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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: Non-homologous end joining (NHEJ) is the main DNA repair mechanism for the repair of double strand breaks (DSBs) throughout the course of the cell cycle. DSBs are generated in developing B and T lymphocytes during V(D)J recombination to increase the repertoire of B and T cell receptors. DSBs are also generated during the class switch recombination (CSR) process in mature B lymphocytes, providing distinct effector functions of antibody heavy chain constant regions. Thus, NHEJ is important for both V(D)J recombination and CSR. NHEJ comprises core Ku70 and Ku80 subunits that form the Ku heterodimer, which binds DSBs and promotes the recruitment of accessory factors (e.g., DNA-PKcs, Artemis, PAXX, Mri) and downstream core factors (XLF, Lig4 and XRCC4). In recent decades, new NHEJ proteins have been reported, increasing complexity of this molecular pathway. Numerous *in vivo* mouse models have been generated and characterized to identify the interplay of NHEJ factors and their role in development of adaptive immune system. This review summarizes the currently available mouse models lacking one or several NHEJ factors, with a particular focus on early B cell development. We also underline genetic interactions and redundancy in the NHEJ pathway in mice.

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, e.g., during V(D)J recombination in developing B and T lymphocytes, and class switch recombination (CSR) in activated mature B cells [1,2].

The DNA damage response (DDR) pathway is initiated upon the induction of DSBs. *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. The Shieldin complex also promotes DNA repair [1,3]. Accumulation of DSBs results in ATM-dependent activation of

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checkpoint kinases 1 and 2 (Chk1 and Chk2), which arrests the cell cycle, followed by phosphorylation and stabilization of p53, which triggers apoptosis [4].

In mammalian cells, the non-homologous end joining (NHEJ) pathway repairs the majority of DSBs [5-7]. There are evolutionary conserved NHEJ factors referred to as "core" factors, as well as accessory factors that may be dispensable for DNA repair in wild type cells. Ku70, Ku80, X-ray repair cross-complementing protein 4 (XRCC4), DNA Ligase 4 (Lig4) and XRCC4-like factor (XLF) are core factors [8-12], while DNA-dependent protein kinase catalytic subunit (DNA-PKcs) [13-16], Artemis nuclease [17], paralogue of XRCC4 and XLF (PAXX) [18-21], and modulator of retroviral infection (Mri) [22,23] are usually considered accessory factors.

During the last years, several models have been proposed to explain how the two free DNA ends are brought back together through synapsis. In particular, Loparo´s group [7] suggested a two-stage model of NHEJ synaptic complex assembly, where DNA ends are initially tied in a long-range complex, followed by transition into a short-range complex. In this model, Ku70, Ku80 and DNA-PKcs (DNA-PK) first participate in the formation of the initial long-range complex, where DNA ends are held sufficiently distant. Then, the short-range complex is formed by DNA-PK, XLF, Lig4 and XRCC4. PAXX and Mri have not been implicated in this model because their functions have not been identified [7]. Another model proposed by Lieber´s group [24] suggests that there are two major structural complexes formed during the NHEJ synapsis. Ku70, Ku80, XRCC4 and Lig4 form the flexible synaptic (FS) complex, where XRCC4 and Lig4 bind to each DSB through interaction of Lig4 with Ku heterodimer. Subsequently, DSBs are brought together through interaction of XRCC4, giving rise to two Ku-XRCC4-Lig4-DNA complexes. XLF and PAXX both promote transition from FS to the second synaptic complex, called close synapsis (CS), although XLF stabilizes CS to a greater extent. XLF stimulates and impacts the general synapsis efficiency mediated by Ku-XRCC4-Lig4. It is suggested that DNA-PKcs is not required for the formation of either FS, or CS. This latter model explains the evolutionarily central synaptic role of the core NHEJ factors, Ku70, Ku80, XRCC4, Lig4 and XLF [24].

For practical purposes, NHEJ can be divided into three major stages: DSB recognition, stabilization-processing, and end-ligation [25]. Initially, DSBs are recognized by the heterodimer Ku, which is formed by Ku70 and Ku80. Ku assists the recruitment of DNA-PKcs [13-16], forming the DNA-PK holoenzyme. Subsequently, Artemis nuclease [17], PAXX [18-21], and Mri [22,23] are recruited to the DSB sites. Finally, XLF, XRCC4 and Lig4 mediate the Ku-dependent DNA end-ligation [1].

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. Both Ig and TCR provide antigen-binding specificity required for an efficient immune response. The proteins encoded by the recombination activating genes 1 and 2 (RAG1,2) form an endonuclease (RAG) that recognizes recombination signal sequences (RSSs) flanking the *V*, *D* and *J* gene segments [26].

CSR takes place in mature B cells, when constant regions of immunoglobulins switch from IgM to IgG, IgA, or IgE. Immunoglobulins, or antibodies, play a crucial role in immune response through their effector functions. CSR is initiated by 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 [27]. The 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 [27]. The joining of DSBs during the CSR is performed both by the classical-NHEJ (C-NHEJ) and alternative end-joining (A-EJ) [28]. Strikingly, the A-EJ can maintain up to 50% of CSR activity in the absence of core C-NHEJ factors, such as Ku70, XRCC4, Lig4 and XLF [1].

Other proteins, such as members of the DNA polymerase X family and terminal deoxynucleotidyl transferase enzyme (TdT), can also be involved in C-NHEJ within B cells [29,30]. For example, DNA polymerase proteins Pol λ and Pol μ promote DNA end-joining through the processing of DSBs, while TdT increases the antibody and TCR repertoire by adding non-template

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nucleotides prior to ligation of DNA ends during V(D)J recombination [29,30]. Proteins such as nipped-B-like protein (NIPBL) and breast cancer 1 (BRCA1) have also been shown to play a role in NHEJ [31,32].

2. Non-Homologous End Joining-Deficient Mice

Before transgenic mice became popular, an inbred strain of immunodeficient mice with severe combined immunodeficiency (SCID) was identified [33]. First characterized by *Bosma et al*., SCID mice carry a recessive mutation in the *Dna-pkcs* gene, which results in impaired V(D)J recombination and a subsequent lack of mature B and T lymphocytes in homozygous mice [33]. Later, transgenic mouse models deficient for *Dna-pkcs* gene were generated by several groups [13-16]. DNA-PKcs-deficient mice (*Dna-pkcs*$^{-/-}$) are live-born and possess a SCID phenotype due to inefficient coding-end (CE) joining during the V(D)J recombination [13] (Figure 1). *Artemis*$^{-/-}$ mice have also been observed to exhibit a SCID phenotype due to lack of CE joining [17]. Thus, both DNA-PKcs and Artemis are required for processing of RAG-mediated hairpin-sealed DNA ends (CEs) during V(D)J recombination, although repair of blunt signal ends (SEs) remains efficient in mice lacking Artemis [17] or DNA-PKcs [13-15]. Inactivation of *Ku70* [11] or *Ku80* [10] in mice results in reduced body weight and a SCID phenotype. Lack of B and T lymphocytes in *Ku70*$^{-/-}$ and *Ku80*$^{-/-}$ mice is explained by inefficient joining of both RAG-induced blunt SEs and hairpin-sealed CEs [10,11].

In contrast, inactivation of *Lig4* [8] or *Xrcc4* [9] results in embryonic lethality in mice, presenting challenges for *in vivo* studies. However, cell studies show that inactivation of *Lig4* or *Xrcc4* led to inefficient joining of both SEs and CEs, resembling Ku-deficient phenotypes in mice. This suggests that such *in vivo* models would yield an immunodeficient animal due to ablated V(D)J recombination and lack of B and T cells, if one could be generated [8,9].

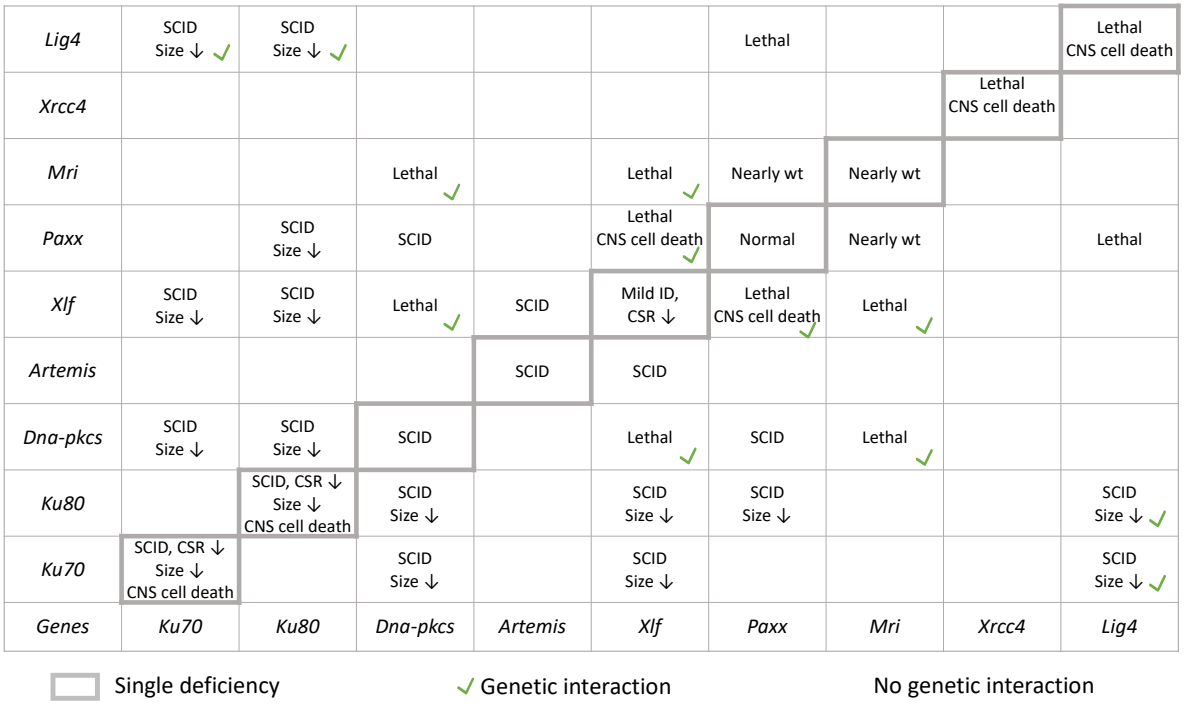

Figure 1. Genetic interaction between the NHEJ factors in mice. The phenotypes of mice are indicated. Genetic interactions are marked as (V). The lack of interaction is marked as (X).

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 [34,35] possess both mature B and T cells, despite being characterized by modest lymphocytopenia, and reduced repertoires of B cell receptors (BCRs) and TCRs [34,35]. *Xlf*$^{-/-}$ lymphocytes support efficient V(D)J recombination *in vitro*, including both SE and CE repair [34,35]. Mice lacking either PAXX

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[18,19,21,36] or Mri [22,23] possess normal counts of mature B and T cells, efficiently supporting both SE and CE repair during the V(D)J recombination. However, more complex mouse models have revealed that XLF, PAXX and Mri are required for V(D)J recombination, although their functions are compensated by each other and additional proteins due to the extensive genetic interaction inside the NHEJ pathway, as well as interaction between the NHEJ and DDR pathways [1,37].

Xlf$^{-/-}$ [34,35] and *Dna-pkcs*$^{-/-}$ [13] mice show notable radiosensitivity, but result in viable mice. However, double-deficient *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice are characterized by perinatal lethality and increased genomic instability, due to nearly no NHEJ [38]. While lymphocytes lacking either XLF or DNA-PKcs are capable of repairing RAG-induced blunt SEs, *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ cells are unable to join SEs [38]. Embryonic lethality of *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice is p53- and Ku-dependent, and triple-deficient *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$*Trp53*$^{+/-}$ [39,40] and *Ku70*$^{-/-}$*Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ [40] mice are live-born, although they possess reduced body weight comparable to one of *Ku70*$^{-/-}$ mice [39,40].

Xlf$^{-/-}$*Dna-pkcs*$^{-/-}$ was the first mouse model that highlighted genetic interaction between *Xlf* and accessory NHEJ factors *in vivo* (Figure 1). Mice lacking both XLF and Artemis are live-born, fertile, and have a SCID phenotype resembling single-deficient *Artemis*$^{-/-}$ mice [38], suggesting no genetic interaction between *Xlf* and *Artemis in vivo*. Later, genetic interaction between *Xlf* and *Paxx* was characterized *in vivo* independently by four research groups [18,19,36,39]. Mice lacking both XLF and PAXX possess late embryonic lethality, increased genomic instability, and immunodeficiency; and *Xlf*$^{-/-}$*Paxx*$^{-/-}$ lymphocytes are unable to sustain repair of both SEs and CEs generated during V(D)J recombination [18,19,36,41]. The embryonic lethality of *Xlf*$^{-/-}$*Paxx*$^{-/-}$ mice is p53-dependent, and both *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{+/-}$ and *Xlf*$^{-/-}$*Paxx*$^{-/-}$*Trp53*$^{-/-}$ mice are live-born, but possess significant weight reduction and a leaky SCID phenotype with nearly no mature B and T lymphocytes [39,42].

Furthermore, *Xlf*$^{-/-}$*Mri*$^{-/-}$ double knockout mice are embryonic lethal [23], but can be rescued by inactivation of one or two alleles of *Trp53* [42]. Mice lacking both XLF and Mri are characterized by leaky SCID with nearly no mature B and T lymphocytes due to the V(D)J recombination defect [23]. In particular, the lymphocytes lacking both XLF and Mri are unable to efficiently ligate both RAG-mediated SEs and CEs *in vitro* [23]. Both the double-deficient mouse model lacking XLF and PAXX, as well as the model lacking XLF and Mri, are characterized by leaky SCID with low but detectable levels of mature B and T lymphocytes [42]. This phenotype is likely possible due to residual NHEJ activity in developing *Xlf*$^{-/-}$*Paxx*$^{-/-}$ and *Xlf*$^{-/-}$*Mri*$^{-/-}$ lymphocytes *in vivo* [39,42].

Thus, there is evidence that *Xlf* genetically interacts with *Dna-pkcs* [38-40], *Paxx* [18,19,36,39,42] and *Mri* [23,42]. Further transgenic mouse studies address whether *Dna-pkcs* genetically interacts with *Paxx* and *Mri*, and whether *Paxx* genetically interacts with *Mri*. *Dna-pkcs*$^{-/-}$*Paxx*$^{-/-}$ mice are live-born, fertile, and indistinguishable from *Dna-pkcs*$^{-/-}$ littermates [39]. Furthermore, *Mri*$^{-/-}$*Paxx*$^{-/-}$ mice possess nearly no detectable phenotype, similar to single-deficient *Mri*$^{-/-}$ and *Paxx*$^{-/-}$ mice [42]. In contrast, *Dna-pkcs*$^{-/-}$*Mri*$^{-/-}$ mice are embryonic lethal [42]. Therefore, there is no genetic interaction between *Dna-pkcs* and *Paxx*, or *Mri* and *Paxx*; however, *Dna-pkcs* and *Mri* interact genetically (Figure 1).

Inactivation of *Ku*, *Trp53* or *Atm* rescues embryonic lethality of *Lig4*$^{-/-}$ mice. In particular, *Ku80*$^{-/-}$*Lig4*$^{-/-}$ [43] and *Ku70*$^{-/-}$*Lig4*$^{-/-}$ [44] mice are not distinguishable from Ku-deficient mice, possessing reduced body weight, increased levels of genomic instability and a 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 SEs and CEs during V(D)J recombination [45]. Moreover, *Lig4*$^{-/-}$*Trp53*$^{+/-}$ mice present a more severe phenotype than *Lig4*$^{-/-}$*Trp53*$^{-/-}$ littermates, likely due to an incomplete block of DNA damage-induced apoptosis in the presence of one *Trp53* allele [45]. In addition, inactivation of one or two alleles of *Atm* rescued lethality in *Lig4*-deficient mice [46]. Both *Lig4*$^{-/-}$*Atm*$^{+/-}$ and *Lig4*$^{-/-}$*Atm*$^{-/-}$ mice displayed impairment in lymphocytic development, growth retardation, and short lifespan (up to 2 days postnatally) [46]. Further, inactivation of one or two alleles of *Trp53* rescues embryonic lethality of *Xrcc4*$^{-/-}$ mice [47]. The *Xrcc4*$^{-/-}$*Trp53*$^{+/-}$ mice possess a more severe phenotype when compared to the *Xrcc4*$^{-/-}$*Trp53*$^{-/-}$ littermates, although both models are characterized by reduced body weight, increased genomic instability, and SCID due to inability to repair RAG-induced DSBs in developing B and T cells [47].

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Several complex mouse models have also revealed a lack of genetic interaction between different pairs of NHEJ genes (Figure 1). In particular, mice lacking Ku80 and either XLF (*Ku80*$^{-/-}$*Xlf*$^{-/-}$) [19], PAXX (*Ku80*$^{-/-}$*Paxx*$^{-/-}$) [19], or DNA-PKcs (*Ku80*$^{-/-}$*Dna-pkcs*$^{-/-}$) [48] are indistinguishable from *Ku80*$^{-/-}$ mice, whereas *Ku70*$^{-/-}$*Xlf*$^{-/-}$ and *Ku70*$^{-/-}$*Dna-pkcs*$^{-/-}$ mice are indistinguishable from *Ku70*$^{-/-}$ littermates [40]. Finally, embryonic lethality of *Lig4*$^{-/-}$ mice is not rescued by the inactivation of *Paxx*$^{-/-}$ (*Lig4*$^{-/-}$*Paxx*$^{-/-}$) [19] (Figure 1).

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 for the by-pass of V(D)J recombination to determine the impact of specific factors on CSR, even though these factors are required for earlier stages of B cell development (e.g., Artemis, DNA-PKcs). Knocking-in pre-assembled *heavy* and *light* chains of the *immunoglobulin* gene ("HL") allows for the development of mature B cells in mice that otherwise lack the capacity for V(D)J recombination [49]. For example, lack of Artemis or DNA-PKcs [49] moderately affects the CSR in mature B cells. Additionally, it was found that CSR levels were reduced 2-3 fold in cells lacking XLF [34,35], Ku70 [44], Ku80 [44], Lig4 [28], or XRCC4 [28]. PAXX seems to be dispensable for CSR in wild-type cells [18,19,21,36,50], while inactivation of *Mri* results in modest CSR defects [22,23,42].

DNA-PKcs and XLF are functionally redundant in CSR [38]. PAXX is also functionally redundant with XLF (*Xlf*$^{-/-}$*Paxx*$^{-/-}$ cell lines) [51], but not with Mri (*Mri*$^{-/-}$*Paxx*$^{-/-}$ mice) [42].

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*$^{-/-}$) [46] (Figure 2). Combined inactivation of *Atm* and *Dna-pkcs* in cells results in more severe CSR defects than in single-deficient controls [52]. Moreover, *Atm*$^{-/-}$*Dna-pkcs*$^{-/-}$ pro-B cells lack repair of both SEs and CEs during the attempted V(D)J recombination [53].

Rag2				SCID ✓		
53bp1				SCID, Size ↓ ✓		
Mdc1				Lethal ✓		
H2ax				Lethal ✓		
Atm	Lethal ✓	Lethal ✓	Lethal ✓	SCID, CSR ↓ Size ↓ ✓	Nearly *Atm*$^{-/-}$	Size ↓ CNS cell death Alive ✓
Genes	*Ku70*	*Ku80*	*Dna-pkcs*	*Xlf*	*Mri*	*Lig4*

✓ Genetic interaction No genetic interaction

Figure 2. Genetic interactions between NHEJ factors, DDR factors and *Rag2* in mice. The phenotypes of mice are indicated. Genetic interactions are marked as (V). The lack of interaction is marked as (X).

Furthermore, *Xlf* genetically interacts with *Atm*, *53bp1*, *H2ax* and *Mdc1* [37,54-56]. 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 (Figure 2) [1,54]. *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

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recombination in developing lymphocytes (Figure 2) [55,56]. Synthetic lethality does arise with *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 [37,54]. Finally, *Atm* does not interact genetically with *Paxx* and *Mri*. *Atm*$^{-/-}$*Paxx*$^{-/-}$ cells possess DNA repair levels similar to single deficient controls [19,41,57], while *Atm*$^{-/-}$*Mri*$^{-/-}$ mice are indistinguishable from *Atm*$^{-/-}$ littermates [23] (Figure 2). Taken together, evidence shows that *Atm* genetically interacts with *Ku70*, *Ku80*, *Dna-pkcs* and *Xlf*, but not with *Paxx* or *Mri*. Correspondingly, *Xlf* genetically interacts with *Atm*, *H2ax*, *Mdc1* and *53bp1*.

2.3. Genetic interacion between Xlf and Rag

Xlf has also been shown to genetically interact with *Rag2* [58]. Mutation in the *Rag2* gene results in the truncated protein "core Rag 2", which continues to support DSB formation and DNA repair in developing B and T lymphocytes. However, in XLF-deficient cells, this "core Rag2" activity is lost, and V(D)J recombination does not proceed. This finding suggests a potential role for RAG in both the induction of DSBs and DNA repair, as the RAG complex supports tethering of DNA ends before ligation [58] (Figure 2).

2.4. 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 1). XRCC4-deficient mice exhibit massive post-mitotic neuronal apoptosis with severe acellularity in the intermediate zone in the neurocortex [9,47]. Similarly, Lig4-deficient mice also exhibit apoptosis of post-mitotic neurons [8]. While *Ku70*$^{-/-}$ mice are live-born, they possess modestly increased levels of neuronal cell death, which is nevertheless lower than that in *Xrcc4*$^{-/-}$ or *Lig4*$^{-/-}$ mice [11,43,59]. Inactivation of *Trp53* in *Xrcc4*$^{-/-}$ (*Xrcc4*$^{-/-}$*Trp53*$^{-/-}$) and *Lig4*$^{-/-}$ mice (*Lig4*$^{-/-}$*Trp53*$^{-/-}$) significantly reduces neuronal apoptosis in the cortex [45,47], similar to what is reported in *Lig4*$^{-/-}$*Atm*$^{-/-}$ mice[46]. Inactivation of *Ku80* rescues the lethality of *Lig4*$^{-/-}$ mice [43], and brains from *Lig4*$^{-/-}$*Ku80*$^{-/-}$ mice are similar to those from *Ku80*$^{-/-}$ mice [43]. XLF-deficient mice show no neuronal defects [34,35], although mutations in human *XLF* are associated with microcephaly [12].

Single deficiency for *Dna-pkcs* [9,59], *Paxx* [18,19,21,36] or *Mri* [22,23] results in mice with no obvious neurological defects. However, mice that are homozygous for *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 [60].

Strikingly, combined deficiency for XLF and PAXX (*Xlf*$^{-/-}$*Paxx*$^{-/-}$) [18,19,36], and XLF and Mri (*Xlf*$^{-/-}$*Mri*$^{-/-}$) [23] results in synthetic lethality, which correlates with massive neuronal apoptosis in the neocortex.

Hence, deficiencies in NHEJ often result in neuronal apoptosis, likely due to 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.

2.5. NHEJ in mouse and human

Mutations in several NHEJ genes have been identified in humans [61,62]. For instance, patients with mutations in *XLF*, *DNA-PKCS*/*PRKDC* and *LIG4* genes display severe clinical features, characterized mainly by SCID, delayed growth and neurological abnormalities [61,62]. In mice, XLF deficiencies lead to a modest lymphocytopenia and defect in CSR [34,35]; DNA-PKcs-deficiencies lead to a SCID phenotype but no neural complications [59]; and Lig4-deficiencies are embryonic lethal [8]. In the same manner, *ARTEMIS*/*DCLRE1C*-deficient patients are characterized by SCID but not neurological defects [62]; similar to Artemis-deficient mice [17]. On the other hand, *Xrcc4*$^{-/-}$ mice are embryonic lethal [9], unlike XRCC4-deficient patients who only display neurological problems [63]. Mutations in several NHEJ genes have not yet been found in human immunodeficient patients up to the present. These genes include *Ku70*, *Ku80*, *PAXX* and *MRI*. Ku70 and Ku80 might be essential in human cells, and therefore mutations in *KU70* and *KU80* genes might be identified only by

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analyzing embryonic samples. However, mutations in accessory factor genes *PAXX* and *MRI* might present without clinical features, based on the knowledge we have obtained from mouse models [18,19,21-23,36]. In the latter case, XLF might compensate for deficiencies in PAXX and Mri in human cells. Sometimes, dramatic differences in phenotypic presentation between mice and humans lacking the same NHEJ factor can be explained, for example, by minor sequence changes between species, resulting in significant changes in protein-protein and protein-DNA interactions, and inability for other factors to compensate the protein loss.

3. Potential reasons for genetic interactions between the NHEJ factors

There are several types of genetic interaction between the DNA repair factors and several potential explanations for them, although detailed mechanisms have not been elucidated yet.

Why does inactivation of *Ku70* [11] or *Ku80* [10] result in viable mice, while inactivation of *Lig4* [8] or *Xrcc4* [9] results in embryonic lethality? This cannot simply be due to lack of NHEJ activity, because cells lacking any of these factors are characterized by similar genomic instability [38,40,57]. The Ku70/Ku80 complex seems to be toxic for the cells when the NHEJ pathway is blocked due to defects in downstream factors. It is possible that Ku may block access to DSB sites from other DNA repair pathway proteins, preventing DNA ligation and eventually resulting in the accumulation of DSBs, activation of p53 and apoptosis. Interestingly, Ku-deficient cells rely on other DNA repair pathways, such as homologous recombination and alternative end-joining. This could also explain why inactivation of *Ku70* or *Ku80* rescues embryonic lethality of *Lig4*$^{-/-}$ mice [43,46]. Similarly, lack of *Ku* rescues embryonic lethality in mice lacking XLF/DNA-PKcs [38,40], as well as in mice with an inactivating DNA-PKcs point mutation [60]. Following the same logic, one can predict that inactivation of *Ku70* or *Ku80* would also have the ability to rescue synthetic lethality between *Xlf* and *Paxx*, and between *Xlf* and *Mri*. We can also predict that inactivation of all NHEJ genes in a mouse would result in a phenotype similar to those of *Ku70*$^{-/-}$ or *Ku80*$^{-/-}$ mice [22], and suggest that all NHEJ genes function in a purely Ku-dependent manner.

Synthetic lethality between *Xlf* and *Dna-pkcs* [38-40], *Xlf* and *Paxx* [18,19,36,39,42*]* and *Xlf* and *Mri* [23,42] results in phenotypes similar to *Lig4*$^{-/-}$ and *XRCC4*$^{-/-}$. In all these cases, it is likely that the DNA ligation step is impaired, while Ku70/Ku80 remains functional. There are several potential explanations for the functional redundancy observed between XLF and other factors in NHEJ and DDR [1,38,54,56]. First, XLF and the second factor could have identical functions, such as having a role in stabilizing the DNA repair complex. A second explanation could be that XLF and the second factor could have purely complementary functions; for example, one protein stimulates DNA ligation while another one is required for DNA end tethering. However, the question of XLF's functionally redundancy with so many other factors [1,18,19,23,36,38-40,42,54] is still an enigma in the field of DNA repair.

ATM and DNA-PKcs are both protein kinases. Synthetic lethality between *Atm* and *Dna-pkcs* [46] is reasonable to predict because these two proteins can partially compensate for each other's activity when one is inhibited, but no other protein can compensate combined ATM/DNA-PKcs deficiency [52,53,64]. DNA-PKcs is part of the DNA-PK holoenzyme, which includes Ku70 and Ku80. Both *Ku70* and *Ku80* are synthetic lethal with *Atm* [46], meaning that ATM is functionally redundant with the Ku70/Ku80/DNA-PKcs complex, and DNA-PKcs will likely be inactive in cells lacking Ku70 or Ku80.

Deficiency in *Atm* partially rescues lethality of mice lacking *Lig4* [46]. This may be related to the function of ATM, which translates critical accumulation of DSBs to apoptosis via Chk1, Chk2 and p53. Similarly, inactivation of one or both alleles of *Trp53* (encoding for p53) rescues embryonic lethality of *Lig4*$^{-/-}$ [45], *Xrcc4*$^{-/-}$ [47], *Xlf*$^{-/-}$*Dna-pkcs*$^{-/-}$ [39,40], *Xlf*$^{-/-}$*Paxx*$^{-/-}$ [39,42] and *Xlf*$^{-/-}$*Mri*$^{-/-}$ [42] mice. We can infer that the dose-dependent reduction of apoptosis from ATM- and p53-deficiency could ensure survival for NHEJ-deficient mice lacking DNA ligation capabilities, albeit these alterations would likely lead to tumorigenesis and cancer [39,40,42,45-47].

3. Conclusion

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Overall, there are complex genetic interactions between the genes of the NHEJ pathway, and between NHEJ and DDR factors. Genetically modified mouse models and murine cell lines have 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. Only a portion of genetic interaction is analyzed today, and empty cells represent potential future studies (Figures 1 and 2).

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