaseh2bKOF mice (C57BL/6) were purchased from the International Knockout Mouse consortium and were genotyped by PCR as recommended by the vendor. For generation of the conditional Rnaseh2bFLOX mouse line, Rnaseh2bFLOX mice were bred to the Flpe-deleter mouse strain (Rodríguez et al., 2000) and successful deletion of the frt-flanked gene trap cassette was demonstrated by PCR. All mouse strains were on the C57BL/6 background. Mice were housed at the Experimental Centre at University of Technology Dresden, Medical Faculty Carl-Gustav Carus, under specific pathogen-free conditions. All procedures were in accordance with institutional guidelines on animal welfare and were approved by the Landesdirektion Dresden.

**Cell culture.** Upon cesarean section, E14.5 or 18.5 mouse embryos were decapitated and internal organs were removed. Tissue was minced, digested with 1× trypsin (2.5%; Invitrogen) for 15 min at 37°C, and disaggregated by pipetting. The cell suspension was seeded into 10-cm Petri dishes in DME (Gibco) containing 10% fetal bovine serum (Biochrom), 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom), 1× nonessential amino acids (Biochrom), and 100 µM β-mercaptoethanol (Sigma-Aldrich).

**Cell cycle analysis.** Embryonic fibroblasts were seeded into a 6-well plate (5 × 10⁴ cells/well) and incubated for 3 d. Cells were harvested, washed once with PBS, fixed in 70% ice-cold ethanol, and chilled for at least 12 h at −20°C, followed by washing in PBS and resuspension and 10-min incubation at room temperature in 0.5 µg/ml RNase A (Sigma-Aldrich) and 2.5 µg/ml propidium iodide (Sigma-Aldrich). Cells were analyzed on a MACSQuant flow cytometer (Miltenyi Biotec).

**3H-thymidine incorporation.** Embryonic fibroblasts were seeded into flat-bottom 96-well plates (5 × 10⁴ cells/well) and incubated for 48 h in complete DME (see Cell culture). 3.7 × 10⁴ Bq 3H-thymidine (HARTMANN ANALYTICS) was added to each well, and after a further 4 h of culture, cells were harvested and washed. Incorporation of 3H-thymidine was determined using a 1450 MicroBeta TriLux scintillation counter (Perkin Elmer).

**B cell in vitro stimulation.** Total splenocytes were cultivated in 6-well plates at a density of 3 × 10⁴ cells/ml in RPMI 1640 supplemented with 10% FCS, 50 µg/ml penicillin, 50 µg/ml streptomycin, 100 µM β-mercaptoethanol, and 10% FCS.
fetal bovine serum (Biochrom) and 100 U/ml penicillin and 100 µg/ml streptomycin, 2 mM L-selenyl-L-glutamine (Biochrom), 10 µM β-mercaptoethanol, and 25 µg/ml lipo polysaccharide from Salmonella minnesota Re 595 (Sigma-Aldrich). For DNA content analysis, cells were harvested after 3 d. For proliferation assays, 3.7 × 10^6 B^4-Thymidine per well was added after 48 h and the cells were incubated for an additional 24 h, followed by harvesting and quantification of B^4-Thymidine incorporation.

**Quantitative PCR.** Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer’s instructions. For qRT-PCR, RNA was digested with DNase (New England Biolabs, Inc.) and purified using the RNasey Mini kit (QiAGEN). 200 ng–1 µg RNA was reverse transcribed using a poly(dT) primer of the RevertAid H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer’s recommendations. 1 µl of the cDNA product served as template in the subsequent qRT-PCR using the Maxima SYBR green/ROX qPCR Master Mix (Thermo Fisher Scientific) using 0.6 µM target specific primers, initial denaturation at 95°C for 15 min, 40 cycles of denaturation (15 s at 95°C), annealing (30 s at 58°C), and elongation (30 s at 72°C). Incorporation of SYBR green was analyzed using the MX3005P QPCR system (Agilent Technologies) and the MxPro QPCR Software. RT-PCR primers were as follows: p21 up, 5′-GCAATCCACAGCGATATCC-3′; p21 down, 5′-CAACTGTCTACGTTCGGACCG-3′ (Kanatsu-Shinohara et al., 2010); Zmat3 up, 5′-GGTACACTCATCTTCCGACTCCG-3′; Zmat3 down, 5′-GAGGGGCTTAAAGTGAAGGGG-3′; Phlda3 up, 5′-TTCCGGCCCGCATCAAGCGGT-3′; Phlda3 down, 5′-AGGGGGAGGGGGAAGTCGAT-3′ (Brady et al., 2011); Rnaseh2b up, 5′-GAGTCACTCATTCTCGGACTCCGC-3′; Rnaseh2b down, 5′-GTCAATGAGGCGACGACGTTGGTGTTGAAGG-3′; β-actin up, 5′-TGACCCAGATCATGTT-3′; and β-actin down, 5′-GGATGCCATCAACATGCTCCTGTTG-3′. Measured transcript levels were normalized to β-actin. All samples were run in triplicate.

**Microarray analysis of fetal liver of RNaseH2b KO mice.** Fetal livers of E14.5 embryos were homogenized using the gentleMACS Dissociator (Miltenyi Biotec). RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 2006). RNA quality of E14.5 embryos were homogenized using the gentleMACS Dissociator (Miltenyi Biotec). RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 2006). RNA quality was determined on a Bioanalyzer instrument (Agilent Technologies). The RNA was reverse transcribed, amplified, and biotin labeled using the Message Amp II-Biotin Enhanced kit (Ambion). Hybridization, staining, and scanning of the GeneChip Mouse Genome 430 2.0 microarrays were performed according to the GeneChip Expression Analysis Technical Manual P/N 702232 Rev. 3 (Affymetrix). Microarray data were analyzed using GeneSpring GX software. Deregulated genes of interest were validated by qRT-PCR. All microarray data were deposited in the GEO database (accession no. GSE66887).

**Nick translation assay for ribonucleotide content of genomic DNA.** 200 ng mouse or yeast genomic DNA (quantified using an ND-1000 Nanodrop Technologies) was digested with 0.5 U E. coli RNase HII (New England Biolabs, Inc.) in a total volume of 50 µl and the recommended buffer at 37°C for 2.5 h, precipitated in 0.3 M sodium acetate, pH 5.2, and ethanol. DNA was dissolved in water and nick translation reagents were added to final concentrations of 1X buffer 2 (New England Biolabs, Inc.) and 20 µM unlabeled dATP, dGTP, and dTTP (Thermo Fisher Scientific). 5 U E. coli DNA polymerase I (New England Biolabs, Inc.) and 3.7 × 10^7 Bq 32P-dCTP (Hartmann Analytics) were added, and the reaction volume was adjusted to 20 µl. The reaction was incubated at 16°C for 35 min. Labeled high molecular weight DNA was separated from unincorporated nucleotides by electrophoresis on a 0.1% TAE agarose gel and visualized by autoradiography (Phosphorimager St. Molecular Dynamics) of dried gels (GD 2000 Vacuum Gel Dryer system; GE Healthcare).

**Generation of chimeric mice.** Fetal liver cells were isolated from E14.5 embryos and homogenized by repeated aspiration through an 18G needle followed by aspiration through a 20G needle. Cell numbers were determined without lysis of red blood cells. Lethally irradiated (7 Gy) CD45.1/2 congenic recipient mice were reconstituted with 2.5 × 10^6 or 4 × 10^6 total fetal liver cells (1.25 or 20× radioprotective dose, respectively) by retroorbital intravenous injection. Chimerism was assessed in CD11b+ Gr1+ neutrophilic granulocytes by flow cytometry FACSCalibur (BD) at various time points starting at week 4 after transfer.

**Staining for phosphorylated histone H2AX (γ-H2AX).** Cytoplasm of 10^5 E18.5 fetal liver or E18.5 fetal thymocytes were washed in Tris-buffered saline (TBS). Cells were fixed in 4% formaldehyde for 30 min at RT and permeabilized in TBS containing 0.25% Triton X-100 for an additional 10 min at room temperature, followed by blocking with 3% bovine serum albumin (Serva) in TBS for at least 1 h at room temperature, incubation with a 1:500 dilution of the anti-γ-H2AX antibody (clone JBW301; Millipore) in blocking buffer, washing, and detection using a 1:1,000 dilution of Alexa Fluor 488 goat anti-mouse IgG (Life Technologies) in blocking buffer. Nuclei were counterstained with DAPI.

**Assay for RNase H2 activity.** Cell lysates were prepared and assayed for specific cleavage of an 18-bp double-stranded DNA substrate containing a single ribonucleotide in one strand as previously described (Reijns et al., 2011). RNase H2–specific activity was determined by subtracting the cellular activity against a sequence-matched DNA duplex without ribonucleotides. Cell lysate protein concentration was determined and lysates were added to the reaction mix at a final protein concentration of 100 ng/µl for fetal liver and fibroblast lysates. Lysates of B cells contained less protein and were added to reactions to final concentration of 5 ng/µl.

**Statistical analysis.** Data are shown as means ± SD. Statistical analysis was performed with the Student’s t test. The authors thank Martin Reijns and Andrew Jackson for performing enzyme activity assays and for sharing unpublished data. We are grateful to Thomas Kunkel for providing RNase H2–deficient yeast strains, Julia Jarrels and Britta Schilling for microarray analyses, Dora Schreier for ES cell transfection and morula injections, Min-Åke Lee-Kirsch, Werner Müller, Michael Bachmann, Rolf Jessebring, Frank Buchholz, Dragomir Krastev, and Alexander Gerbaulet for advice and discussions, and Christina Hillier and Tobias Häring for expert technical assistance.

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