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Generation of a Mouse Model Lacking the Non-Homologous End-Joining Factor Mri/Cyren

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Abstract: Classical non-homologous end joining (NHEJ) is a molecular pathway that detects, processes and ligates DNA double-strand breaks (DSBs) throughout the cell cycle. Mutations in several NHEJ genes result in neurological abnormalities and immunodeficiency both in humans and mice. The NHEJ pathway is required for the V(D)J recombination in developing B and T lymphocytes, and for class switch recombination in mature B cells. Ku heterodimer formed by Ku70 and Ku80 recognizes DSBs and facilitates recruitment of accessory factors (e.g., DNA-PKcs, Artemis, Paxx and Mri/Cyren) and downstream core factors subunits XLF, XRCC4 and Lig4. Accessory factors might be dispensable for the process depending on the genetic background and DNA lesion type. To determine the physiological role of Mri in DNA repair and development, we introduced frame-shift mutation in the Mri gene in mice. We then analyzed development of Mri-deficient mice as well as wild type and immunodeficient controls. Mice lacking Mri possessed reduced levels of class switch recombination in B lymphocytes and slow proliferation of neuronal progenitors when compared to wild type littermates. Human cell lines lacking Mri were as sensitive to DSBs as WT controls. Overall, we concluded that Mri/Cyren is largely dispensable for DNA repair and mouse development.

Keywords: NHEJ; double-strand breaks; mouse model; lymphocyte; neurodevelopment

1. Introduction

Non-homologous end-joining (NHEJ) is a molecular pathway that recognizes, processes and repairs DNA double-strand breaks (DSBs) throughout the cell cycle [1]. Core NHEJ factors Ku70 and Ku80 form heterodimer (Ku) that is rapidly associated with the DSB sites facilitating recruitment of downstream factors, including core X-ray cross-complementing 4 (XRCC4) and DNA ligase 4 (Lig4). XRCC4-like factor (XLF) is also a core factor that binds XRCC4 and stimulates Lig4-dependent DNA ligation. A number of accessory NHEJ factors are required for specific DNA end processing and DNA complex stabilization, i.e. DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), nucleosome Artemis, and structural components, a paralogue of XRCC4 and XLF (PAXX) and modulator of retroviral infection (Mri) [2, 3]. Mice lacking Ku70, Ku80, DNA-PKcs or Artemis possess severe combined immunodeficient phenotype (SCID), while inactivation of both alleles of Xlf gene results
in 2-3 folds reduced B and T cell count [4-8]. Mice lacking PAXX or Mri possess no or very modest phenotype due to functional redundancy with XLF [9-13]. Contrary, mice lacking either XRCC4 or Lig4 demonstrate p53- and Ku-dependent embryonic lethality, which correlates with massive neuronal apoptosis in central nervous system [1, 14-18].

Combined inactivation of Xlf and Dna-pkcs results in p53- and Ku70-dependent perinatal lethality in mice [11, 19, 20]. Moreover, deficiency or haploinsufficiency for Trp53 rescues synthetic lethality between Xlf and Paxx [11]. XLF is also functionally redundant in mouse development with Mri [21], recombination activating gene 2, RAG2 [22], and a number of DNA damage response (DDR) factors, including Ataxia telangiectasia mutated (ATM) [7], histone H2AX [7, 23], Mediator of DNA damage checkpoint protein 1 (MDC1) [11], and p53-binding factor (53BP1) [8, 24].

Development of B and T lymphocytes depends on programmed DSBs induced by RAG during the V(D)J recombination, and NHEJ pathway, which is used for error-prone DNA repair [4]. Moreover, mature B cells replace constant regions of immunoglobulins during the somatic recombination process known as class switch recombination (CSR), when DSBSs are initiated by activation-induced cytidine deaminase (AID) and Uridine-N-glycosylase (UNG), and NHEJ is used for DNA repair [1, 25, 26]. Furthermore, the NHEJ process is required for neurodevelopment by preventing neuronal apoptosis [1, 27].

Mri was initially described as an open reading frame at human chromosome 7 (C7orf49), a factor reversing the resistance to retroviral infection in cell lines [28]. Mri was found to enhance NHEJ [29] and possess an N-terminal Ku-binding motif (KBM) [30]. Later, Mri/Cyren was suggested to inhibit NHEJ at telomeres during the S and G2 phases of the cell cycle [31], and finally confirmed to be a bona fide NHEJ factor, which is functionally redundant with XLF in mouse development, including the V(D)J recombination and development of central nervous system [21]. However, it was not clear whether XLF and Mri functionally overlap during the early stages of neurodevelopment, e.g. supporting proliferation and self-renewal of neuronal stem cells. Moreover, due to the lack of viable mouse model deficient for both XLF and Mri, impact of Mri on B and T lymphocyte development in vivo is not fully understood.

Here, we introduced a frame-shift mutation to exon 2 of the murine Mri gene. By interbreeding heterozygous parents, we obtained Mri+/m, Mri+/- and Mri+/- mice at nearly expected ratios. Mri-deficient mice possessed normal body size and number of B and T lymphocytes; however, we detected that stimulated primary mature Mri+/- B cells had reduced levels of IgG1, and Mri+/- neurospheres showed reduced proliferation rate when compared to Mri+/- controls.

2. Materials and Methods

2.1. Mouse models

All experiments involving mice were performed according to the protocols approved by the Animal Resources Care Facility of Norwegian University of Science and Technology (NTNU, Norway). Ung+/- mice were described previously [32]. Mri+/- mice were generated on request and described here for the first time.

2.2. Generation of Mri+/- mice

MRI-deficient (Mri+/-) mice were generated through a CRISPR/Cas9 gene-editing approach in 2017 by Horizon Discovery (Saint Louis, USA) upon request from the Oksenych group (IKOM, Faculty of Medicine and Health Science, NTNU, Norway). Single-guide RNA (sgRNA) GGG CTG TCA TCC AAG AGG GGA GG was designed to target exon 2 of Mri gene in C57BL/6 mice. The 14 bp deletion resulted in a premature stop codon (Figure 1A). Cas9 and sgRNAs were injected into single-cell fertilized embryos, which were then transferred back into pseudopregnant females for gestation. Live-born pups were screened for indel mutation by DNA sequencing. Homozygous pups were used for back-crossing with wild type C57BL/6 mice. Heterozygous Mri+/- mice were obtained from Horizon Discovery.
2.3. Mouse genotyping

Two polymerase chain reactions (PCRs) were designed to determine mouse genotypes. The first PCR was performed using TCGGTCCTGACCTACACTGA and TGCGTGGGTCTTCCTGTGA primers, detecting both wild type (428 bp) and null (414 bp) alleles (Figure 1B). The second PCR performed with TCGGTCCTGACCTACACTGA and AGAGGGAGGACCC primers was used to validate the presence of the WT allele (234 bp, Figure 1B). The PCRs were performed using 50 ng of genomic DNA extracted from murine tissues (e.g., ears, tails), in a final reaction volume of 25 μL, using the Taq 2x Master Mix Kit (New England Biolabs® Inc., Ipswich, Massachusetts, USA; #M0270L). The 2.5% agarose gel was used to separate 428 bp and 414 bp PCR products during 18 h at 4°C, 90 V; 0.7% agarose gel was used to detect 234 bp PCR product (75 min, room temperature, 124 V). Genomic DNA isolated from Mri<sup>wt</sup> and Mri<sup>−/−</sup> cells, as well as samples with no genomic DNA, were used as PCR controls (Figure 1B).

2.4. Fluorescence-activated cell sorting, splenocyte and thymocyte count

Fluorescence-activated cell sorting (FACS) analysis was performed as previously described [12, 33]. Briefly, spleens and thymi were isolated from 2-month-old mice, splenocytes and thymocytes were counted using Countess™ Automated Cell Counter (Invitrogen); the cell suspension was spun down and diluted with PBS to get a final cell concentration of 2.5 x 10<sup>6</sup> cells/200 μl. Doxorubicin (Selleckchem, Houston, TX, USA; #H8943) was used to induce DSBs, and PrestoBlue™ Cell Viability Reagent (Thermo Fisher, USA; #A13263) was used to estimate cellular metabolism levels. Briefly, 2000 cells per well were seeded into 96 well plates, in 100 μL of IMDM medium (day 0). On day 1, 50 μL of the medium was replaced with 50 μL of fresh medium containing doxorubicin, bleomycin or etoposide, when indicated. Each experimental condition was performed in triplicates. On day 4, 11 μL of 10x PrestoBlue reagent was added to the wells and incubated for 30 min at 37°C. The cellular viability was estimated according to manufacturer’s instructions, using the excitation/emission wavelengths set at 544/590 nm.

2.5. Class switch recombination

Class switch recombination (CSR) from IgM to IgG1 was performed as previously described [12]. Naïve B lymphocytes were purified from spleens of 2-month-old mice, using EasySep mouse B cell enrichment kit (STEMCELL Technology, Vancouver, Canada; #19854) according to the manufacturers’ instructions. For each CSR assay, 2 x 10<sup>6</sup> cells/200 μL were used in duplicates. The cells were stimulated with LPS (Lipopolysaccharides, 40 μg/ml; Sigma Aldrich, St. Louis, MO, USA; #437627-5MG) and IL-4 (Interleukin 4, 20 ng/ml; PeproTech, Stockholm, Sweden; #214-14) for 96 h. Then the cells were blocked with Fc receptor antibody (2.4G2) and normal mouse serum (Invitrogen, Carlsbad, California, USA; #10410). The cells were washed in PermWash™ (BD Biosciences, New Jersey, USA; #553142). The cells were then incubated with fluorochrome-conjugated antibodies (see below) and sorted.

2.6. Double strand break sensitivity assay

DSBs sensitivity assay was performed as previously described [11, 33, 34]. Human nearly-haploid HAPI cells were generated by Horizon Discovery Group (Waterbeach, UK, #HZGHC005061c001 and #HZGHC005061c004) and are commercially available. HAPI cells were cultured according to the manufacturer’s instructions. Doxorubicin (Selleckchem, Houston, TX, USA; #S1208), bleomycin (Selleckchem; #S1214) and etoposide (Sigma-Aldrich, USA; #E1383) were used to induce DSBs, and PrestoBlue™ Cell Viability Reagent (Thermo Fisher, USA; #A13262) was used to estimate cellular metabolism levels. Briefly, 2000 cells per well were seeded into 96-well plates, in 100 μL of IMDM medium (day 0). On day 1, 50 μL of the medium was replaced with 50 μL of fresh medium containing doxorubicin, bleomycin or etoposide, when indicated. Each experimental condition was performed in triplicates. On day 4, 11 μL of 10x PrestoBlue reagent was added to the wells and incubated for 30 min at 37°C. The cellular viability was estimated according to manufacturer’s instructions, using the excitation/emission wavelengths set at 544/590 nm.
2.7. Brain isolation and neural stem progenitor cell culture

The brain was isolated from postnatal day one mouse after the cerebellum was removed. The isolated brain was mechanically disrupted in proliferation medium consisting of Dulbecco Modified Eagle Medium, Nutrient Mixture F12 (DMEM/F12; Thermo Fisher, USA; #11330-057), supplemented with penicillin/streptomycin (Thermo Fisher, USA; #15140122), B27 without vitamin A (Thermo Fischer Scientific, USA; #12587001), EGF (10 ng/ml; PeproTech, Stockholm, Sweden; #AF-100-15) and bFGF (20ng/ml; PeproTech; #100-18B). Neural stem progenitor cells (NSPC) form free-floating globular structures referred to as neurospheres. The neurospheres were formed during the incubation at 37°C, 5% CO2 and 95% humidity in order to perform proliferation and self-renewal assay (Figure 3A) [35].

2.8. Neural stem progenitor cell proliferation and self-renewal assays

Early passage NSPCs (P3–P10) were used throughout all the NSPC experiments. A PrestoBlue™ Cell Viability Assay was used to investigate the neurosphere proliferation rates, following the manufacturer’s instructions during each incubation day 1 to 7. The capacity of neural stem cells to maintain their multipotency ex vivo was assessed by determining the number and two-dimensional size of neurospheres [35]. Single NSPCs were plated onto 6-well suspension plates in the proliferation medium on day 0. During days 8 and 10 in culture, images of the entire wells were captured using EVOS microscope. Only areas between 50 and 1500 pixels were included in the analyses.

2.9. Antibodies

The following antibodies were used for FACS. Rat anti-mouse anti-CD16/CD32 (Fc Block, BD Biosciences, USA; #553141, 1 : 50); anti-CD4-PE-Cy7 (Thermo Scientific, USA, #25-0042-81, 1 : 100); anti-CD8-PE-Cy5 (BD Biosciences, USA, #553034, 1 : 100); anti-CD19-PE-Cy7 (Biolegends, USA, #115520, 1 : 100). Hamster anti-mouse anti-CD3-APC (Biolegends, USA, #100312, 1 : 100).

3. Results

3.1. Generation of Mri−/− mice

To investigate the impact of Mri on mouse development, we generated a mouse model with 14 bp frame-shift deletion in Mri exon 2 on a C57BL/6 background (Figure 1A). Purified sgRNA and Cas9 RNA were introduced to fertilized oocytes, resulting in complete inactivation of the Mri gene. Mri status (WT, wild type, +/+; heterozygous, +/-; and null, -/-) was confirmed for every experiment by PCR screening (Figure 1B). Mri−/−, Mri+/− and Mri−/− mice were born from Mri−/+ parents at ratios close to 1:2:1 (Figure 1C). Thirty-day old Mri−/− mice possessed an average body weight of 15.0 g, which was slightly lower but not significantly different from Mri+/− controls, with a bodyweight of 17.5 g, on average (Figure 1D). The lifespan of Mri−/− and Mri+/− mice was monitored for up to 12 months, according to the local regulations. During this time frame, both Mri+/− and Mri−/− mice were fertile and had no cancer incidence, similarly to the Mri+/− controls.
Figure 1. Generation of Mri<sup>+</sup> mice. (A) Top. Schematic diagram of murine Mri locus indicating the frame-shift mutation in the exon 2, induced by the sgRNA and resulting in a 14 bp deletion. Bottom. Resulting Mri<sup>-/-</sup> locus lacking part of the exon 2. (B) Top. PCR-based genotyping strategy reveals the Mri<sup>WT</sup> allele (428 bp) and Mri null allele (414 bp). Bottom. WT gene validation PCR reveals the Mri<sup>WT</sup> wild type allele (234 bp). (C) Genotype Observed | Expected (1:2:1) | Mri<sup>+/+</sup> | 29 | 35 | Mri<sup>+/−</sup> | 75 | 70 | Mri<sup>−/−</sup> | 36 | 35 | Total: 140 | 140 | (D) Analyses of 140 pups born from Mri<sup>+</sup>/− parents reveals expected genetic distribution of Mri<sup>+/+</sup> (29), Mri<sup>+/−</sup> (75) and Mri<sup>−/−</sup> (36) mice, which is close to the Mendelian distribution 1:2:1. (D) Body weight of six to eight week old Mri<sup>+/+</sup> mice (n=6) is not distinguishable from Mri<sup>−/−</sup> mice (n=7), p=0.4242. (E) The weight of spleens isolated from Mri<sup>+/+</sup> (n=8) and Mri<sup>−/−</sup> (n=11) mice is not distinguishable, n.s., p=0.7713. (F) Thymus weight of six to eight week old Mri<sup>+/+</sup> mice (n=6) is not distinguishable from Mri<sup>−/−</sup> mice (n=7), p=0.6796.
significantly different, p=0.8551. Spleen size in immunodeficient Dna- pkcs<sup>−/−</sup> mice (n=10) is reduced when compared to the Mri<sup>+/+</sup> and Mri<sup>−/−</sup> mice, p<0.0001. (F) Splenocyte count is not affected in the Mri<sup>−/−</sup> mice (n=11) when compared to the Mri<sup>+/+</sup> (n=10), p=0.7713. A number of splenocytes in immunodeficient Dna- pkcs<sup>−/−</sup> mice (n=6) is significantly reduced when compared to Mri<sup>+/+</sup> and Mri<sup>−/−</sup> mice, p<0.0001. (G) The weight of thymus from Mri<sup>+/+</sup> (n=11) and Mri<sup>−/−</sup> (n=11) mice is similar, p=0.6796. Thymus size in immunodeficient Dna- pkcs<sup>−/−</sup> mice (n=7) is significantly reduced when compared to Mri<sup>+/+</sup> and Mri<sup>−/−</sup> mice, p<0.0001. (H) Thymocyte count is nearly identical in Mri<sup>+/+</sup> (n=8) and Mri<sup>−/−</sup> (n=6) mice, p=0.5285. A number of thymocytes in immunodeficient Dna- pkcs<sup>−/−</sup> mice (n=6) is significantly reduced when compared to Mri<sup>+/+</sup> and Mri<sup>−/−</sup> mice, p<0.0001.

3.2. Mri<sup>−/−</sup> mice develop normal spleens and thymis

The NHEJ is required for the V(D)J recombination in developing B and T lymphocytes, and for CSR in mature B cells [1]. To determine specific functions of Mri in B and T cell development, we first analyzed spleens and thymis isolated from Mri-deficient and WT mice. The average weights of spleens (91 mg) and thymis (69 mg), as well as average count of splenocytes (121 million) and thymocytes (173 million), was not affected in Mri<sup>−/−</sup> mice when compared to Mri<sup>+/+</sup> controls (90 mg; 71 mg; 118 million; 186 million, respectively). These numbers were significantly different from immunodeficient controls, Dna- pkcs<sup>−/−</sup> mice (23 mg; 10 mg; 6 million; 5 million, respectively) (Figure 1E-H). Moreover, proportions of CD19<sup>+</sup> B cells in spleens of six to eight weeks old Mri<sup>−/−</sup> mice were on average 60%, which was similar to the proportion of CD19<sup>+</sup> in Mri<sup>+/+</sup> mice (55%, p=0.0668), and significantly higher than background levels detected in immunodeficient Dna- pkcs<sup>−/−</sup> controls (p<0.0001; Figure 2A). The average proportion of CD3<sup>+</sup> T splenocytes in Mri<sup>−/−</sup> mice (21%) was also similar to the one observed in Mri<sup>+/+</sup> controls (22%, p=0.8228), and higher than in Dna- pkcs<sup>−/−</sup> controls (1%, p<0.0001; Figure 2A). Mri<sup>+/+</sup> and Mri<sup>−/−</sup> mice had similar proportions of CD4<sup>+</sup> T cells (p=0.8876) and CD8<sup>+</sup> T cells (p=0.7026) in the spleens, while proportions of CD4<sup>+</sup> and CD8<sup>+</sup> T splenocytes in Dna- pkcs<sup>−/−</sup> controls were 4-5 folds reduced (p<0.0001, Figure 2B). In thymi of six- to eight-week-old Mri<sup>+/+</sup> and Mri<sup>−/−</sup> mice, proportions of CD4<sup>+</sup>, CD8<sup>+</sup> and CD4/CD8<sup>+</sup> T cells was similar (p>0.5589), while only background levels were detected in Dna- pkcs<sup>−/−</sup> controls (p<0.0001, Figure 2C).

3.3. CSR to IgG1 is reduced in Mri<sup>−/−</sup> mice.

To determine whether Mri deficiency affects CSR, we isolated B cells from the spleens of Mri<sup>+/+</sup> and Mri<sup>−/−</sup> mice and stimulated the cells with LPS and IL-4. After 96 h, we detected that average IgG1 levels were 33% in Mri<sup>−/−</sup> mice, which was significantly lower (p=0.0031) than in Mri<sup>+/+</sup> controls (average 39%; Figure 2D). B lymphocytes isolated from Ung<sup>−/−</sup> mice were used as negative control and possessed on average only 2% of IgG1 at the end of the experiment (96 h), which is lower than in Mri<sup>+/+</sup> or Mri<sup>−/−</sup> mice (p<0.0001).
Figure 2. Lymphocyte development in Mri\(^{+/+}\) mice. (A) Proportions of T (CD3\(^+\)) and B (CD19\(^+\)) cells in the spleens of Mri\(^{+/+}\) (n=6), Mri\(^{+/-}\) (n=3) and Dna-pkcs\(^{-/-}\) (n=4) mice. Proportions of T and B cells are similar in Mri\(^{+/+}\) and Mri\(^{+/-}\) mice, p>0.0667, and are only background levels in immunodeficient Dna-pkcs\(^{-/-}\) mice, p<0.0001. (B) Proportions of CD4\(^+\) and CD8\(^+\) T splenocytes in Mri\(^{+/+}\) (n=6), Mri\(^{+/-}\) (n=3) and Dna-pkcs\(^{-/-}\) (n=4) mice. Mri\(^{+/-}\) mice possess similar proportions of CD4\(^+\) T helper and CD8\(^+\) T cytotoxic cells when compared to Mri\(^{+/+}\) mice, p=0.8876 and p=0.7026, respectively. Only background levels of CD4\(^+\) and CD8\(^+\) T cells are present in immunodeficient Dna-pkcs\(^{-/-}\) spleens, p<0.0001. (C) Proportions of CD4\(^+\), CD8\(^+\) and CD4\(^+\)CD8\(^+\) thymocytes in Mri\(^{+/+}\) (n=6), Mri\(^{+/-}\) (n=3) and Dna-pkcs\(^{-/-}\) (n=4) mice. Proportions of T cell types in Mri\(^{+/-}\) mice are similar to the ones detected in Mri\(^{+/+}\) mice, p>0.5589 and higher than in Dna-pkcs\(^{-/-}\) mice, p<0.0001. (D) CSR to IgG1 in primary B splenocytes isolated from the Mri\(^{+/-}\) mice (n=4) is reduced when compared to the cells from the Mri\(^{+/+}\) mice (n=3), p=0.0032. CSR to IgG1 was significantly reduced in Ung\(^{-/-}\) B cells (n=3) when compared to the Mri\(^{+/+}\) and Mri\(^{+/-}\), p<0.0001.
3.4. Lack of Mri results in the reduced proliferation rate of neuronal stem progenitor cells

Previous studies have shown that single knockout of NHEJ DNA repair genes, e.g. Xrcc4, Lig4, Ku70, results in impaired nervous system development in mice [4, 14, 15]. To determine the impact of Mri on the developing nervous system, we used NSPC isolated from Mri+/+ and Mri−/− mice at postnatal day 1. We performed four independent experiments using two cell lines from two mice of each genotype. The average proliferation rate of Mri−/− neurospheres was approximately 35% lower than that in WT controls, p=0.0043 (Figure 3B).

3.5. Normal self-renewal capacity of Mri-deficient neuronal stem progenitor cells

To analyze the capacity of NSPCs to maintain features of stem cells throughout cell divisions and numerous propagations (self-renewal capacity), we counted the number of neurospheres formed in cell culture. In four independent experiments, we plated 10,000 single NSPCs and cultured for 8 days. In total, we counted 5123 neurospheres that originated from Mri+/+, and 4608 from Mri−/− mice. On average, there were 256 neurospheres in each of 20 Mri+/+ samples analyzed, and 230 neurospheres in each of 20 Mri−/− samples (p=0.7254, n.s., Figure 3C). In addition, the images of neurospheres were collected and the surface was calculated using ImageJ software. Inactivation of Mri did not affect the average diameter of neurospheres, which was 461 px² on average in Mri+/+ and 427 px² in Mri−/− neurospheres, p=0.4915 (Figure 3D). We concluded that Mri is dispensable for the self-renewal capacity of NSPC.

Figure 3. Characterization of neurogenesis in Mri−/− mice. For each experiment, four independent cell lines isolated from two mice were used (n=8). (A) Neurospheres isolation diagram from Mri+/+ and Mri−/− mice at postnatal day 1. (B) Neurospheres proliferation isolated from the Mri−/− mice is reduced when compared to

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**Figure 3.** Characterization of neurogenesis in Mri−/− mice. For each experiment, four independent cell lines isolated from two mice were used (n=8). (A) Neurospheres isolation diagram from Mri+/+ and Mri−/− mice at postnatal day 1. (B) Neurospheres proliferation isolated from the Mri−/− mice is reduced when compared to...
the Mri<sup>−/−</sup> mice, p=0.0043. (C) Number of neurospheres formed in cell culture for 8 days. Mri<sup>−/−</sup> and Mri<sup>+/−</sup>
neurospheres possess similar self-renewal capacity, p=0.7254. (D) Neurosphere size isolated from Mri<sup>−/−</sup> and
Mri<sup>+/−</sup> mice are similar, p=0.4915. The surface of neurospheres, pxl. Areas between 50 and 1500 pixels were
included in the analyses. Four independent experiments using two cell lines of each genotype were
performed in all experiments (A-C). p values were calculated using unpaired t-test. The horizontal bars
represent the average.

3.6. Human HAP1 cells lacking Mri possess normal levels of sensitivity to DSBs

Deficiency for XRCC4, LIG4, XLF, or DNA-PKcs results in hypersensitivity to DSBs in human
HAP1 cells [11, 33, 34]. To determine the effect of Mri on DSB sensitivity, we obtained two
independent clones of MRI-deficient HAP1 cells, as well as WT and XRCC4-deficient controls. We
exposed the HAP1 cells to DSBI-inducing agents bleomycin (0 to 0.4 μM), doxorubicin (0 to 4 nM) and
etoposide (0 to 160 nM), and evaluated the cell viability 4 days later (Figure 4). We observed no
hypersensitivity of HAP1 cells lacking Mri when compared to WT controls. However, cells lacking
XRCC4 were hypersensitive to all indicated compounds, bleomycin, doxorubicin, and etoposide
(p<0.0001, Figure 4).

![Figure 4](image)

**Figure 4.** Sensitivity to DSBs in Mri-deficient HAP1 cells. Sensitization of WT, two independent Mri-
deficient clones, Mri<sup>+/−</sup> and Mri<sup>−/−</sup>, and XRCC4<sup>−/−</sup> HAP1 cells to bleomycin (A), doxorubicin (B), and
etoposide (C) at indicated concentrations. Results are from the mean (SD) of three repeats. Cell viability (%)
represents the relative proportion of the fluorescence normalized to untreated cells. Comparisons between
eyery two groups were made using one-way ANOVA, GraphPad Prism 8. WT, Mri<sup>+/−</sup> and Mri<sup>−/−</sup> vs XRCC4<sup>−/−</sup>,
p<0.0001 (****); WT vs Mri<sup>+/−</sup>, p=0.9983 (n.s); WT vs Mri<sup>−/−</sup>, p=0.1295 (n.s); Mri<sup>+/−</sup> vs Mri<sup>−/−</sup>, p=0.1791 (n.s).

4. Discussion

We have generated a new knockout mouse model with 14 bp deletion in exon 2 of the Mri gene,
Mri<sup>−/−</sup>. While we were characterizing our mouse model, another group reported an independently-
generated Mri-deficient mouse [21], which possessed a similar phenotype. Thus, our observations are
confirmatory to the findings observed by the Sleekman group [21].

The mice lacking Mri are live-born at expected ratios and demonstrate normal growth and
development of lymphoid organs. Mri<sup>−/−</sup>, Mri<sup>+/−</sup> and Mri<sup>+/+</sup> mice possess similar sizes of spleens and
thymi, a similar number of splenocytes and thymocytes, and proportions of B and T cells (Figure 1). Similarly to MRI-deficient mice, Paxx<sup>−/−</sup> mice did not have detectable phenotype [9-13]. However,
inactivation of other NHEJ factors resulted in reduced number of B and T cells (Xlf<sup>−/−</sup> mice, [5-8, 19, 22,
24]), block in B and T cell development, e.g. Artemis<sup>−/−</sup> [36], DNA-PKcs<sup>−/−</sup> [37], Ku70<sup>−/−</sup> [38], Ku80<sup>−/−</sup> [39]; or
even embryonic lethality in Xrc4<sup>−/−</sup> [40] and Lig4<sup>−/−</sup> [41].

The CSR to IgG1 was reduced in primary B cells isolated from Mri<sup>−/−</sup> mice when compared to WT
controls (Figure 2), which suggests that Mri is involved in specific DNA repair-mediated event.
Furthermore, we isolated neuronal stem progenitor cells from Mri<sup>−/−</sup> brains and found that these cells
proliferate slower when compared to Mri<sup>+/+</sup> controls. Reduced proliferation rates of MRI-deficient
neuronal stem progenitor cells could be explained, as one option, by lower expression or lack of
factors functionally redundant with Mri in these cell types. Future studies would be required to
address this option. Moreover, future studies may address questions such as neurological defects and
cognitive functions in mice lacking Mri, as well as whether the Mri-deficient mice are prone to infections.

In addition, we found that human nearly haploid HAP1 cell lines lacking Mri possessed no proliferation defect or hypersensitivity to DNA damaging agents, such as etoposide, doxorubicin and bleomycin (Figure 4). Previously, it was reported that murine embryonic fibroblasts (MEF) lacking Mri are hypersensitive to ionizing radiation when compared to WT controls, although the sensitivity is less obvious than in XLF-deficient MEFs [21]. The discrepancy between our and previously published data could be due to the usage of different cell types, difference between species, as well as distinct ways to induce DNA damages (e.g., chemicals vs irradiation). Further studies involving various cell type models originated from different species, and using various DNA damaging strategies, would deepen our understanding of the specific functions of Mri in DNA repair in mammalian cells. Overall, we concluded that the lack of Mri has rather a minor effect on murine and human cells.

Combined inactivation of Mri and Xlf [21], however, revealed an important role of Mri in mouse development, which was overlooked due to its functional redundancy with XLF. Synthetic lethality between Mri and Xlf complicated studies of genetic interaction between these factors in vivo. There are several potential ways to overcome this challenge. One option is to use conditional knockouts of Xlf or Mri genes. Moreover, there might be another genetic-based approach. Inactivation of one or two alleles of Trp53 partially rescued synthetic lethality between Xlf and Dna-pkcs [11, 19, 20] and Xlf and Paxx [11]. In addition, deficiency or haploinsufficiency for Trp53 rescued embryonic lethality of Lig4+ and Xrc4+ mice [14, 15]. Inactivation of the Atm gene rescued embryonic lethality of Lig4− mice [42]. Finally, inactivation of both alleles of Ku70 rescued embryonic lethality of Lig4− mice [18], and inactivation of Ku70 rescued synthetic lethality between Xlf and Dna-pkcs [20]. Based on these data, one can speculate that inactivation of Trp53, Atm, Ku70 or Ku80 will rescue synthetic lethality between Xlf and Mri. Moreover, given critical roles of Ku70 and Ku80 in initiation of classical NHEJ, one could propose that mice lacking all known NHEJ factors, e.g., Ku70−/−Ku80−/−Dna-pkcs−/−Artemis−/−Xlf−/−Paxx−/−Mri−/− will be viable, indistinguishable from Ku-deficient mice, and serve as a suitable in vivo model to investigate alternative end-joining, A-EJ.

5. Conclusions

A new Mri-deficient mouse model was generated. Mri−/− deficient mice possessed normal body size and number of B and T lymphocytes; however, Mri is required for efficient class switch recombination process in mature B cells. Mri−/− neurospheres showed reduced proliferation rate, but similar self-renewal capacity, when compared to Mri+/+ controls.

Author Contributions: V.O. designed and performed experiments, contributed key reagents and analyzed the data. A.S. performed CSR assay using primary B cells. M.T. performed DSBs sensitivity assay using human HAP1 cells. S.C.Z., C.H., O.R., Q.Z., A.L., J.W., N.B.L. performed lymphocyte analyzes. O.R., W.W., and P.J. performed and analyzed neurosphere-based experiments. R.G.F developed Mri−/− genotyping strategy. M.B. and V.O. interpreted the results. The paper was written by O.R., S.C.Z. and V.O.; all the authors read and approved the manuscript.

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Abbreviations:
ATM - Ataxia-telangiectasia mutated
CSR - Class switch recombination
DDR - DNA damage response
DNA-PKcs - DNA-dependent protein kinase
DSBs - DNA double-strand breaks
GFAP - Glial fibrillary acid protein
HAP1 - A near-haploid human cell line derived from KBM-7 cell line
IL-4 - Interleukin 4
Lig4 - DNA ligase IV
LPS - Lipopolysaccharides
Mri - Modulator of retroviral infection
NHEJ - Non-homologous end-joining
NSPC - Neuronal stem progenitor cell
PAXX - Paralogue of XRCC4 and XLF
PCR - Polymerase chain reaction
XLF - XRCC4-like factor
XRCC4 - X-ray repair cross-complementing protein 4

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