# Leaky severe combined immunodeficiency in mice lacking non-homologous end joining factors XLF and MRI

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**Abstract**

Non-homologous end joining (NHEJ) is a DNA repair pathway that is required to detect, process, and ligate DNA double-stranded breaks (DSBs) throughout the cell cycle. The NHEJ pathway is necessary for V(D)J recombination in developing B and T lymphocytes. During NHEJ, core factors Ku70 and Ku80 form a heterodimer called Ku, which recognizes DSBs and promotes recruitment and function of downstream factors PAXX, MRI, DNA-PKcs, Artemis, XLF, XRCC4, and LIG4. Mutations in several known NHEJ genes can result in immunodeficient phenotypes, including severe combined immunodeficiency (SCID). Inactivation of *Mri, Paxx* or *Xlf* in mice results in normal or mild phenotype, while combined inactivation of *Xlf/Mri, Xlf/Paxx,* or *Xlf*/*Dna-pkcs* leads to late embryonic lethality. Here, we demonstrated that deletion of pro-apoptotic factor *Trp53* rescues embryonic lethality in mice with combined deficiency of *Xlf* and *Mri*. Furthermore, these $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice possessed reduced body weight, severely reduced mature B and T cell counts in the spleen and thymus, and accumulation of progenitor B cells in the bone marrow. Combined inactivation of *Mri* and *Paxx* results in live-born mice with modest B cell phenotype. In contrast, combined inactivation of *Mri* and *Dna-pkcs* results in embryonic lethality. Therefore, we conclude that MRI is functionally redundant with XLF during B and T lymphocyte development *in vivo,* and that $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice possess a leaky SCID phenotype.

## 1. Introduction

Non-homologous end-joining (NHEJ) is a DNA repair pathway that recognizes, processes and ligates DNA double-stranded breaks (DSB) throughout the cell cycle. NHEJ is required for lymphocyte development; in particular, to repair DSBs induced by the recombination activating genes (RAG) 1 and 2 in developing B and T lymphocytes, and by activation-induced cytidine deaminase (AID) in mature B cells [1]. NHEJ is initiated when core subunits Ku70 and Ku80 (Ku) are recruited to the DSB sites. Ku, together with DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), form the DNA-PK holoenzyme [2]. Subsequently, the nuclease Artemis is recruited to the DSB sites to process DNA hairpins and overhangs [3]. Finally, DNA ligase IV (LIG4), X-ray repair cross-complementing protein 4 (XRCC4) and XRCC4-like factor (XLF) mediate DNA end ligation. The NHEJ complex is stabilized by a paralogue of XRCC4 and XLF (PAXX) and a modulator of retroviral infection (MRI) [4, 5].

Inactivation of *Ku70*, *Ku80*, *Dna-pkcs* or *Artemis* results in severe combined immunodeficiency (SCID) characterized by lack of mature B and T lymphocytes [2, 3, 6-8]. Deletion of both alleles of *Xrcc4* [9] or *Lig4* [10] results in late embryonic lethality in mice, which correlates with increased apoptosis in the central nervous system (CNS). Inactivation of *Xlf* (*Cernunnos*) only results in modest immunodeficiency in mice [11-13], while mice which lack *Paxx* [14-17] or *Mri* [5, 18] display no overt phenotype.

The mild phenotype observed in mice lacking XLF could be explained by functional redundancy between XLF and multiple DNA repair factors, including *Ataxia telangiectasia* mutated (ATM), histone H2AX [19], Mediator of DNA Damage Checkpoint 1 (MDC1) [20, 21], p53-binding protein 1 (53BP1) [17, 22], RAG2 [23], DNA-PKcs [20, 24, 25], PAXX [4, 14, 15, 20, 26-28] and MRI [5]. However, combined inactivation of *Xlf* and *Paxx* [4, 14, 15, 20], as well as *Xlf* and *Mri* [5], results in late embryonic lethality in mice, presenting a challenge to the study of B and T lymphocyte development *in vivo*. It has also been shown that both embryonic lethality and increased levels of CNS neuronal

apoptosis in mice with deficiency in *Lig4* [9, 10, 29, 30], *Xrcc4* [9, 31], *Xlf* and *Paxx* [20], or *Xlf* and *Dna-pkcs* [24, 25] is p53-dependent.

In this study, we rescue synthetic lethality from *Xlf* and *Mri* by inactivating one or two alleles of *Trp53*. We also show that the resulting $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice possess a leaky SCID phenotype with severely reduced mature B and T lymphocyte counts in the spleen, low mature T cell counts in the thymus, and accumulated progenitor B cells in bone marrow. Finally, we demonstrate that MRI functions in B and T lymphocyte development *in vivo*, and that its roles are compensated by XLF.

## 2. Materials and Methods

### *2.1. Mice*

All experiments involving mice were performed according to the protocols approved by the Comparative Medicine Core Facility (CoMed) at the Norwegian University of Science and Technology (NTNU, Trondheim, Norway). $Xlf^{+/-}$ [11] and $Dna\text{-}pkcs^{+/-}$ [2] mice were imported from the laboratory of Professor Frederick W. Alt at Harvard Medical School. $Trp53^{+/-}$ mice [32] were imported from Jackson Laboratories. $Paxx^{+/-}$ [16] and $Mri^{+/-}$ [18] mice were generated by the Oksenych group and described previously.

### *2.2. Lymphocyte development*

Lymphocyte populations were analyzed by flow cytometry as described previously [16, 18, 19, 22]. In summary, cells were isolated from the spleen, thymus, and femur of 5-7-week-old mice and treated with red blood cell lysis buffer Hybri-Max™ (Sigma Aldrich, St. Louis, MO, USA; #R7757). The cells were resuspended in PBS (Thermo Scientific, Basingstoke, UK; #BR0014G) containing 5% Fetal bovine serum, FCS (Sigma Life Science, St. Louis, Missouri, United States; #F7524), and counted using a Countess™ II Automated Cell Counter (Invitrogen, Carlsbad, CA, United States; #A27977). Then, the cell suspension was diluted with PBS to get a final cell concentration of $2.5 \times 10^7$ cells/mL.

Finally, surface markers were labeled with fluorochrome-conjugated antibodies and the cell population was analyzed using flow cytometry.

*2.3. Class switch recombination*

Spleens were isolated from 5-7-week-old mice and stored in cold PBS. Splenocytes were obtained by mincing the spleens, and naïve B cells were negatively selected using an EasySep Isolation kit (Stemcell™, Cambridge, UK; #19854). Lipopolysaccharide (LPS; 40 µg/mL; Sigma Aldrich, St. Louis, MO, USA; #437627-5MG) and interleukin 4 (IL-4; 20 ng/mL; PeproTech, Stockholm, Sweden; #214-14) were used to induce CSR to IgG1. Expression of IgG1 was analyzed by flow cytometry.

*2.4. Antibodies*

The following antibodies were used for flow cytometry analysis: rat anti-CD4-PE-Cy7 (BD Pharmingen™, Allschwil, Switzerland, #552775; 1:100); rat anti-CD8-PE-Cy5 (BD Pharmingen™, Allschwil, Switzerland, #553034; 1:100); anti-CD19-PE-Cy7 (Biolegend, San Diego, CA, USA, #115520; 1:100); hamster anti-mouse anti-CD3-FITC (BD Pharmingen™, Allschwil, Switzerland, #561827; 1:100); rat anti-mouse anti-CD43-FITC (BD Pharmingen™, Allschwil, Switzerland, #561856; 1:100); rat anti-mouse anti-CD45R/B220-APC (BD Pharmingen™, Allschwil, Switzerland; #553092; 1:100); rat anti-mouse anti-IgM-PE-Cy7 (BD Pharmingen™, Allschwil, Switzerland, #552867; 1:100); rat anti-mouse IgG1-APC (BD Pharmingen™, Allschwil, Switzerland; #550874; 1:100). A LIVE/DEAD™ fixable violet dead cell stain kit (ThermoFisher Scientific, Waltham, MA, USA; #L34955; 1:1000) was used to identify dead cells.

*2.5. Statistics*

Statistical analyses were performed using one-way ANOVA, GraphPad Prism 8.0.1. 244 (San Diego, CA, USA). In all statistical tests, $p<0.05$ were taken to be significant (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$).

**3. Results**

*3.1. Inactivation of Trp53 gene rescued embryonic lethality in mice lacking XLF and MRI*

Combined inactivation of *Xlf* and *Mri* has previously been shown to result in synthetic lethality [5]. To generate $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice, we intercrossed an $Mri^{-/-}$ strain [18] with an $Xlf^{-/-}Trp53^{+/-}$ [20] strain. Next, we selected and intercrossed triple heterozygous ($Xlf^{+/-}Mri^{+/-}Trp53^{+/-}$), and later, $Xlf^{-/-}Mri^{+/-}Trp53^{+/-}$ mice. With PCR screening, we identified $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ (n=11), $Xlf^{-/-}Mri^{-/-}Trp53^{-/-}$ (n=2), and $Xlf^{-/-}Mri^{-/-}Trp53^{+/+}$ (n=1) (Fig. 1) among the resulting offspring. Mice lacking both XLF and MRI possessed reduced weight (12 g on average, $p<0.0001$) when compared with gender- and age-matched WT (19 g), $Xlf^{-/-}$ (19 g) and $Mri^{-/-}$ (20 g) controls (Fig. 1A,B). We used these XLF/MRI-deficient mice to further characterize the development of B and T lymphocytes *in vivo*.

*3.2. Leaky SCID in $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice*

To determine the roles of XLF and MRI in lymphocyte development *in vivo*, we isolated the thymus, spleen, and femur from $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice, as well as from $Xlf^{-/-}$, $Mri^{-/-}$, $Trp53^{+/-}$ and WT controls. Combined deficiency for XLF and MRI resulted in a 3-fold reduction in thymus size (32 mg on average, $p<0.0001$) and a 9-fold reduction in thymocyte count ($1.9x10^7$, $p<0.0001$) when compared to single deficient or WT controls (Fig. 1C). Similarly, both average spleen weight (22 mg, $p<0.0001$) and splenocyte count ($2.0x10^7$, $p<0.0001$) in $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice decreased approximately 4-5 fold when compared with WT and single deficient controls (Fig. 1D). We did not detect any direct influence of *Trp53* genotype on lymphocyte development. The reduced number of splenocytes in XLF/MRI-deficient mice could be explained by decreased populations of B and T lymphocytes observed in the $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice (Fig. 1E-G). Specifically, CD3+ T cells were reduced 4-fold ($p<0.0001$), while CD19+ B cells were reduced 20-fold ($p<0.0001$) when compared with single deficient and WT controls (Fig. 1E-G). Likewise, counts of CD4+, CD8+ and CD4+CD8+ T cells in the thymus, as well as counts of CD4+ and CD8+ T cells in the spleen, were all dramatically reduced when compared with single deficient and WT controls (about 4-fold, $p<0.0001$; Fig. 1E,G,H).

From these observations, we conclude that XLF and MRI are functionally redundant during B and T lymphocytes development in mice.

*3.3. Leaky SCID in mice lacking XLF and PAXX*

Combined inactivation of XLF and PAXX has been shown to result in embryonic lethality in mice [4, 14, 15, 20]. To determine the impact of XLF and PAXX on B and T cell development *in vivo*, we rescued the synthetic lethality by inactivating one allele of *Trp53*, as described previously [20]. The resulting $Xlf^{-/-}Paxx^{-/-}Trp53^{+/-}$ mice possessed 30- to 40-fold reduced thymocyte count ($4.0x10^6$, $p<0.0001$) when compared to WT ($1.3x10^8$), $Xlf^{-/-}$ ($1.4x10^8$) and $Paxx^{-/-}$ ($1.7x10^8$) mice. This is reflected in decreased levels of double-positive CD4+CD8+ cells, as well as decreased levels of single-positive CD4+ and CD8+ T cells (Fig. 1,2). Spleen development was dramatically affected in mice lacking XLF and PAXX compared to WT and single-deficient controls, due to the lack of B cells and decreased T cell count (Fig. 1,2). When compared with the WT and single knockout controls, $Xlf^{-/-}Paxx^{-/-}Trp53^{+/-}$ mice had a 400- to 600-fold reduction in CD19+ B splenocyte count ($0.1x10^6$, $p<0.0001$) and a 70- to 90-fold reduction in CD3+ splenocyte count (to $0.3x10^6$) (Fig. 1F-H and Fig. 2). From these results, we concluded that XLF and PAXX are functionally redundant during the B and T lymphocyte development in mice.

*3.4. Early B cell development is abrogated in mice lacking XLF and MRI, or XLF and PAXX*

Reduced counts and proportions of mature B lymphocytes in $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice suggest a blockage in B cell development in the bone marrow. To investigate this further, we isolated the bone marrow cells from femora of mice lacking XLF, MRI or both XLF/MRI, and analyzed the proportions of B220+CD43+IgM- progenitor B cells and B220+CD43-IgM+ immature and mature B cells. We detected only background levels of B220+CD43-IgM+ B cells in bone marrows isolated from $Xlf^{-/-}Mri^{-/-}Trp53^{+/-}$ mice (Fig. 3A,B). However, these mice exhibited a 2- to 3-fold higher proportion of pro-B cells when compared with WT, $Mri^{-/-}$ and $Xlf^{-/-}$ controls (Fig. 3A,C). Similarly, $Xlf^{-/-}Paxx^{-/-}Trp53^{+/-}$ mice also possessed background levels of IgM+ B cells ($p<0.0001$; Fig. 3A,B) while having 3- to 4-fold

higher proportion of pro-B cells when compared with WT, *Paxx*$^{-/-}$ and *Xlf*$^{-/-}$ controls ($p$<0.0001; Fig. 3A,C). Therefore, we concluded that B cell development is blocked at the pro-B cell stage of *Xlf*$^{-/-}$ *Mri*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ mice.

*3.5. Paxx*$^{-/-}$*Mri*$^{-/-}$ *mice* possess a modest phenotype

Both PAXX and MRI are NHEJ factors that are functionally redundant with XLF in mice. Combined inactivation of *Paxx* and *Xlf* [4, 14, 15, 20], or *Mri* and *Xlf* ([5]; this study) results in synthetic lethality in mice, as well as in abrogated V(D)J recombination in vAbl pre-B cells [4, 5, 14, 15, 27]. To determine if *Paxx* genetically interacts with *Mri*, we intercrossed mice that are heterozygous or null for both genes (e.g., *Paxx*$^{+/-}$*Mri*$^{+/-}$). We found that resulting *Paxx*$^{-/-}$*Mri*$^{-/-}$ mice are live-born, fertile, and are similar in size to WT littermates (17 g, $p$>0.9999) (Fig. 4A,B). Specifically, we observed that *Paxx*$^{-/-}$*Mri*$^{-/-}$ mice had normal thymocyte and splenocyte counts. Furthermore, *Paxx*$^{-/-}$ *Mri*$^{-/-}$ mice underwent normal T cell development that was indistinguishable from the WT, *Paxx*$^{-/-}$, and *Mri*$^{-/-}$ controls (Fig. 1G,H and 4C). However, *Paxx*$^{-/-}$*Mri*$^{-/-}$ mice had a reduction in the CD19+ B cell number (Fig. 1F) when were compared to WT, *Paxx*$^{-/-}$ and *Mri*$^{-/-}$ controls ($p$<0.0025). Moreover, CD19+ B cell counts were similar in *Paxx*$^{-/-}$*Mri*$^{-/-}$ and *Xlf*$^{-/-}$ mice (p>0.9270). *Paxx* inactivation did not affect Ig switch to IgG1 in MRI-deficient B cells (Fig. 4D,E). The quantity of IgG1+ cells after CSR stimulation was similar between *Paxx*$^{-/-}$*Mri*$^{-/-}$ and *Mri*$^{-/-}$ naïve B cells (p>0.48), although both were lower than that of the WT control, both at 72 h and at 96 h (p<0.05). From this, we can conclude that there is a genetic interaction between *Paxx* and *Mri in vivo,* and it is only detected in B cells.

*3.6. Synthetic lethality between Mri and Dna-pkcs in mice*

Both MRI and DNA-PKcs are functionally redundant with XLF in mouse development [5, 24]. Combined inactivation of *Paxx* and *Mri* (this study), or *Paxx* and *Dna-pkcs* [20] genes results in live-born mice that are indistinguishable from single deficient controls. To determine if *Mri* genetically interacts with *Dna-pkcs*, we crossed *Mri*$^{+/-}$ and *Dna-pkcs*$^{+/-}$ mouse strains, then intercrossed the double-heterozygous and *Mri*$^{-/-}$*Dna-pkcs*$^{+/-}$ strains (Fig. 5A). We identified 12 *Mri*$^{-/-}$*Dna-pkcs*$^{+/+}$ mice

and 12 *Mri*$^{-/-}$*Dna-pkcs*$^{+/-}$ mice, but no *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice (out of 6 expected). To determine if double-deficient *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ embryos are present at day E14.5, we intercrossed *Mri*$^{-/-}$*Dna-pkcs*$^{+/-}$ mice and analyzed embryos (Fig. 5B). We identified two *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ embryos (63mg), which were about 40% lighter than *Mri*$^{-/-}$ embryos (108mg) (Fig. 5C,D). A Chi-Square test ($\chi 2$) was performed to determine if the embryonic distribution data fits the mendelian ratio of 1:2:1 that is expected from *Mri*$^{-/-}$*Dna-pkcs*$^{+/-}$ parents. With DF=2 and $\chi 2$=1.8, the corresponding p-value lies within the range $0.25<p<0.5$. This affirms that our data fit the expected 1:2:1 distribution and suggests that *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ is synthetic lethal. Therefore, we can conclude that there is genetic interaction between *Mri* and *Dna-pkcs in vivo*.

**4. Discussion**

Genetic inactivation of *Xlf* [11], *Paxx* [4, 14-16], or *Mri* [5, 18] in mice leads to development of modest or no detectable phenotype. However, inactivation of other NHEJ factors, such as *Ku70*$^{-/-}$ [6], *Ku80*$^{-/-}$ [7], *Artemis*$^{-/-}$ [3], *Dna-pkcs*$^{-/-}$ [2] results in blockage of B and T cell development in mice, while inactivation of *Xrcc4* [9] and *Lig4* [10] results in embryonic lethality. Moreover, combined inactivation of *Xlf* and *Mri* [5], *Xlf* and *Paxx* [4, 14, 15], or *Xlf* and *Dna-pkcs* [24, 25] also results in embryonic lethality, which is correlated with increased levels of neuronal apoptosis in the central nervous system (Fig. 6). *Xlf* also genetically interacts with *Rag2* [23] and DDR factors, such as *Atm*, *53bp1*, *H2ax,* and *Mdc1* [17, 19-22, 33]. *Xlf*$^{-/-}$*Rag2c* mice almost completely lack mature B cells and have significantly fewer mature T cells than single deficient controls [23]. *Xlf*$^{-/-}$*Atm*$^{-/-}$ and *Xlf*$^{-/-}$*53bp1*$^{-/-}$ mice are live-born and exhibit reduced body weight, increased genomic instability, and severe lymphocytopenia as a result of V(D)J recombination impairment in developing B and T cells [1, 17, 19, 22]. *Xlf*$^{-/-}$*H2ax*$^{-/-}$ and *Xlf*$^{-/-}$*Mdc1*$^{-/-}$, on the other hand, are embryonic lethal [19-21]. There are several possible explanations for the functional redundancy observed between DNA repair genes. For instance, the two factors could have identical (e.g., if both proteins are involved in ligation or DNA end tethering) or complementary (e.g., if one protein stimulates ligation while the other is

required for DNA end tethering) functions. To date, XLF has been shown to genetically interact with multiple DNA repair factors [1, 4, 5, 14, 15, 19, 20, 24, 25], and this list is likely to grow [33, 34]. However, no clear genetic interaction has been shown between *Xlf* and *Artemis* or *Xrcc4* in the context of mouse development and V(D)J recombination [24], meaning that it remains difficult to predict genetic interactions without developing and characterizing genetic models.

Inactivation of one or two alleles of *Trp53* rescues the embryonic lethality of *Xrcc4*$^{-/-}$ [9, 31], *Lig4*$^{-/-}$ [10, 30], *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$ [20] mice (Fig. 6). Recent findings suggest that MRI forms heterogeneous complexes involving PAXX or XLF, which function during DNA DSB repair by NHEJ [5]. Additionally, combined inactivation of *Xlf* and *Mri* in vAbl pre-B cells results in a severe block in V(D)J recombination and accumulation of unrepaired DSBs *in vitro,* although it is unclear whether this combined inactivation will lead to a deficiency in B lymphocytes when translated to a mouse model [5]. Similarly, double deficient vAbl pre-B cells lacking *Xlf* and *Paxx* are also unable to sustain V(D)J recombination. Importantly, the lack of a progenitor T cell model system left the question of T cell development in *Xlf*$^{-/-}$*Mri*$^{-/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$ mice completely unexplored. Inactivation of *Trp53* resulted in live-born mice lacking XLF/PAXX [20]. These *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{-/-}$ mice had nearly no B and T cells, reduced size of spleen and hardly detectable thymus [20] (Fig. 6). Moreover, a conditional knockout mouse model, which results in double-deficiency of XLF/PAXX in early hematopoietic progenitor cells, was also able to overcome the embryonic lethality of *Xlf*$^{-/-}$*Paxx*$^{-/-}$ mice [35]. With this model, impairment of V(D)J recombination in *Xlf*$^{-/-}$*Paxx*$^{-/-}$ cells, as well as the resulting depletion of mature B cells and lack of a visible thymus could also be observed *in vivo* [35].

We have demonstrated that mice lacking XLF, MRI and p53, although live-born, possess a leaky SCID phenotype. *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$ mice have a clear fraction of mature B cells in the spleens (CD19+) and bone marrow (B220+CD43-IgM+) (Fig. 1,3,6), as well as clear fractions of double- and single-positive T cells in the thymus (CD4+CD8+, CD4+, CD8+) and single-positive T cells in the spleen (CD4+ and CD8+) (Fig. 1). However, the cell fractions from these mice are noticeably smaller than

those of WT or single-deficient mice. Similarly, *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ mice are also live-born and possess a very small number of mature B cells in the spleen and bone marrow, as well as very minor fractions of single positive T cells in thymus and spleen (Fig. 2,3,6). Due to the smaller presence of mature B and T cells in these mice, we categorize the observed immunodeficient phenotypes as "leaky SCID", which has previously been described in mice lacking other NHEJ factors, such as *Ku70*$^{-/-}$ [6], *Artemis*$^{-/-}$ [3], *Lig4*$^{-/-}$*Trp53*$^{-/-}$ , *Xrcc4*$^{-/-}$*Trp53*$^{-/-}$ [9, 31], *Xlf*$^{-/-}$*Atm*$^{-/-}$ [19] *and Xlf*$^{-/-}$*Rag2*$^{-/-}$ [23].

Both mature B and T cells are present in mice lacking XLF/PAXX and XLF/MRI. This can be explained by incomplete block in NHEJ and V(D)J recombination, in which the process is dramatically reduced but still possible. We also detected more mature T cells than B cells in these double-deficient mice. Potential explanations include longer lifespan of T cells, which accumulate over time following low efficiency of V(D)J recombination, while B cells are eliminated faster from the pool due to the different physiology [36, 37]. It is also possible that T cells we detected are a resultant subpopulation that is descended from the few cells that were able to bypass V(D)J recombination [12]. In this case, the repertoire of T cells based on T cell receptor in mice lacking XLF/PAXX and XLF/MRI would be significantly lower than in control mice, even if normalized to the total cell count.

It is important to note that inactivation of *Trp53* is not always sufficient to rescue embryonic lethality in mice; for example, PLK1-interacting checkpoint helicase (PICH)-deficient mice possess developmental defects in the presence or absence of p53 [38], and ATR mutants (Seckel syndrome) are not completely rescued from embryonic lethality with the inactivation of *Trp53* [39]. Embryonic lethality of XLF/PAXX and XLF/MRI double-deficient mice can be explained by the presence of Ku70/Ku80 heterodimer at the DSBs sites, which blocks DNA repair by alternative end-joining pathway(s), leading to massive apoptosis and cell cycle arrest [33]. Previously, it was shown that embryonic lethality of LIG4-deficient [40] and XLF/DNA-PKcs double-deficient mice [25] could be rescued by inactivating *Ku70* or *Ku80* genes. Similarly, we propose that inactivation of either *Ku70* or

*Ku80* gene will rescue the embryonic lethality of XLF/PAXX and XLF/MRI double-deficient mice and will result in mice indistinguishable from Ku70- or Ku80-deficient controls.

We also found that mice with combined inactivation of *Paxx* and *Mri* (*Paxx*$^{-/-}$*Mri*$^{-/-}$) are live-born, fertile, and undergo almost normal B and T cell development (Fig. 4), where only the number of splenic B cells is affected, giving rise to a modest phenotype. Moreover, inactivation of *Paxx* did not affect the CSR efficiency in *in vitro* stimulated MRI-deficient B cells (Fig. 4), thereby confirming our observations *in vitro*. It has been shown that combined inactivation of *Paxx* and *Mri* genes in vAbl pre-B cells lead to similar V(D)J recombination efficiency to single deficient *Mri*$^{-/-}$, *Paxx*$^{-/-}$ and WT controls [5]. Furthermore, combined inactivation of *Paxx* and *Ku80* (*Paxx*$^{-/-}$*Ku80*$^{-/-}$), or *Paxx* and *Atm* (*Paxx*$^{-/-}$*Atm*$^{-/-}$) [15], as well as *Paxx* and *Dna-pkcs* (*Paxx*$^{-/-}$*Dna-pkcs*$^{-/-}$) [20] lead to a phenotype similar to their single deficient controls, *Ku80*$^{-/-}$, *Atm*$^{-/-}$ and *Dna-pkcs*$^{-/-}$, correspondingly. Thus, we conclude that there is a genetic interaction between *Paxx* and *Mri*.

Both *Mri* an *Dna-pkcs* genetically interact with *Xlf*. Strikingly, we found that combined inactivation of *Mri* and *Dna-pkcs* (*Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$) leads to embryonic lethality, and that E14.5 *Mri*$^{-/-}$ *Dna-pkcs*$^{-/-}$ murine embryos were about 40% smaller than single-deficient siblings. DNA-PKcs is associated with the N-terminus of the MRI and Ku heterodimer in the process of recognizing DSBs [5], which may account for genetic interaction between *Mri* and *Dna-pkcs*. Thus, inactivation of *Trp53, Ku70* or *Ku80* may be a viable method to rescue synthetic lethality from *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice.

*Xlf* and *Mri* interact genetically, and mice lacking XLF/MRI are embryonic lethal. It was demonstrated by *Hung et al. (2018)* [5] previously, and largely confirmed in this study; however, we were able to identify one *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/+}$ mouse at day P30 post-birth. This mouse resembled *Xlf*$^{-/-}$ *Mri*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{-/-}$ mice of similar age with respect of B and T cell development, although this mouse was generally sicker than its littermates and had to be euthanized (Fig. 1, 6). Similarly, one live-born *Xlf*$^{-/-}$*Paxx*$^{-/-}$ mouse was reported by *Balmus et al. (2016)* [15], indicating that,

exceptionally, embryonic lethality in NHEJ-deficient mice can be overcome, likely due to activity of alternative end-joining.

In conclusion, we have developed and described several complex genetic mouse models (Fig. 6). *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ mice possessed severely impaired B and T lymphocyte development; *Paxx*$^{-/-}$*Mri*$^{-/-}$ mice develop a modest B cell phenotype; and *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice are embryonic lethal.

## Author contributions

VO, SCZ, QZ, AL and MFB designed the study, analyzed and interpreted the results. SCZ, QZ, AL and MFB performed most of the experiments. VO wrote the paper with the help of SCZ, QZ and RY. All the authors contributed to writing of the final manuscript.

## Conflict of interest statement

The authors declare no conflict of interest.

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## Figure legends

**Fig. 1. B and T cell development in *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$ mice.** (A) Comparison of body size, thymi and spleens of XLF/MRI-deficient and XLF-deficient mice of the same age. (B) Weights of WT, *Xlf*$^{-/-}$, *Mri*$^{-/-}$, *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$ mice. (C, D) Number ($\times 10^6$) of thymocytes (C) and splenocytes (D) in WT, *Xlf*$^{-/-}$, *Mri*$^{-/-}$, *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$ mice. (E) Flow cytometric analysis of thymic and splenic T cell subsets and splenic

CD19+ B cells. (F,G,H) Number ($\times 10^6$) of splenic CD19+ B cells (F), splenic CD3+ T cells (G) and thymic CD4+CD8+ double positive (DP) T cells (H) in WT, *Xlf*$^{-/-}$, *Mri*$^{-/-}$, *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$, *Paxx*$^{-/-}$, *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ and *Paxx*$^{-/-}$*Mri*$^{-/-}$ mice. *Dna-pkcs*$^{-/-}$ mice were used as an immunodeficient control. Comparisons between every two groups were made using one-way ANOVA, GraphPad Prism 8.0.1. *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{-}$ is a combination of *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{-/-}$. *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{-}$ is a combination of *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{-/-}$. Not shown in the graph for (F): WT vs *Paxx*$^{-/-}$*Mri*$^{-/-}$, $p<0.0001$ (****), *Paxx*$^{-/-}$ vs *Paxx*$^{-/-}$*Mri*$^{-/-}$, $p<0.0001$ (****), *Mri*$^{-/-}$ vs *Paxx*$^{-/-}$*Mri*$^{-/-}$, $p<0.0025$ (**), *Xlf*$^{-/-}$ vs *Paxx*$^{-/-}$*Mri*$^{-/-}$, $p=0.9270$ (n.s), *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{-}$ vs *Paxx*$^{-/-}$*Mri*$^{-/-}$, $p<0.0001$ (****), and *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{-}$ vs *Paxx*$^{-/-}$*Mri*$^{-/-}$, $p<0.0001$ (****).

**Fig. 2. B and T cell development in *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ mice**. Examples of flow cytometric analysis of thymic and splenic T cell subsets and splenic CD19+ B cells.

**Fig. 3. B cell development is abrogated in bone marrow of *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ mice.** (A) Flow cytometric analysis of developing B cells. Upper left box marks progenitor B cell population and lower right box marks the IgM+ B cells. (B, C) Frequencies (%) of IgM+ B cells (B) and progenitor B cells (C) in WT, *Xlf*$^{-/-}$, *Mri*$^{-/-}$, *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$, *Paxx*$^{-/-}$, *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ and *Paxx*$^{-/-}$*Mri*$^{-/-}$ mice. Comparisons between groups were made using one-way ANOVA, GraphPad Prism 8.0.1. *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{-}$ is a combination of *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Mri*$^{-/-}$*Trp53*$^{-/-}$. *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{-}$ is a combination of *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{-/-}$.

**Fig. 4. B and T cell development in *Paxx*$^{-/-}$*Mri*$^{-/-}$ mice.** (A) Number of thirty-day-old mice (P30) of indicated genotypes. Parents were *Paxx*$^{+/-}$*Mri*$^{+/-}$ and *Paxx*$^{-/-}$*Mri*$^{+/-}$. (B) Example of thirty-day-old *Paxx*$^{-/-}$*Mri*$^{-/-}$ and WT male littermates with their respective thymi and spleens. (C) Example of flow cytometry analyzes of B and T cells in *Paxx*$^{-/-}$*Mri*$^{-/-}$ and WT mice. (D,E) Class switching analyzes of *in vitro* activated naïve B cells of indicated genotypes.

**Fig. 5. Genetic interaction between *Mri* and *Dna-pkcs in vivo***. (A) No live-born *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice were detected. (B,C) *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ embryos were detected at day E14.5. (D) Body weight in

milligrams (mg) from two E14.5 *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ and *Mri*$^{-/-}$*Dna-pkcs*$^{+/-}$ embryos from the same litter. The mendelian ratio 1:2:1 in embryos was verified by the Chi-Square test ($\chi 2$). The $\chi 2$ was 1.8 and its corresponding probability was between 25 and 50%. *Expected distribution assuming lethality.

**Fig. 6. Mutations in NHEJ genes result in different phenotypes. Suggested models.** Inactivation of *Paxx* or *Mri* results in live-born mice with nearly no DNA repair defects. Inactivation of *Xlf* or *Dna-pkcs* results in live-born mice with increased levels of genomic instability due to reduced NHEJ activity. Combined inactivation of *Xlf/Paxx*, *Xlf/Mri* and *Xlf/Dna-pkcs* leads to embryonic lethality in mice that correlate with high levels of genomic instability and nearly no NHEJ. Accumulated DSBs activate the DNA damage response (DDR) pathway that triggers cell cycle arrest and apoptosis. Alternative end-joining is blocked by presence of Ku70/Ku80. Inactivation of one or two alleles of *Trp53* rescues embryonic lethality of *Xlf/Paxx*, *Xlf/Mri* and *Xlf/Dna-pkcs* mice. This embryonic lethality is likely to be rescued by inactivation of *Ku70* or *Ku80*.

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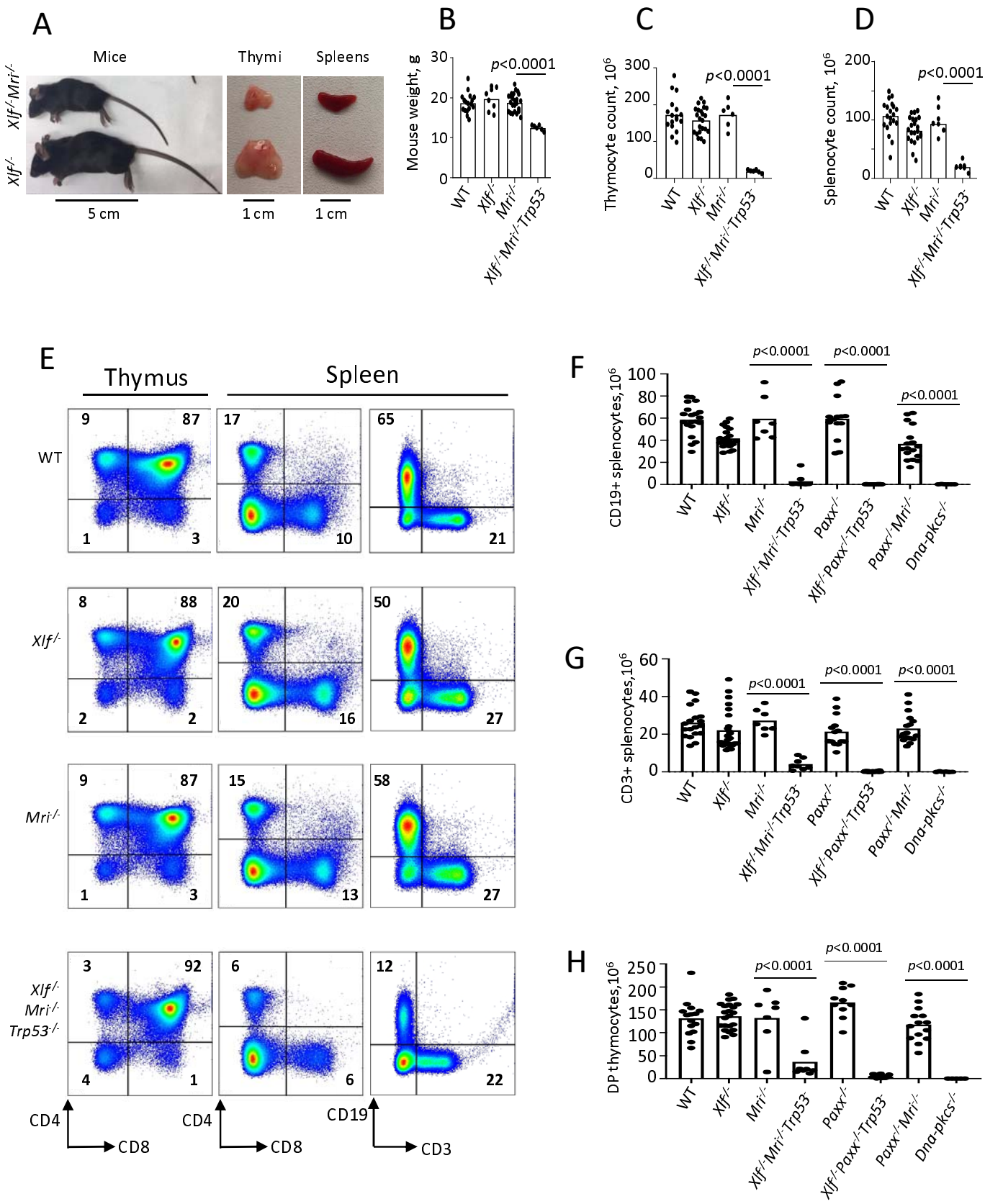
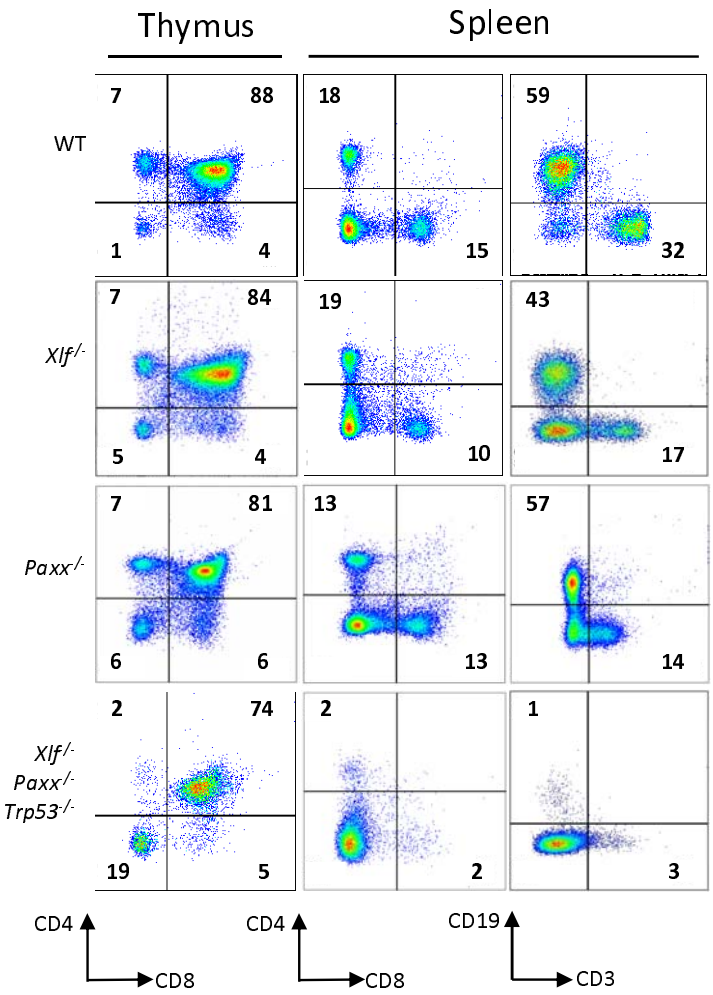
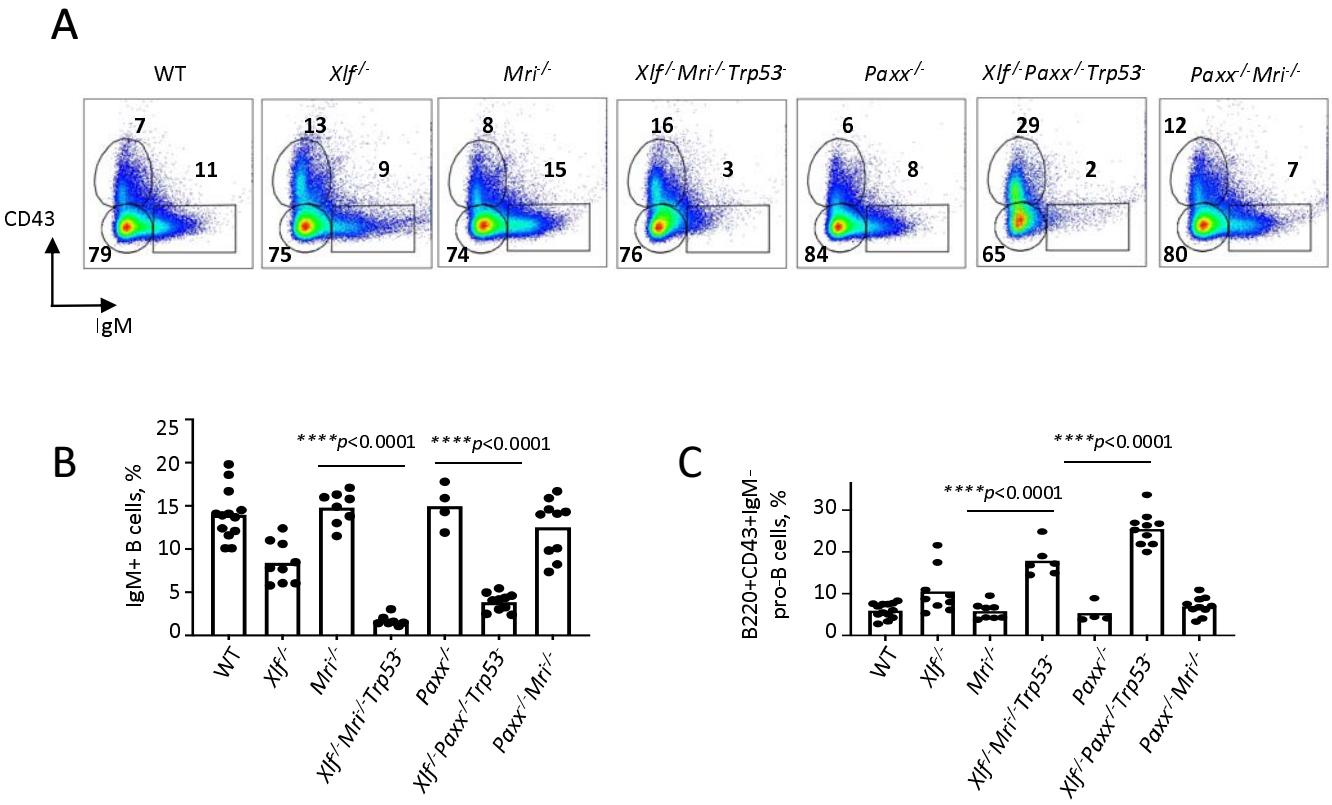
# Figure 1



# Figure 2



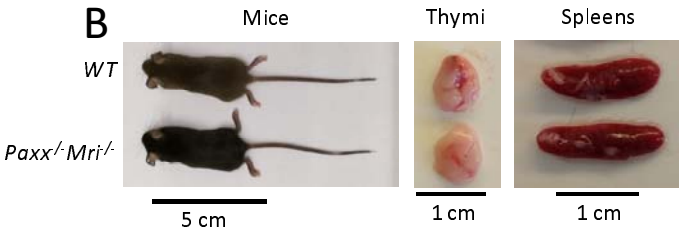
# Figure 3

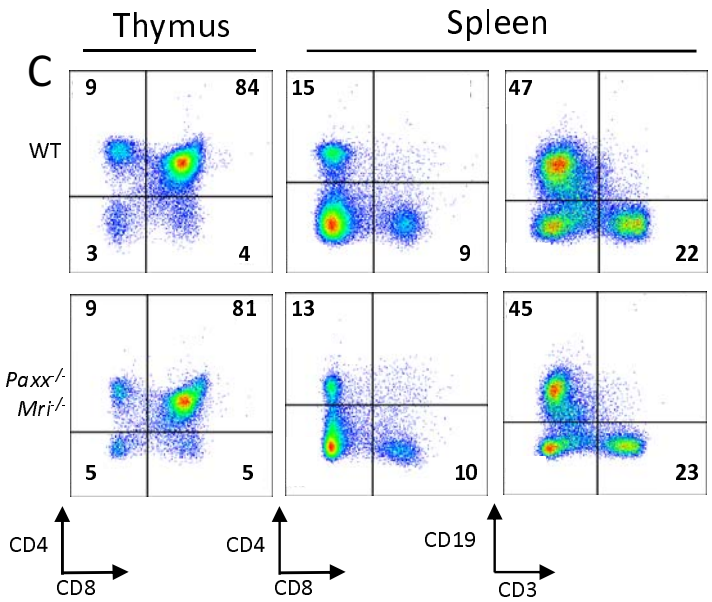


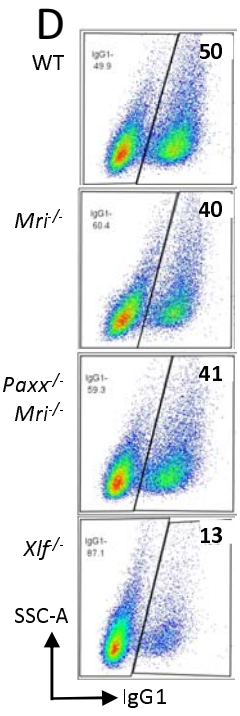
# Figure 4

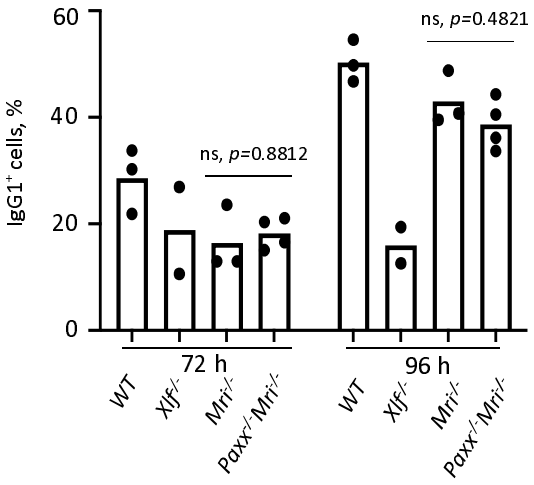
A

| | Offsprings, P30 | Expected, 1:2:1 |
|---|---|---|
| *Paxx*$^{-/-}$*Mri*$^{+/+}$ | 2 | 3.25 |
| *Paxx*$^{-/-}$*Mri*$^{+/-}$ | 4 | 6.50 |
| *Paxx*$^{-/-}$*Mri*$^{-/-}$ | 7 | 3.25 |
| Total | 13 | 13.00 |



# Figure 5

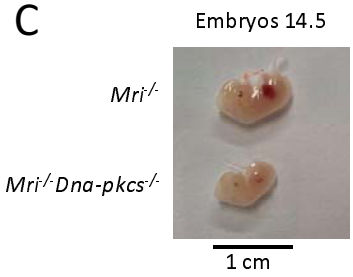
A

| | Offsprings, P30 | Expected, 1:2:1 | *Expected, 1:2:0 |
|---|---|---|---|
| *Mri*$^{-/-}$*Dna-pkcs*$^{+/+}$ | 12 | 6 | 8 |
| *Mri*$^{-/-}$*Dna-pkcs*$^{+/-}$ | 12 | 12 | 16 |
| *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ | **0** | 6 | 0 |

B

| | Offsprings, E14.5 | Expected, 1:2:1 | *Expected, 1:2:0 |
|---|---|---|---|
| *Mri*$^{-/-}$*Dna-pkcs*$^{+/+}$ | 1 | 2.5 | 3.3 |
| *Mri*$^{-/-}$*Dna-pkcs*$^{+/-}$ | 7 | 5.0 | 6.7 |
| *Mri*$^{-/-}$*Dna-pkcs*$^{-/-}$ | **2** | 2.5 | 0.0 |

C

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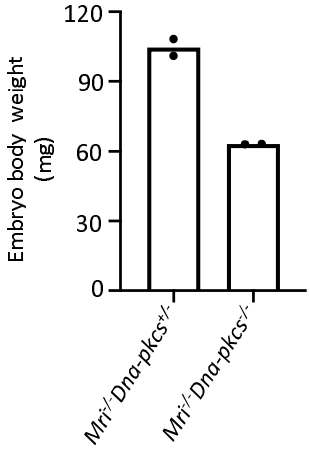

# Figure 6