Normal development of mice lacking PAXX, the paralogue of XRCC4 and XLF

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DNA repair consists of several cellular pathways which recognize and repair damaged DNA. The classical nonhomologous DNA end-joining (NHEJ) pathway repairs double-strand breaks in DNA. It is required for maturation of both B and T lymphocytes by supporting V(D)J recombination as well as B-cell differentiation during class switch recombination (CSR). Inactivation of NHEJ factors Ku70, Ku80, XRCC4, DNA ligase 4, DNA-PKcs, and Artemis impairs V(D)J recombination and blocks lymphocyte development. Parologue of XRCC4 and XLF (PAXX) is an accessory NHEJ factor that has a significant impact on the repair of DNA lesions induced by ionizing radiation in human, murine, and chicken cells. However, the role of PAXX during development is poorly understood. To determine the physiological role of PAXX, we deleted part of the Paxx promoter and the first two exons in mice. Further, we compared Paxx−/− knockout mice with wild-type (WT) and NHEJ-deficient controls including Ku80−/− and Dna-pkcs-null and severe combined immunodeficiency mice. Surprisingly, Paxx−/− deficient mice were not distinguishable from the WT littermates; they were the same weight and size, fertility status, had normal spleen, thymus, and bone marrow. Paxx−/− deficient mice had the same number of chromosomal and chromatid breaks as WT mice. Moreover, Paxx−/− deficient primary B lymphocytes had the same level of CSR as lymphocytes isolated from WT mice. We concluded that PAXX is dispensable for normal mouse development.

Abbreviations
53BP1, p53-binding protein 1; AID, activation-induced cytidine deaminase; ATM, Ataxia telangiectasia mutated; CSR, class switch recombination; DDR, DNA damage response signaling; DNA-PKcs, DNA-dependent protein kinase, catalytic subunit; DNA-PK, DNA-dependent protein kinase (holoenzyme); DSB, DNA double-strand break; HRP, Horseradish peroxidase; Ig, immunoglobulin; IL, interleukin; Lig4, DNA ligase 4; LPS, lipopolysaccharide; NHEJ, nonhomologous end joining; PAXX, paralogue of XRCC4 and XLF; RAG1/2, recombination activating genes 1 and 2; SCID, severe combined immunodeficiency; T-FISH, telomere fluorescence in situ hybridization; UNG, uracil DNA N-glycosylase; XLF, XRCC4-like factor; XLS, XRCC4-like small protein; XRCC4, X-ray cross-complementing protein 4.
Nonhomologous end joining (NHEJ) recognizes and repairs DNA double-strand breaks (DSBs) throughout the cell cycle [1]. NHEJ is required to maintain genomic stability in response to extrinsically and physiologically induced DSBs. The latter includes DNA breaks generated by the recombination activating genes (RAG1/2) during V(D)J recombination in developing B and T lymphocytes. Activation-induced cytidine deaminase (AID) converts cytosine to uracil at the actively transcribed switch regions of immunoglobulin heavy-chain coding regions in mature B lymphocytes, and in cooperation with uracil DNA N-glycosylase (UNG), it produces single-strand breaks in both DNA strands leading to DSBs that are recognized and repaired by NHEJ [1,2]. NHEJ includes core subunits, Ku70 and Ku80 (or X-ray repair cross-complementing proteins, XRCC6 and XRCC5, respectively) that form the Ku heterodimer, which recognizes DSBs and serves as a platform to recruit and stabilize other NHEJ subunits. X-ray cross-complementing protein 4 (XRCC4) and DNA ligase 4 (Lig4) form another heterodimer that ligates DNA ends. There are several known accessory NHEJ factors that likely work downstream of Ku, upstream or in cooperation with XRCC4/Lig4, and are required in specific cases. Among them DNA-dependent protein kinase, catalytic subunit (DNA-PKcs), which is a protein kinase that forms the DNA-PK holoenzyme with Ku70/Ku80 and phosphorylates most NHEJ factors, including itself. DNA-PKcs is specifically required for stabilization and activation of the nuclease Artemis that processes RAG-induced DNA hairpins and overhangs during V(D)J recombination. The inactivation of any core NHEJ factor, as well as DNA-PKcs and Artemis, results in severe immunodeficiency associated with B and T lymphocytopenia, due to the inability of B and T progenitors to perform V(D)J recombination and thus to mature (reviewed in Ref. [1]).

XRCC4-like factor (XLF, also known as Nhej1 or Cernunnos) is considered both a core and an accessory factor in NHEJ. Similar to core NHEJ factors, XLF is evolutionary conserved in eukaryotic cells from yeast to humans. It also suppresses medulloblastoma development in p53-deficient background [3]. On the other hand, Xlf inactivation alone does not lead to a severe phenotype in mice, likely due to its functional overlap with other accessory NHEJ factors [4,5] and potentially with the Ataxia telangiectasia mutated (ATM)-dependent DNA damage response (DDR) pathway [1,6–8]. XLF was also shown to have functional overlap with RAG recombinase, which is likely lymphocyte-specific [9]. Xlf inactivation in combination with, for example, knockout of Atm, histone H2ax, DNA damage response factor p53-binding protein 1 (53BP1), or RAG2 truncation leads to a block in lymphocyte development and thus a severe reduction in B and T lymphocyte numbers [1,5,9]. It is very likely that other accessory NHEJ or DDR factors complement the roles of XLF in DNA repair and lymphocyte development.

PAXX (also known as C9Orf142, or XRCC4-like small protein) is an accessory NHEJ factor reported by several research groups in 2015 [10–12]. PAXX-deficient human, murine, and chicken cells displayed an increased sensitivity to DSBs induced by ionizing radiation [10–12]. In experiments based on knockout chicken and murine cells, PAXX was shown to have some functional redundancy with its paralogue XLF [12–16]. In addition, three knockout mouse models were generated at different laboratories and published recently [17–19]. These studies suggest that PAXX has an overlapping function with XLF and is required for embryonic development and maintenance of central nervous system. However, the role of PAXX on the organismal level requires further investigation.

To determine the physiological role of PAXX, we generated a Paxx-deficient knockout mouse model. We compared Paxx null mice with wild-type (WT) and NHEJ-deficient controls, including the Ku80-, Dna-pkcs-deficient, and severe combined immunodeficiency (SCID) mice. We found that Paxx null mice do not differ from WT and heterozygous littermates in viability, lymphoid organ development, class switch recombination (CSR) efficiency, and genomic stability.

**Materials and methods**

**Mouse models**

All experiments involving mice were performed according to the protocols approved by the Norwegian University of Science and Technology (NTNU). Ku80+/− [20], Dnapkcs+/- [21], SCID [22], and Ung+/- [23] mice were described previously. Paxx+/- mice are custom-generated and described here for the first time.

**Generation of Paxx−/− mice**

Paxx-deficient (Paxx−/−) mice were generated upon request as OKS1 project by genOway (Lyon, France). Analysis of the Paxx gene structure showed that another gene Clic3 overlaps with the Paxx. Thus, only the part of Paxx gene that does not overlap with Clic3 was deleted. Two sgRNAs were designed to target the promoter region and the end of exon 2 of the Paxx gene: sgRNA#1, CCC AAC AGG GGC TTT TAC TGC; sgRNA#2, GGC GGC GTG CTG CAG ACT. Fertilized oocytes were collected from superovulated...
female mice previously mated with males. The purified sgRNAs and Cas9 RNA were microinjected into the male pronucleus. Injected zygotes were cultivated overnight to the two-cell stage to assess sgRNAs toxicity. Resulting two-cell embryos were reimplanted into pseudopregnant foster mothers 0.5 day post coitum. A total of 158 injected embryos were reimplanted into foster mothers, leading to the birth of 60 viable pups.

**Mouse screening strategy**

The screening was performed on genomic DNA extracted from mouse skin. Two primers were used to amplify the original or modified part of the Paxx gene. The intact gene results in a 965-bp product and deletion resulted in shorter products ranging from 280 to 412 bp, depending on the size of deletion. Four founder mice were identified in which mutation was confirmed by sequencing at genOway. Three heterozygous Paxx-knockout lines were obtained by backcrossing founders to C57BL6/N WT mice. The primers to detect deletion in murine Paxx gene were as follows: ACA GAG GTG GTG GAC TCA GAC AAT GG and GGA AAT GCT ATT AGA ACC ACT GCC ACG.

**Antibodies**

To detect the PAXX protein by western blot, we used rabbit polyclonal anti-PAXX/C9orf142 IgG (NovusBio, Littleton, CO, USA, NBPI-94172, dilution 1 : 500), which recognizes the C-terminal half of the PAXX protein (amino acids 109-204); anti-PAXX/C9orf142 IgG (Abcam, Cambridge, UK, ab126353, 1 : 200) and swine polyclonal anti-rabbit Ig-HRP (Dako antibodies, #P0260, 1 : 3000). Anti-GAPDH rabbit polyclonal (Sigma, St. Louis, MO, USA, #G9545, developed to recognize 314–333 amino acids of mouse GAPDH, 1 : 2000) and mouse monoclonal anti-β-actin (Abcam, ab8226, 1 : 3000) with rabbit polyclonal anti-mouse Ig-HRP (Dako antibodies, #P0260, 1 : 3000) were used to control protein loading.

**CSR to IgG1, tail fibroblasts, telomere FISH (T-FISH), statistical analyses**

CSR to IgG1 was performed as previously described in Refs [4,8,24]. Briefly, splenic B cells were isolated by negative selection using magnetic immunolabelling with an EasySep™ Mouse B Cell Isolation Kit (Stemcell, Cambridge, UK, #19854), stimulated with IL-4 and lipopolysaccharide (LPS), and analyzed by flow cytometry at day 4. Antibody used for IgG1 detection was anti-IgG1-APC (BD Biosciences, Franklin Lakes, NJ, USA, #550874). Primary murine tail fibroblasts were generated and cultured as previously described [4,5,7]. T-FISH was performed as previously described [4,5,7]. Statistical analyses were performed using GRAPHPAD PRISM 7.03 (La Jolla, CA, USA), one-way ANOVA.

**Histological brain analysis**

We isolated brains of 5-week-old mice, fixed them with 4% formaldehyde for 15 days, paraffin-embedded, and sectioned (4 μm). Nissl staining was performed as described in Ref. [25]. Briefly, after deparaffinization and hydration, the sections were immersed in 0.1% cresyl violet acetate (Sigma) for 5 min. Then, the samples were rinsed with distilled water and dehydrated in ethanol, cleared in Clear-Rite™ 3 (Thermo Fisher Scientific, Waltham, MA, USA), and mounted with Entall™New (Merck Millipore, Burlington, MA, USA). Images were taken with Nikon D5-Fi2 microscope (Nikon, Tokyo, Japan). Figures were analyzed to distinguish between apoptotic cells harboring pyknotic nuclei (strong staining, rounded shape, and smaller size) from proliferative cells (strong staining, flattened cells with a lot of cytoplasm around nuclei). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed by In Situ Cell Death Detection Kit, TMR red Protocol (Roche, Basel, Switzerland) following the manufacturer’s guide. Briefly, the sections were deparaffinized by heating and hydrated with decreasing concentrations of ethanol. Then, the samples were treated with proteinase K for 20 min at 37 °C and washed with 1× PBS (Sigma). Sections were incubated in a terminal deoxynucleotidyl transferase (TdT) reaction mix for 1 h at 37 °C, washed with PBS, and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). Images were taken with Zeiss LSM 510 Meta microscope (Nikon).

**Results**

**Generation of Paxx−/− mice**

To identify the physiological role of PAXX, we generated a mouse model with deletion of part of the Paxx locus on a C57BL6/N background (Fig. 1). For this, the purified sgRNAs and Cas9 RNA were microinjected into the fertilized oocytes, resulting in a locus deletion and complete inactivation of the Paxx gene function. A total of 158 injected embryos were reimplanted into foster mothers leading to 60 viable pups. Four Paxx F0 null founders that carried 538- to 670-bp deletions covering the Paxx promoter and exons 1–2 were identified by PCR screening (Fig. 1B) and DNA sequencing. The founders were backcrossed to C57BL6/N WT mice, and the first heterozygous generation was used to establish colonies. The resulting Paxx-deficient mice (Paxx−/−) showed complete absence of the Paxx protein in tail fibroblasts, spleen, thymus, liver, and lungs when compared to Paxx+/+ and Paxx+/− littermates (Fig. 1C–E). These results we verified using two independent antibodies, one from NovusBio and one from Abcam, both generated to recognize the C-terminal half,
109–204 amino acids of human PAXX protein. Moreover, haploinsufficiency for Paxx resulted in reduced PAXX protein level in Paxx+/+ cells compared to Paxx+/- controls, as shown for tail fibroblasts and liver (Fig. 1C). We concluded that our Paxx+/− mice do not express PAXX.

**Paxx+/- mice are viable and grow normally**

The inactivation of one or both Paxx alleles resulted in viable and fertile mice indistinguishable by size from WT littermates (Fig. 1F–H). The pups from Paxx+/− parents were born at the expected 1 : 2 : 1 proportion, 43 Paxx+/+, 89 Paxx+/-, and 52 Paxx−/− mice (Fig. 1F). At day 30, the average size of Paxx+/+, Paxx+/-, and Paxx−/− mice was not significantly different; all these mice were larger than Ku80−/− mice which are known to have reduced body size (Fig. 1G, H). To further describe our Paxx−/− mouse model, we performed Nissl staining and TUNEL assay on brain sagittal sections of 5-week-old mice. Nissl staining revealed that the morphology of the Paxx−/− brain

![Image](image_url)
was identical to WT littermates. According to this, the presence of apoptotic cell WT brains did not outnum-
ber the apoptotic cells found in Paxx<sup>−/−</sup>. Proliferative cells were found in both genotypes at similar
levels of apoptosis were almost undetectable in both Paxx<sup>−/−</sup> and WT brains. We concluded that
inactivation of Paxx alone did not affect growth, size, fertility, and development of central nervous system in
mice, which is in line with recently published three independent Paxx-knockout mouse models

**Paxx<sup>−/−</sup> mice develop normal lymphoid organs**

Neither spleen nor thymus development was affected in the absence of PAXX (Fig. 2). The average spleen sizes
at 45 days were 70, 67, and 73 mg for Paxx<sup>+/+</sup>, Paxx<sup>+/−</sup>, and Paxx<sup>−/−</sup>, respectively (no significant difference),
while it was reduced in NHEJ-deficient Ku80<sup>−/−</sup> (15 mg) and Dna-pkcs<sup>−/−</sup> (21 mg) mice of the same age,

\[ P < 0.0001 \] (Fig. 2A,C). The average splenocyte counts were similar in Paxx<sup>++</sup>, Paxx<sup>+/−</sup>, and Paxx<sup>−/−</sup> mice
(108, 126, and 108 million, respectively, \( P > 0.1415 \), Fig. 2B). Thymus weight was also comparable between

**Fig. 2. Development of lymphoid organs in Paxx<sup>−/−</sup> mice.** (A) The weight of spleens isolated from Paxx<sup>++</sup> (n = 26), Paxx<sup>+/−</sup> (n = 13), and Paxx<sup>−/−</sup> (n = 17). Six-week-old mice were not significantly different, with \( P > 0.4677 \). Spleen size in immunodefect Ku80<sup>−/−</sup> and Dna-
pkcs<sup>−/−</sup> mice was significantly reduced, correspondingly; both pairs, Paxx<sup>−/−</sup> vs Ku80<sup>−/−</sup>, and Paxx<sup>−/−</sup> vs Dna-pkcs<sup>−/−</sup> have \( P < 0.0001 \). (B) Splenocyte count is not affected in Paxx<sup>−/−</sup> mice when compared to WT littermates, \( P > 0.1566 \). (C) Example of spleens isolated from Dna-
pkcs<sup>−/−</sup>, Paxx<sup>−/−</sup>, Paxx<sup>+/−</sup>, and Paxx<sup>++</sup> mice. (D) The weight of thymus from Paxx<sup>++</sup> (n = 16), Paxx<sup>+/−</sup> (n = 6), and Paxx<sup>−/−</sup> (n = 9) is similar
with \( P > 0.9255 \). (E) Thymocyte count was nearly identical in Paxx<sup>++</sup> (n = 9), Paxx<sup>+/−</sup> (n = 7), and Paxx<sup>−/−</sup> (n = 9) mice, \( P > 0.2649 \). (F) Example of thymi from Paxx<sup>++</sup>, Paxx<sup>+/−</sup>, and Paxx<sup>−/−</sup> mice. (G) Count of total cells in bone marrow was similar in Paxx<sup>++</sup> (n = 9), Paxx<sup>−/−</sup>
(n = 3), and Paxx<sup>+/−</sup> (n = 6) mice, \( P > 0.1546 \). (H) CSR to IgG1 was identical in Paxx<sup>++</sup> (n = 7), Paxx<sup>−/−</sup> (n = 2), and Paxx<sup>+/−</sup> (n = 3) mice. CSR to IgG1 was significantly reduced in Ung<sup>−/−</sup> B cells when compared to Paxx<sup>−/−</sup> (n = 6), \( P < 0.0001 \).
*Paxx*+/+, *Paxx*+/-, and *Paxx*–/– mice (84, 101, and 83 mg, respectively) (Fig. 2D,F). The thymocyte count was also similar between *Paxx*+/+, *Paxx*+/-, and *Paxx*–/– mice (199, 226, 179 million, respectively) (Fig. 2E). Total count of cells in bone marrow was similar in *Paxx*+/+, *Paxx*+/-, and *Paxx*–/– mice (P > 0.1546, Fig. 2G). Thus, we concluded that PAXX is dispensable for development of lymphoid tissue in mice.

**Ex vivo stimulated primary *Paxx*–/– B lymphocytes exhibit normal CSR levels**

Deleting of XLF and XRCC4 results in a twofold to threefold reduction in CSR activity, which can be explained by the activity of alternative end joining (A-EJ) in the *Xrcc4–/–* cells [24] and the residual activity of both classical NHEJ and A-EJ in *Xlf–/–* cells [3,8]. To determine the role of PAXX in CSR, we isolated primary splenic B lymphocytes from *Paxx*+/+, *Paxx*+/-, and *Paxx*–/– mice and stimulated them with bacterial LPS and interleukin 4 to undergo CSR. Four days after stimulation, CSR levels to IgG1 in *Paxx*+/+ and *Paxx*–/– cells ranged from 23.4% to 31.3% and were not significantly different (Fig. 2H), P = 0.5226. B cells from *Ung*–/– mice were used as negative control and switched at background levels, 0.4–1.2% of IgG1 + B cells, in line with original observations [26] (Fig. 2H). We therefore concluded that PAXX is dispensable for CSR in mice.

**Paxx–/– mice exhibit no change in genomic stability**

We have previously demonstrated that inactivation of the core NHEJ factor *Ku70* results in a threefold to sixfold increase of aberrant metaphases in murine tail fibroblasts measured as chromosomal and chromatid breaks compared to WT controls. In addition, inactivation of *Xlf* and *Dna-pkcs* resulted in a significant though moderate increase in the proportion of aberrant metaphases [1,4,5,7]. To determine whether *Paxx* inactivation affects genomic stability, we measured metaphase aberrations in isolated tail fibroblasts from five *Paxx*+/+ and five *Paxx*–/– mice, using three *Ku80*–/– mice as NHEJ-deficient controls. We found that the average proportion of aberrant metaphases was identical in *Paxx*+/+ and *Paxx*–/– mice (8%, P > 0.9999), while significantly increased to 33% in *Ku80*–/– mice (P < 0.0001) (Fig. 3). We concluded that PAXX is dispensable for genomic stability in mice.

**Discussion**

Here, we report the newly generated *Paxx*–/– knockout mouse model. We deleted part of the *Paxx*...
Characterization of Paxx-knockout mouse model
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promoter region, transcription start site, and exons 1–2. We did not delete the entire Paxx locus because it overlaps with other genes. In particular, prostaglandin-H2 D-isomerase (Ptgds) is in sense orientation 5.6 kb upstream of Paxx, chloride intracellular channel protein 3 (Clic3) overlaps with Paxx intron 3, and ATP-binding cassette subfamily A member 2 (Abca2) is in antisense orientation 6.6 kb downstream of Paxx.

Inactivation of both alleles of Paxx in mice resulted in no detectable protein levels in tail fibroblasts, spleen, thymus, liver, and lungs. Furthermore, it did not affect growth, fertility, development of lymphoid organs, or genomic stability of mice, when compared to heterozygous or WT littermates. In addition, PAXX was dispensable for CSR to IgG1 in primary B splenocytes, which is in line with data obtained using knockout B-cell CH12F3 cell lines, where inactivation of Paxx did not affect CSR to IgA [14,27]. Finally, we analyzed brain sections of WT and Paxx−/− mice and found no difference in neurodevelopment using two independent methods, Nissl staining and TUNEL assay (not shown).

While our mouse model was produced and analyzed, three other groups independently reported Paxx-deficient mice [17–19]. In contrast to our mouse model, those mice had the entire Paxx locus deleted. These groups found that PAXX is dispensable for mouse development unless its paralog XLF is also inactivated, which limits further studies of the functional interaction between PAXX and XLF in adult animals. One option to bypass this challenge is to generate conditional knockout genes for Xlf, Paxx, or both genes. This would enable the specific deletion of Xlf and Paxx in organs of interest, such as the spleen, thymus, or brain. However, conditional knockouts are often limited by, for example, incomplete deletion. Previously, it has been demonstrated that the inactivation of Ku80 rescues embryonic lethality in Lig4−/− mice [28], and Ku70 inactivation rescues perinatal lethality in Xlf−/−Dna-pkcs−/− [5]. Thus, the inactivation of upstream NHEJ factors Ku70 or Ku80 might rescue embryonic lethality in Paxx−/−Xlf−/− mice, although it would completely inactivate residual classical NHEJ. Additionally, haploinsufficiency for the Trp53 gene (p53) rescues embryonic lethality of Lig4−/− [29], Xrec−c4−/− [30], and Xlf−/−Dna-pkcs−/− mice [5]. Thus, we may speculate whether a Paxx−/−Xlf−/−Trp53−/− mouse may be viable despite complete absence of PAXX and XLF in all organs and tissues allowing the study of functional interaction between Paxx and Xlf in vivo.

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Author contributions
All authors designed research, analyzed results, performed experiments, and commented on the manuscript. VO wrote the manuscript.

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