INTRODUCTION

DNA double-strand breaks (DSBs) are generated both extrinsically, for example, by chemotherapeutic agents, and physiologically, for example, 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, thereby activating the formation of Really Interesting New Gene (RING) Finger Protein 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.\textsuperscript{1,3} Accumulation of DSBs results in ATM-dependent activation of checkpoint kinases 1 and 2 (CHK1 and CHK2), which arrests the cell cycle, followed by phosphorylation and stabilization of p53, which triggers apoptosis.\textsuperscript{4}

In mammalian cells, the non-homologous end-joining (NHEJ) pathway repairs the majority of DSBs.\textsuperscript{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, XRCC4, Lig4 and XLF.\textsuperscript{18-21} Artemis nuclease,\textsuperscript{17} paralogue of XRCC4 and Lig4 form the flexible synaptic (FS) complex, where XRCC4 interacts with Ku, which is formed by Ku70 and Ku80. Ku assists the recruitment of DNA-PKcs\textsuperscript{13-16} forming the DNA-PK holoenzyme. Subsequently, Artemis nuclease,\textsuperscript{17} PAXX\textsuperscript{18-21} and MRI\textsuperscript{22,23} are recruited to the DSB sites. Finally, XLF, XRCC4 and Lig4 mediate the Ku-dependent DNA end ligation.\textsuperscript{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.\textsuperscript{26}

Class switch recombination 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 deamidates deoxyctydine resulting in deoxyuracil (dC > dU). The dUs are excised by the uracil DNA N-glycosylase (UNG) enzyme, leaving an abasic (aprymidinic/apurinic [AP]) site.\textsuperscript{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.\textsuperscript{27} The joining of DSBs during the CSR is performed both by the classical NHEJ (C-NHEJ) and alternative end joining (A-EJ).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 nucleotides prior to ligation of DNA ends during V(D)J recombination.\textsuperscript{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.\textsuperscript{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.\textsuperscript{33} First characterized by Bosma et al.,\textsuperscript{33} SCID mice carry a recessive mutation in the Dnapkcs gene, which results in impaired V(D)J recombination and a subsequent lack of mature B and T lymphocytes in...
homozygous mice. Later, transgenic mouse models deficient for \textit{Dna-pkcs} gene were generated by several groups.\textsuperscript{13-16} DNA-PKcs−/− mice possess both mature B and T cells, despite being characterized by modest lymphocytopenia, and reduced repertoires of B cell receptors and TCRs.\textsuperscript{34,35} Xlf−/− lymphocytes support efficient V(D)J recombination \textit{in vitro}, including both SE and CE repair.\textsuperscript{34,35} Mice lacking either PAXX\textsuperscript{18,19,21,36} or MRI\textsuperscript{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.\textsuperscript{1,37}

\textbf{FIGURE 1} Genetic interactions between the NHEJ factors in mice. The phenotypes of mice are indicated. Genetic interactions are marked as $\checkmark$. The lack of interaction is marked as $\times$.
NHEJ factors in vivo (Figure 1). Mice lacking both XLF and Artemis are live-born, are fertile and have a SCID phenotype resembling single-deficient Artemis−/− mice, 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. 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. 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.

Furthermore, Xlf−/−Mri−/− double knockout mice are embryonic lethal, but can be rescued by inactivation of one or two alleles of Trp53. 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. In particular, the lymphocytes lacking both XLF and MRI are unable to efficiently ligate both RAG-mediated SEs and CEs in vitro. Both the double-deficient mouse lacking XLF and PAXX and the model lacking XLF and MRI are characterized by leaky SCID with low but detectable levels of mature B and T lymphocytes. This phenotype is likely possible due to residual NHEJ activity in developing Xlf−/−Paxx−/− and Xlf−/−Mri−/− lymphocytes in vivo.

Thus, there is evidence that Xlf genetically interacts with Dna-pkcs and Paxx, and the model lacking XLF and MRI is synthetically lethal in CSR. 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−/−), PAXX (Ku80−/−Paxx−/−), or DNA-PKCs (Ku80−/−Dna-pkcs−/−) are indistinguishable from Ku80−/− mice, whereas Ku70−/−Xlf−/− and Ku70−/−Dna-pkcs−/− mice are indistinguishable from Ku70−/− littermates. Finally, embryonic lethality of Lig4−/− mice is not rescued by the inactivation of Paxx−/− (Lig4−/−Paxx−/−) mice.

2.1 Class switch recombination

Class switch recombination occurs in mature B cells following the efficient V(D)J recombination in vivo. However, several experimental models allow for the bypass 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. For example, lack of Artemis or DNA-PKcs moderately affects the CSR in mature B cells. Additionally, it was found that CSR levels were reduced two- to threefold in cells lacking XLF, and Ku70−/− and Ku80−/− mice. However, Lig4−/− or XRCC4−/− mice are embryonic lethal. 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−/− and Ku70−/−/Lig4−/− 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. 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. In addition, inactivation of one or two alleles of Atm rescued lethality in Lig4-deficient mice. Both Lig4−/−/Atm−/− and Lig4−/−/Atm−/− mice displayed impairment in lymphocytic development, growth retardation and short lifespan (up to 2 days postnatally). Further, inactivation of one or two alleles of Trp53 rescues embryonic lethality of Xrcc4−/− mice. 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.

2.2 Genetic interaction between NHEJ and DDR factors

DNA damage response factors genetically interact with NHEJ components. Atm is synthetically lethal with Ku70...
(Atm−/−Ku70−/−), Ku80 (Atm−/−Ku80−/−) and Dna-pkcs (Atm−/−Dna-pkcs−/−) (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

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 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 pro-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−/− and Xlf−/−Paxx−/− cells possess DNA repair levels similar to single-deficient controls,19,41,57 while Atm−/−Mri−/− mice are indistinguishable from Atm−/− littermates23 (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 interaction 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 Rag2’, 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 ligation53 (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 (CNS), 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 neocortex.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,43,45 similar to what is reported in Lig4−/−Atm−/− mice.56 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 Paxx18,19,21,36 or Mri22,23 results in mice with no obvious neurological defects. However, mice that are homozygous for Dna-pkcs with point mutation D3922A (kinase dead, Dna-pkcsKD/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.

### FIGURE 2

Genetic interactions between NHEJ factors, DDR factors and Rag2 in mice. The phenotypes of mice are indicated. Genetic interactions are marked as ✓. The lack of interaction is marked as ×.
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. 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. In mice, XLF deficiencies lead to a modest lymphocytopenia and defect in CSR; DNA-PKcs deficiencies lead to a SCID phenotype but no neural complications; and Lig4-deficiencies are embryonic lethal. 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. Similarly, lack of Ku rescues embryonic lethality in mice lacking XLF/DNA-PKcs as well as in mice with an inactivating DNA-PKcs point mutation. 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, and suggest that all NHEJ genes function in a purely Ku-dependent manner.

Synthetic lethality between Xlf and Dna-pkcs, Xlf and Paxx, and Xlf and Mre 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. 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 strand annealing and alternative end joining. This could also explain why inactivation of one or both alleles of Trp53 (encoding for p53) rescues embryonic lethality of Lig4−/− mice. Similarly, inactivation of one or both alleles of Xrcc4−/− 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.
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 analysed today, and empty cells represent potential future studies (Figures 1 and 2).

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
SCZ and VO wrote most of the manuscript, while all the authors contributed to the initial and revised versions of the manuscript.

ORCID
Sergio Castañeda-Zegarra https://orcid.org/0000-0002-2808-6788
Marion Fernandez-Berrocal https://orcid.org/0000-0002-5839-9355
Nikki Lyn Esnardo Upfold https://orcid.org/0000-0002-6312-5156
Valentyn Oksenych https://orcid.org/0000-0002-5088-3791

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