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Review

Genetic Interaction Between the Non-homologous End Joining Factors during B and T Lymphocyte Development: *In Vivo* Mouse Models

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Abstract: The non-homologous end joining (NHEJ) DNA repair pathway is the main mechanism to repair DNA double strand breaks (DSBs) throughout the whole cell cycle. During NHEJ, core Ku70 and Ku80 subunits form the Ku heterodimer; Ku binds DSBs and promotes the recruitment of accessory factors (e.g., DNA-PKcs, PAXX, Mri) and downstream core factors (XLF, Lig4 and XRCC4). DSBs are induced during the V(D)J recombination in developing B and T lymphocytes to increase the repertoire of B and T cell receptors. Furthermore, DSBs are generated during the class switch recombination (CSR) in mature B lymphocytes, providing distinct effector functions of antibody heavy chain constant regions. The NHEJ is required for both V(D)J recombination and CSR. During the last decades, new NHEJ proteins have been reported, increasing the complexity of the molecular pathway. Multiple *in vivo* mouse models were generated and characterized to identify specific functions of NHEJ factors in the adaptive immune system. Here, we are summarizing available mouse models lacking one or several NHEJ factors, with a particular focus on early B cell development.

Keywords: B cell; V(D)J recombination; mouse model; NHEJ; DNA repair

1. Introduction

DNA double strand breaks (DSBs) are generated both extrinsically, e.g. by chemotherapeutic agents; and physiologically, for example, during the V(D)J recombination in developing B and T lymphocytes, and class switch recombination (CSR) in activated mature B cells [1,2].

DNA damage response (DDR) pathway operates upon the DSBs induction. *Ataxia telangiectasia* mutated (ATM) is a DDR regulator protein kinase that phosphorylates multiple substrates in response to the DSBs, including histone H2AX, modulator of DNA damage checkpoint 1 (MDC1), and p53-binding protein 1 (53BP1). Phosphorylated H2AX facilitates the recruitment of MDC1, following the activation of really interesting protein (RING) finger 8 (RNF8) and RNF168, which are ubiquitin ligases. Phosphorylated and ubiquitinated H2AX facilitates recruitment of 53BP1, which in turn mediates recruitment of RIF1 and interacts with Rev7. Moreover, Shieldin complex promotes DNA repair [1,3]. Accumulation of DSBs results in ATM-dependent activation of checkpoint kinases 1 and 2 (Chk1 and Chk2) that arrest the cell cycle, and then phosphorylation and stabilization of p53, which trigger apoptosis [4].

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In mammalian cells, the non-homologous end joining (NHEJ) pathway repairs approximately 75% of DSBs [5]. NHEJ consists of the DSB recognition, stabilization-processing, and end-ligation stages. There are evolutionary conserved NHEJ factors referred to as "core", and accessory NHEJ factors, which might be dispensable for the DNA repair in wild type cells (Figure 1A). The Ku70, Ku80, X-ray repair cross-complementing protein 4 (XRCC4), DNA Ligase 4 (Lig4) and XRCC4-like factor (XLF) are core factors [6-10]. Initially, DSBs are recognized by the heterodimer Ku, which is formed by the Ku70 and Ku80. Ku assists the recruitment of the accessory factor DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [11], forming the DNA-PK holoenzyme. Subsequently, Artemis nuclease [12], paralogue of XRCC4 and XLF (PAXX) [13-16], and modulator of retroviral infection (Mri) [17,18] are recruited to the DSB sites. Finally, the core factors XLF, XRCC4 and Lig4 mediate the Ku-dependent DNA end-ligation [1].

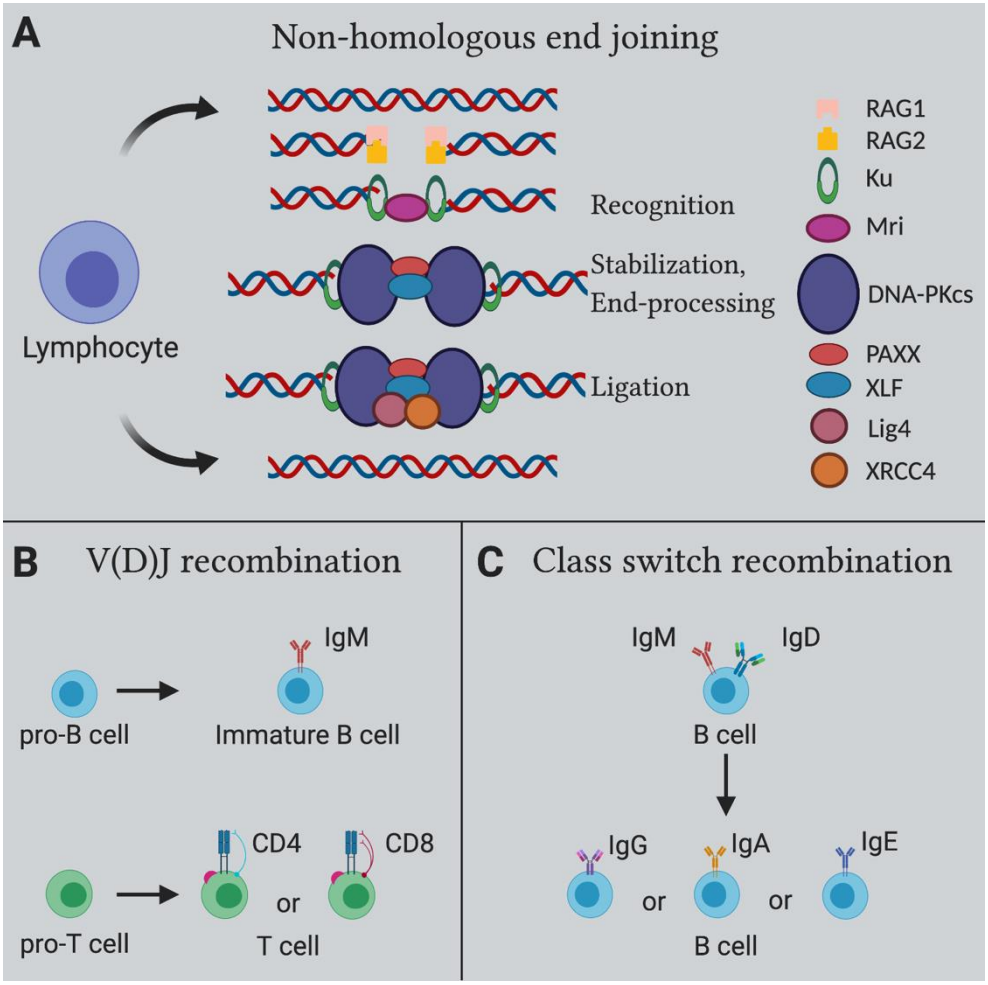

Figure 1. NHEJ and lymphocyte development. (A) Simplified overview of the NHEJ. During the V(D)J recombination, RAG1/RAG2 induce DSBs, which are recognized by the Ku (Ku70 and Ku80) heterodimer that facilitates recruitment of downstream factors, including DNA-PKcs, Mri, PAXX, XLF, Lig4 and XRCC4, in order to recognize, stabilize, process and ligate the DNA ends. **(B)** V(D)J recombination takes place in developing B and T cells, e.g. progenitor B and T cells (pro-B and pro-T cells), resulting in the surface expression of IgM and IgD (B cells) or TCR (T cells). **(C)** CSR in mature B cells results in the immunoglobin constant region switching from IgM and IgD to IgG, IgA, or IgE.

During the early stages of B and T cell development, NHEJ is required for the V(D)J recombination assembling *immunoglobulin (Ig)* and *T cell receptor (TCR)* genes using *V*, *D*, and *J* gene segments (Figure 1B). Both Ig and TCR provide antigen-binding specificity required for an efficient immune response. The recombination activating genes 1 and 2 proteins form an endonuclease (RAG) that recognizes the recombination signal sequences (RSSs) flanking the *V*, *D* and *J* gene segments [19].

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The CSR takes place in mature B cells, when constant regions of immunoglobulins switch from IgM to IgG, IgA, or IgE (Figure 1C). The immunoglobulins, or antibodies, possess a crucial role in the immune responses through their effector functions. The CSR is initiated by the activation-induced cytidine deaminase (AID). In repetitive *switch* regions of *Igh* gene, AID deaminates deoxycytosine resulting in deoxyuracil (dC>dU). The dUs are excised by the uracil DNA N-glycosylase (UNG) enzyme leaving an abasic (apyrimidinic/apurinic [AP]) site [20]. The abasic (AP) sites are cut by AP endonuclease (APE)1 or APE2, producing DNA single-strand breaks (SSB). Two SSBs on the opposite DNA strands form DSBs, initiating the CSR [20]. The joining of DSBs during the CSR is performed both by the classical-NHEJ (C-NHEJ) and alternative end-joining (A-EJ) [21]. Strikingly, the A-EJ maintains up to 50% of CSR activity in the absence of core C-NHEJ factors, e.g. Ku70, XRCC4, Lig4 [1].

Several mutations in genes encoding NHEJ factors were identified in human patients. The features of these patients vary from mild immunodeficiency when *XLF/CERNUNNOS* is mutated, to severe combined immunodeficiency, delayed growth and neurological abnormalities in patients with defects in *XLF*, *DNA-PKCS/PRKDC*, *ARTEMIS/DCLRE1C*, *XRCC4*, and *LIG4* [22]. Mutations in several NHEJ genes have not been found in immunodeficient patients up to the present. These genes include *Ku70*, *Ku80*, *PAXX* and *MRI*. If genes are essential for human life, e.g. *Ku70* and *Ku80*, the mutations would be identified by analyzing early embryonic samples. However, mutations in accessory factor genes, e.g. PAXX and MRI, might be tolerated without leading to clinical features.

2. Non-homologous end joining deficient mice

Before the transgenic mice became broadly-used models, *Bosma et al.* identified inbred immunodeficient mice, which are known today as mice with severe combined immunodeficiency (SCID) [23]. These mice got a spontaneous mutation in *Dna-pkcs* gene, which resulted in an immunodeficient phenotype in the homozygous state. SCID mice possess no B and T lymphocytes due to the impaired V(D)J recombination in pro-B and pro-T cells [23]. Later, a transgenic mouse model null for the *Dna-pkcs* gene (*Dna-pkcs*$^{-/-}$) was generated [11]. The *Dna-pkcs*$^{-/-}$ mice were live-born and possessed SCID phenotype lacking B and T lymphocytes due to the inefficient coding-end (CE) joining during the attempted V(D)J recombination [11] (Figure 2). Lack of CE joining resulting in SCID phenotype was also observed in *Artemis*$^{-/-}$ mice [12]. Thus, both DNA-PKcs and Artemis are required for processing of the RAG-mediated hairpin-sealed DNA ends (CEs) during the V(D)J recombination, while the repair of blunt signal ends (SEs) was efficient in the mice lacking either Artemis or DNA-PKcs [11,12]. Inactivation of Ku70 [9] or Ku80 [8] resulted in mice possessing reduced body weight and SCID phenotype. Lack of B and T lymphocytes in *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ mice is explained by the inefficient joining of both RAG-induced blunt SEs and hairpin-sealed CEs [8,9].

Differently, inactivation of *Lig4* [6] or *Xrcc4* [7] resulted in embryonic lethality in mice, challenging the *in vivo* studies. However, the cells lacking *Lig4* or *Xrcc4* possessed inefficient joining of both SEs and CEs, resembling the Ku-deficient phenotypes and suggesting that *in vivo* models, if available, would be immunodeficient due to the ablated V(D)J recombination and lack of B and T cells [6,7].

Several single-deficient mouse models initially suggested that XLF, PAXX and Mri are dispensable for the V(D)J recombination. Particularly, mice lacking XLF/Cernunnos [24,25] possessed both mature B and T cells, although being characterized by modest lymphocytopenia and reduced repertoires of B cell receptors (BCRs) and TCRs [24,25]. *Xlf*$^{-/-}$ lymphocytes supported efficient V(D)J recombination *in vitro*, including both SEs and CEs repair [24,25]. Mice lacking either PAXX [13,14,16,26] or Mri [17,18] possessed normal counts of mature B and T cells, efficiently supporting both SEs and CEs repair during the V(D)J recombination. Nevertheless, more complex mouse models revealed that XLF, PAXX and Mri are required for the V(D)J recombination, although their functions are compensated by each other and additional proteins due to extensive genetic interaction inside the NHEJ pathway, and between the NHEJ and DDR pathways [1,27].

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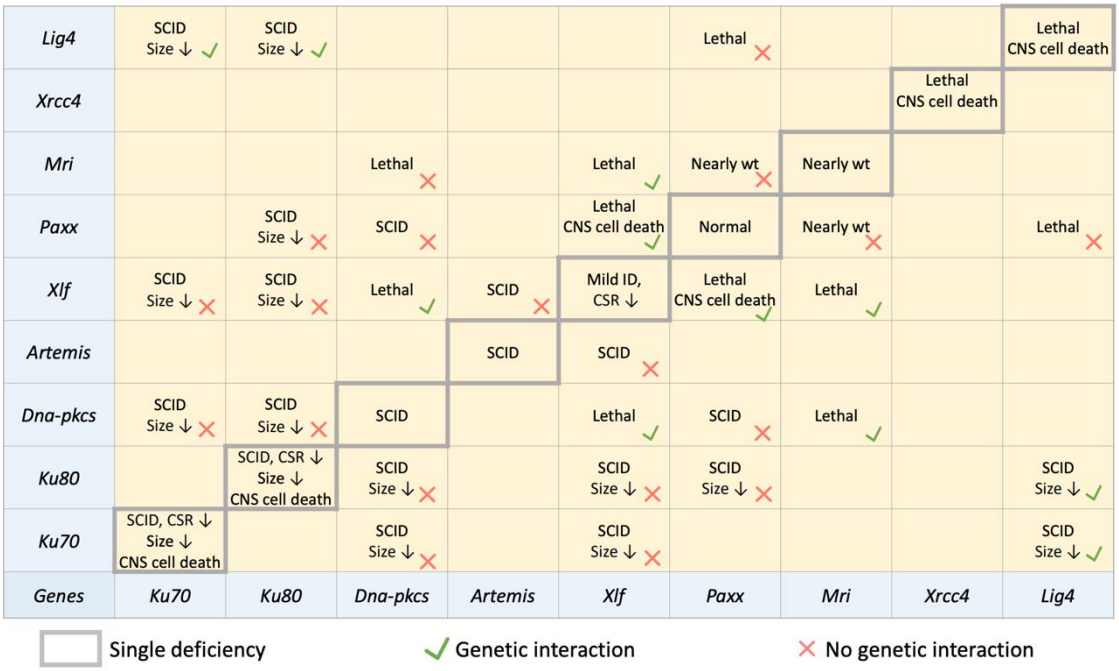

Figure 2. Genetic interactions between the NHEJ factors in mice. Single and double knockouts are indicated. Embryonic lethality, SCID, defects in CSR and death of neurons in CNS are marked. Genetic interactions are indicated as (V). The lack of interaction is marked as (X).

Until 2013, it was thought that both XLF and DNA-PKcs are dispensable for DSB ligation [1]. Surprisingly, double-deficient *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice were characterized by perinatal lethality and increased genomic instability in the cells due to nearly no NHEJ [28]. While lymphocytes lacking either XLF or DNA-PKcs were capable to repair RAG-induced blunt SEs, *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ cells were unable to join SEs [28]. Embryonic lethality of *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice is p53- and Ku-dependent, and triple-deficient *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$*Trp53*$^{+/-}$ [29,30] and *Ku70*$^{-/-}$*Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ [30] mice are live-born, although possess reduced body weight, which is comparable to one of *Ku70*$^{-/-}$ mice [29,30].

Xlf$^{-/-}$*Dna-pkcs*$^{-/-}$ was the first mouse model pointing out genetic interaction between *Xlf* and accessory NHEJ factor *in vivo* (Figure 2). Contrary, mice lacking both XLF and Artemis were live-born, fertile, and possessed SCID phenotype resembling the single-deficient *Artemis*$^{-/-}$ mice [28], suggesting no genetic interaction between *Xlf* and *Artemis in vivo*. A few years later, genetic interaction between *Xlf* and *Paxx in vivo* was characterized independently by four research groups [13,14,26,29]. Mice lacking both XLF and PAXX possess late embryonic lethality, increased genomic instability, and immunodeficiency. The *Xlf*$^{-/-}$*Paxx*$^{-/-}$ lymphocytes are unable to support DNA repair of both SEs and CEs generated during the attempted V(D)J recombination [13,14,26,31]. The embryonic lethality of *Xlf*$^{-/-}$*Paxx*$^{-/-}$ mice is p53-dependent. Both *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{-/-}$ mice are live-born, although possess significant weight reduction and leaky SCID phenotype with nearly no mature B and T lymphocytes [29,32].

Furthermore, *Xlf* and *Mri* also interact genetically. *Xlf*$^{-/-}$*Mri*$^{-/-}$ mice possess embryonic lethality [18], which is rescued by inactivation of one or two alleles of *Trp53* [32], Mice lacking both XLF and Mri are characterized by leaky SCID with nearly no mature B and T lymphocytes detected, due to the V(D)J recombination defect [18]. In particular, the lymphocytes lacking both XLF and Mri are unable to efficiently ligate both RAG-mediated SEs and CEs *in vitro* [18]. Collectively, both double deficient mouse models lacking XLF and PAXX, or XLF and Mri, are characterized by leaky SCID with low but detectable levels of mature B and T lymphocytes [32]. This phenotype is likely possible due to the residual NHEJ activity in developing *Xlf*$^{-/-}$*Paxx*$^{-/-}$ and *Xlf*$^{-/-}$*Mri*$^{-/-}$ lymphocytes *in vivo* [29,32].

Thus, *Xlf* genetically interacts with *Dna-pkcs*, *Paxx* and *Mri* [13,14,18,26,28,29,32]. Further questions were whether *Dna-pkcs* genetically interacts with *Paxx* and *Mri*, and whether *Paxx*

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genetically interacts with *Mri*? The *Dna-pkcs*$^{-/-}$*Paxx*$^{-/-}$ mice are live-born, fertile, and indistinguishable from *Dna-pkcs*$^{-/-}$ littermates [29]. Furthermore, *Mri*$^{-/-}$*Paxx*$^{-/-}$ mice possess nearly no detectable phenotype [32]. Strikingly, *Dna-pkcs*$^{-/-}$*Mri*$^{-/-}$ mice are embryonically lethal [32]. In summary, there is no genetic interaction between *Dna-pkcs* and *Paxx*, and between *Mri* and *Paxx*. However, *Dna-pkcs* and *Mri* interact genetically (Figure 2).

Inactivation of *Ku* or *Trp53* rescues embryonic lethality of *Lig4*$^{-/-}$ mice. In particular, *Ku80*$^{-/-}$*Lig4*$^{-/-}$ [33] and *Ku70*$^{-/-}$*Lig4*$^{-/-}$ [34] mice are not distinguishable from Ku-deficient mice, possessing reduced body weight, increased levels of genomic instability and SCID phenotype. Furthermore, *Lig4*$^{-/-}$*Trp53*$^{+/-}$ and *Lig4*$^{-/-}$*Trp53*$^{-/-}$ mice, although live-born, possess reduced body weight, increased levels of genomic instability, and SCID, due to inability to repair both SEs and CEs during the attempted V(D)J recombination [35]. Moreover, *Lig4*$^{-/-}$*Trp53*$^{+/-}$ mice possess a more severe phenotype than *Lig4*$^{-/-}$*Trp53*$^{-/-}$ littermates, likely due to an incomplete block of DNA damage-induced apoptosis in the presence of one allele of *Trp53* [35]. Further, inactivation of one or two alleles of *Trp53* rescues embryonic lethality of *Xrcc4*$^{-/-}$ mice [36]. Similarly, *Xrcc4*$^{-/-}$*Trp53*$^{+/-}$ mice possess more severe phenotype when compared to the *Xrcc4*$^{-/-}$*Trp53*$^{-/-}$ littermates, although both models are characterized by the reduced body weight, increased genomic instability and SCID due to inability to repair RAG-induced DSBs during the attempted V(D)J recombination in pro-B and pro-T cells [36].

Several complex mouse models revealed no genetic interaction between different pairs of NHEJ genes (Figure 2). In particular, the mice lacking Ku80 and XLF (*Ku80*$^{-/-}$*Xlf*$^{-/-}$), or PAXX (*Ku80*$^{-/-}$*Paxx*$^{-/-}$) [14], or DNA-PKcs (*Ku80*$^{-/-}$*Dna-pkcs*$^{-/-}$) [37] were indistinguishable from *Ku80*$^{-/-}$ mice, whereas *Ku70*$^{-/-}$*Xlf*$^{-/-}$ and *Ku70*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice were indistinguishable from *Ku70*$^{-/-}$ littermates [30]. The embryonic lethality of *Lig4*$^{-/-}$ mice was not rescued by the inactivation of *Paxx*$^{-/-}$ (*Lig4*$^{-/-}$*Paxx*$^{-/-}$) [14] (Figure 2).

2.1. Class swtich recombination

Class switch recombination (CSR) occurs in mature B cells following the efficient V(D)J recombination *in vivo*. However, several experimental models allow to by-pass the V(D)J recombination and determine the impact of specific factors on CSR, even though these factors are required for earlier B cell development stages (e.g., Artemis, DNA-PKcs). Knocking-in of pre-assembled *heavy* and *light* chains of *immunoglobulin* gene ("HL") allows to obtain mature B cells in the mice lacking V(D)J recombination [38]. In addition, CH12F3 murine lymphoma cell line allows to investigate the switching from IgM to IgA after inactivating the genes essential for the V(D)J recombination [39,40]. Thus, lack of Artemis or DNA-PKcs [38] moderately affects the CSR in mature B cells. Furthermore, the CSR levels were 2-3 folds reduced in the cells lacking one of the following factors: XLF [24,25], Ku70 [34], Ku80 [34], Lig4 [21], or XRCC4 [21]. PAXX seems to be dispensable for CSR in wild-type cells [13,14,16,26,39], while inactivation of *Mri* results in modest CSR defect [17,18,32].

Moreover, XLF and DNA-PKcs are functionally redundant during the CSR [28]. Similarly, in CSR assays, PAXX is functionally redundant with XLF (*Xlf*$^{-/-}$*Paxx*$^{-/-}$ cells) [40], but not with Mri (*Mri*$^{-/-}$*Paxx*$^{-/-}$ mice) [32].

2.2. Genetic interaction between NHEJ and DDR factors

DDR factors genetically interact with NHEJ components. *Atm* is synthetically lethal with *Ku70* (*Atm*$^{-/-}$*Ku70*$^{-/-}$), *Ku80* (*Atm*$^{-/-}$*Ku80*$^{-/-}$), and *Dna-pkcs* (*Atm*$^{-/-}$*Dna-pkcs*$^{-/-}$) [41]. Combined inactivation of *Atm* and *Dna-pkcs* in cells results in more severe CSR defects than in single-deficient controls [42]. Moreover, *Atm*$^{-/-}$*Dna-pkcs*$^{-/-}$ pro-B cells lack DNA repair of both SEs and CEs during the attempted V(D)J recombination [43].

Furthermore, *Xlf* genetically interacts with *Atm*, *53bp1*, *H2ax* and *Mdc1* [27,44-46]. In particular, mice lacking XLF and ATM (*Xlf*$^{-/-}$*Atm*$^{-/-}$) are live-born, possess reduced body weight and CSR, increased genomic instability and lack of V(D)J recombination in developing B and T cells, resulting in severe lymphocytopenia [1,44]. *Xlf*$^{-/-}$*53bp1*$^{-/-}$ mice are live-born, possess reduced body weight, increased genomic instability and reduced B and T cell count due to the block in V(D)J recombination in developing lymphocytes [45,46]. Differently, there is synthetic lethality between *Xlf* and *H2ax* as

well as *Xlf* and *Mdc1* in mice, and double deficient pre-B cells, *Xlf*$^{-/-}$*H2ax*$^{-/-}$ and *Xlf*$^{-/-}$*Mdc1*$^{-/-}$, possess reduced efficiency of V(D)J recombination [27,44]. Finally, *Atm* does not interact genetically with *Paxx*, and *Atm*$^{-/-}$*Paxx*$^{-/-}$ cells possess DNA repair levels similar to single deficient controls [14,31,47]. To summarize, *Atm* genetically interacts with *Ku70*, *Ku80*, *Dna-pkcs* and *Xlf,* but not *Paxx*. Moreover, *Xlf* genetically interacts with *Atm*, *H2ax*, *Mdc1* and *53bp1*.

2.3. Neuronal phenotype of NHEJ-deficient mice

Genomic instability in NHEJ-deficient mice gives rise to increased cell death in the central nervous system, including the brain, which correlates with embryonic lethality (Figure 2). XRCC4-deficient mice possess massive post-mitotic neuronal apoptosis with severe acellularity in the intermediate zone in the neurocortex [7,36]. Similarly, Lig4-deficient mice possess apoptosis of post-mitotical neurons [6]. While *Ku70*$^{-/-}$ mice a live-born, they possess modestly increased levels of neuronal cell death, which is nevertheless lower than that in *Xrcc4*$^{-/-}$ or *Lig4*$^{-/-}$ mice [9,33,48]. Inactivation of *Trp53* in *Xrcc4*$^{-/-}$ (*Xrcc4*$^{-/-}$*Trp53*$^{-/-}$) and *Lig4*$^{-/-}$ mice (*Lig4*$^{-/-}$*Trp53*$^{-/-}$) significantly reduces the neuronal apoptosis in the cortex [35,36]. Inactivation of *Ku80* rescues the lethality of *Lig4*$^{-/-}$ mice [33], and brains from *Lig4*$^{-/-}$*Ku80*$^{-/-}$ mice are similar to the ones from *Ku80*$^{-/-}$ mice [33]. XLF-deficient mice show no neuronal defects [24,25], although mutations in human *XLF* are associated with microcephaly [10].

Single deficiency for *Dna-pkcs*, *Paxx* or *Mri* results in mice with no obvious neurological defects [7,11,13,14,16-18,26]. However, homozygous mice carrying *Dna-pkcs* with point mutation D3922A (kinase dead, *Dna-pkcs*$^{KD/KD}$) possess p53- and Ku-dependent embryonic lethality, as well as increased neuronal apoptosis, with an overall phenotype resembling *Xrcc4*$^{-/-}$ and *Lig4*$^{-/-}$ mice [49].

Strikingly, combined deficiency for XLF and PAXX (*Xlf*$^{-/-}$*Paxx*$^{-/-}$), and XLF and Mri (*Xlf*$^{-/-}$*Mri*$^{-/-}$) results in synthetic lethality, which correlates with massive neuronal apoptosis in neocortex [13,14,18,26].

Hence, a deficient NHEJ results in neuronal apoptosis likely due to the accumulation of DSBs in post-mitotic neurons. Inactivation of p53 prevents neuronal apoptosis, for example, by allowing A-EJ to repair DSBs in NHEJ-deficient cells.

3. Conclusion

Overall, there is a complex genetic interaction between the genes of NHEJ pathway, and between NHEJ and DDR factors. Genetically modified mouse models and murine cell lines helped to uncover specific functions of DNA repair factors previously hidden due to the functional redundancy. Further studies will uncover additional genetic interactions between the DNA repair factors and pathways.

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