Synthetic lethality between PAXX and XLF in mammalian development

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PAXX was identified recently as a novel nonhomologous end-joining DNA repair factor in human cells. To characterize its physiological roles, we generated Paxx-deficient mice. Like Xlf−/− mice, Paxx−/− mice are viable, grow normally, and are fertile but show mild radiosensitivity. Strikingly, while Paxx loss is epistatic with Ku80, Lig4, and Atm deficiency, Paxx/Xlf double-knockout mice display embryonic lethality associated with genomic instability, cell death in the central nervous system, and an almost complete block in lymphogenesis, phenotypes that closely resemble those of Xrcc4−/− and Lig4−/− mice. Thus, combined loss of Paxx and Xlf is synthetic-lethal in mammals.

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DNA double-strand breaks (DSBs) are extremely toxic lesions that must be repaired for an organism to pass on its genetic material intact to the next generation (Jackson and Bartek 2009). Cells have evolved two principal DSB repair pathways to address this challenge: homologous recombination (HR) and nonhomologous end-joining (NHEJ). HR is restricted to S and G2 phases of the cell cycle because it requires a sister chromatid as the template for repair. In contrast, NHEJ is the dominant DSB repair pathway throughout intersphase in mammalian cells, although it is restrained during DNA replication (Beucher et al. 2009; Karanam et al. 2012). NHEJ is initiated by Ku, a ring-shaped heterodimeric protein complex consisting of Ku70 and Ku80 subunits that specifically recognizes DSB ends (Grundy et al. 2014). Ku forms a platform for the downstream recruitment of core NHEJ factors such as DNA ligase IV (LIG4); its stable binding partner, XRCC4; and XLF, a protein structurally related to XRCC4 (Ochi et al. 2014).

Most DSBs in vertebrate cells are generated by agents such as ionizing radiation (IR) or molecules that directly damage DNA by chemically reacting with it or through processing of other DNA lesions during DNA replication or mitosis. Additionally, some DSBs are induced deliberately by enzymatic cleavage in certain cell types at various stages of development; for example, to generate immune receptor diversity in B and T lymphocytes during immune receptor diversity in B and T lymphocytes during B cell development, to generate immunoglobulin and T cell receptor diversity (Zhang et al. 2013). Consequently, the lack of significant immunodeficiency in these mice is surprising because DSB repair is critical for cellular resistance to DSB-inducing agents and is rapidly recruited to DNA damage sites, where it stabilizes NHEJ factors on chromatin and promotes DNA repair by NHEJ. In vitro, Mouse models of NHEJ deficiency show both overlapping and unique features. Ku-deficient mice are subviable and fertile but have profound growth defects, increased neuronal cell death, and immunodeficiency (Nussenzweig et al. 1996; Zhu et al. 1996; Ouyang et al. 1997; Gu et al. 2000]. Loss of Xrcc4 or Lig4 results in a more severe phenotype with embryonic growth defects, blocked lymphogenesis, and late embryonic lethality associated with a large increase in cell death in the developing central nervous system (CNS) [Barnes et al. 1998; Frank et al. 1998; Gao et al. 1998]. In contrast, Xlf−/− mice have a relatively mild phenotype with no growth defects, neuronal cell death, or overt immunodeficiency despite XLF being a core NHEJ factor [Ahnesorge et al. 2006; Buck et al. 2006; Li et al. 2008]. Furthermore, Xlf−/− B cells perform V(D)J recombination at almost wild-type levels, which explains the lack of significant immunodeficiency in these mice and suggests that compensatory mechanisms can mitigate loss of XLF in developing lymphocytes. One of these mechanisms comprises the ATM–H2AX–53BP1 axis of DSB repair, as combined loss of any one of these factors with XLF deficiency causes profound defects in V(D)J recombination and lymphocyte development even though loss of any of these proteins individually is not significantly detrimental to these processes [Zha et al. 2011; Liu et al. 2012; Oksenech et al. 2012].

Recently, we and others identified a third XRCC4-like NHEJ protein, called PAXX (Cranxton et al. 2015; Ochi et al. 2015; Xing et al. 2015]. PAXX is required for cellular resistance to DSB-inducing agents and is rapidly recruited to DNA damage sites, where it stabilizes NHEJ factors on chromatin and promotes DNA repair by NHEJ. In vitro, synthetic lethality between PAXX and XLF in mammalian development.

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PAXX can stimulate DNA end ligation in the presence of LIG4, XRCC4, and Ku, with most if not all of these functions requiring its direct binding to Ku. However, it is still unknown whether PAXX functions similarly to other core NHEJ factors in physiological settings or how PAXX loss might impact at the organism level in terms of growth and development.

In this study, we describe the generation and characterization of PAXX-deficient mice. Like Xlf−/− mice, Paxx−/− mice are viable, grow normally, and are fertile. However, these mice are radiosensitive and show a mild reduction in splenic lymphocyte numbers. Strikingly, combined loss of Paxx and Xlf is synthetic-lethal, as the majority of double-knockout embryos dies before birth with significant growth defects, increased genomic instability and subsequent cell death in the developing CNS, and an almost complete block in lymphocyte development, phenotypes that are strongly reminiscent of Xrcc4−/− or Lig4−/− mice. Thus, PAXX and XLF share a redundant function that is critical for DNA repair during mammalian development.

**Results and Discussion**

**Generation of Paxx−/− mice**

To characterize PAXX function in mice, we disrupted the Paxx locus in C57BL/6NTac zygotes using CRISPR–Cas9 [Wright et al. 2016] by injection of wild-type Cas9 mRNA together with two small guide RNAs (sgRNAs) and an oligonucleotide template to collapse the entire Paxx genomic region, resulting in a 1.6-kb deletion [Fig. 1A]. For ease of mutant selection when using Cas9, Paxx sgRNAs were co-injected with an sgRNA to disrupt exon 1 of the tyrosine kinase gene (Tyr) to yield an albino phenotype in successfully targeted mice [Supplemental Fig. S1A,B]. We identified four Paxx F0 founders, all of which showed the albino phenotype [Supplemental Fig. S2A,B]. Upon confirmation of Paxx locus deletion by PCR and sequencing [Supplemental Fig. S2C], we backcrossed founders to the C57BL/6NTac original background for three generations to segregate the Tyr deletion and any off-target mutations. Resulting mice showed complete absence of PAXX protein in tissues such as thymus, lung, and brain that normally highly express Paxx [Fig. 1A]. Paxx−/− mice were born at the expected Mendelian frequencies, were fertile, and displayed no growth abnormalities [Supplemental Fig. S2D,E; Supplemental Table S1]. Paxx−/− mice also showed no increased tumor predisposition up to 400 d [data not shown] and no significant increase in micronucleus formation [Supplemental Fig. S3A], a sensitive method for detecting genomic instability in vivo [Balmus et al. 2015].

Paxx−/− mice are radiosensitive but show no overt immune phenotype

In light of the immune defects of NHEJ-deficient mouse models, we examined whether Paxx−/− mice displayed any overt immunodeficiency by analyzing leukocyte numbers. We detected a modest reduction in cell counts in the spleens of Paxx−/− mice compared with wild-type littermates, but this was not observed in the thymus or bone marrow [Fig. 1B]. Splenic cell reduction was due to decreased B-cell and T-cell populations, while the NK cell lineage was not significantly affected [Fig. 1C]. T-cell development in the thymus [as measured by CD4 and CD8 expression] was essentially normal in Paxx−/− mice [Supplemental Fig. S3B], although they did exhibit a small increase in the number and percentage of mature B cells within the bone marrow [Supplemental Fig. S3C; data not shown]. To test whether Paxx−/− mice were capable of mounting a proper immune response, we immunized Paxx−/− and wild-type littermates with purified fragment C of tetanus toxin and found that immune responses as measured by total immunoglobulin [lg], IgG1, and IgG2a antibody titers were similar in both (Supplemental Fig. S3D). Furthermore, class switch recombination (CSR) was normal in the absence of PAXX [Supplemental Fig. S3E]. As PAXX is important for efficient DSB repair in human cells [Crayton et al. 2015; Ochi et al. 2015; Xing et al. 2015], we treated Paxx−/− mice with IR. Following whole-body IR treatment, we observed significantly increased lethality in Paxx−/− mice when compared with wild-type controls, although this was not as dramatic as that observed in Atm−/− mice [Fig. 1D; Barlow et al. 1996]. Taken together, these data implied that although PAXX is important for effective DSB repair, it does not play an essential role in mammalian development and is unlikely to function as a strong tumor suppressor. In addition, Paxx−/− mice showed only a mild reduction in lymphocyte numbers in the bone marrow (Supplemental Fig. S3C).
numbers and displayed no overt immunodeficiency phenotype. In this regard, Paxx<sup>−/−</sup> mice most closely resemble Xlf<sup>−/−</sup> mice [Li et al. 2008] rather than mice deficient in other core NHEJ factors.

Paxx loss is epistatic with Ku, Lig4, and Atm deficiency

Genetic crosses between NHEJ mutant mice and with other DNA repair-deficient mouse models have revealed both synthetic viability [e.g., Ku80<sup>−/−</sup> Lig4<sup>−/−</sup> mice] and synthetic lethality [e.g., Atm<sup>−/−</sup> Xlf<sup>−/−</sup> mice] relationships [Karanjawala et al. 2002; Zha et al. 2011]. Such genetic crosses can highlight antagonistic roles and/or functional redundancies between DNA repair factors and can help position such factors in NHEJ and other events. We therefore crossed our Paxx<sup>−/−</sup> mice into Ku80-deficient, Lig4-deficient, Atm-deficient, or Xlf-deficient backgrounds. Paxx<sup>−/−</sup> Ku80<sup>−/−</sup> mice were born at frequencies similar to Ku80<sup>−/−</sup> mice and did not show any overt phenotypes compared with Ku80<sup>−/−</sup> animals [Fig. 2A, Supplemental Table S2A], consistent with Paxx being epistatic with Ku80 in regard to NHEJ. Notably, this was similar to what we observed with Xlf<sup>−/−</sup> Ku80<sup>−/−</sup> mice, which also closely resemble Ku80<sup>−/−</sup> mice [Supplemental Fig. S4A, B]. In contrast to Ku80<sup>−/−</sup> mice, Lig4<sup>−/−</sup> mice die during embryonic development in a manner associated with dramatically increased apoptosis in the CNS [Barnes et al. 1998; Frank et al. 1998; Gao et al. 1998]. As Ku80 loss rescues the lethality of Lig4<sup>−/−</sup> mice [Karanjawala et al. 2002] and because PAXX functions in physical contact with Ku in NHEJ, we wondered whether PAXX loss would result in a similar rescue. However, multiple rounds of breeding did not produce any viable Paxx<sup>−/−</sup> Lig4<sup>−/−</sup> offspring, indicating that, unlike Ku80 loss, PAXX loss cannot rescue the embryonic lethality caused by Lig4 deficiency [Fig. 2B, Supplemental Table S2B].

Next, we generated Paxx<sup>−/−</sup> Atm<sup>−/−</sup> mice to explore potential functional relationships between these two genes. Notably, in contrast to the relationship between Atm and Xlf [Zha et al. 2011], Paxx<sup>−/−</sup> Atm<sup>−/−</sup> mice displayed no additional phenotypes compared with Atm<sup>−/−</sup> mice [Fig. 2C, Supplemental Fig. S5; Supplemental Table S2C], indicating that Paxx and Atm are epistatic for immune functions such as CSR and during development.

Paxx loss is synthetic-lethal with Xlf deficiency

We also crossed Paxx<sup>−/−</sup> mice with Xlf<sup>−/−</sup> mice to examine potential functional relationships between these genes. Strikingly, these double-knockout mice were dramatically underrepresented, with only one Paxx<sup>−/−</sup> Xlf<sup>−/−</sup> mouse born out of 25 expected [Fig. 2D, Supplemental Table S2D]. Furthermore, this mouse was born smaller and, at 5 d of age, was clearly distinguishable from its littermate controls [Supplemental Fig. S6A]. By 10 d of age, this Paxx<sup>−/−</sup> Xlf<sup>−/−</sup> mouse failed to thrive and, at necropsy, exhibited a microspleen and a complete absence of a thymus, similar to what is observed in Ku80<sup>−/−</sup> animals [Fig. 2E]. In accord with these findings, there was a significant decrease in splenic cell counts relative to body weight, and, upon red blood cell lysis, no lymphocytes were recovered [Supplemental Fig. S6B,C]. Taken together, these data indicated that while Paxx loss is epistatic with Ku80, Lig4, and Atm in terms of its developmental roles, it is synthetic-lethal in combination with Xlf loss.

To determine when Paxx<sup>−/−</sup> Xlf<sup>−/−</sup> mice die during embryonic development, we established timed matings and performed embryo dissections starting at embryonic day 9.5 (E9.5) when Paxx/Xlf double mutants showed no gross abnormalities [Supplemental Fig. S7A]. By E10.5, although double mutants were recovered at the expected frequencies, more than half of the Paxx<sup>−/−</sup> Xlf<sup>−/−</sup> embryos were smaller than their littermate controls [Fig. 3A, Supplemental Fig. S7B]. Nevertheless, the Paxx/Xlf double mutants were not developmentally delayed at this stage, showing somite numbers similar to those of wild-type, Paxx<sup>−/−</sup>, or Xlf<sup>−/−</sup> embryos [Fig. 3B], thus hinting at a possible cell fate abnormality. Similarly, at E14.5, double-mutant embryos were obtained at the expected Mendelian frequencies [Fig. 3C; Supplemental Table S3A] but were smaller than controls [Fig. 3D,E]. By E18.5, Paxx<sup>−/−</sup> Xlf<sup>−/−</sup> double mutants were no longer obtained at the expected frequencies, and a significant proportion of them was found to have died [Fig. 3F, Supplemental Table S3B]. A few were viable but showed reduced body weight, much smaller spleens, and a drastic involution of the thymus [Fig. 3G–I], thus highlighting a requirement for either PAXX or XLF in lymphatic organ development and for life for more than a few days postnatally.
To determine the cause of embryonic death in Paxx<sup>−/−</sup> Xlf<sup>−/−</sup> mice, we looked for markers of genomic instability (phosphorylated histone H2AX, γH2AX) and apoptosis (cleaved caspase 3) in E10.5 and E14.5 embryos via immunohistochemistry. At both E10.5 and E14.5, we consistently observed an increase in γH2AX-positive cells in the CNS of the Paxx<sup>−/−</sup> Xlf<sup>−/−</sup> double-mutant mice as compared with littermate controls (Fig. 4A,B, Supplemental Fig. S7C). This was associated with increased apoptosis, as evidenced by the accumulation of cleaved caspase 3, especially in the cortical plate region of the cerebral cortex (Fig. 4B, Supplemental Fig. S7C,D). In contrast, significant increases in these markers were not detected in most other tissues [Supplemental Fig. S8A,B]. Together, these findings highlighted a critical requirement for Paxx or Xlf for development of not only lymphatic organs but also the CNS. We thus conclude that lack of effective maintenance of genome stability during development results in increased cell death and embryonic lethality in the absence of both Paxx and Xlf.

In this study, we described the generation of Paxx-deficient mice. We found that while Paxx<sup>−/−</sup> mice are radiosensitive, they are born at Mendelian ratios and display no overt immunodeficiency or developmental defects. In this regard, Paxx<sup>−/−</sup> mice are reminiscent of Xlf<sup>−/−</sup> mice, although the immunological phenotype of the latter is slightly more pronounced (Li et al. 2008). Furthermore, our data support a model in which Paxx functions in NHEJ in a manner connected to and dependent on Ku because Ku<sup>−/−</sup> Paxx<sup>−/−</sup> double-mutant mice were essentially identical to Ku<sup>−/−</sup> mice. Furthermore, we found that Paxx loss could not rescue the embryonic lethality phenotype of LIG4<sup>−/−</sup> mice. This was expected based on previous cell-based studies and biochemical analyses of Paxx, which showed that Paxx functions downstream from Ku in the NHEJ process, being recruited to Ku-bound DNA ends by direct binding of the Paxx C terminus to Ku (Ochi et al. 2015; Xing et al. 2015).

Perhaps surprisingly, we found that combined Paxx and Xlf deficiency is lethal in mice. This was unexpected based on our previous results in human cells, where we found that depletion of XLF in Paxx<sup>−/−</sup> RPE-1 cells caused no additional radiosensitivity (Ochi et al. 2015). Interestingly, we were able to recapitulate this epistasis in mouse embryonic fibroblasts (MEFs) derived from our Paxx/Xlf single-knockout and double-knockout mice exposed to IR and the radiomimetic drug phleomycin [Supplemental Fig. S9A-C]. Due to the extensive cell death in lymphoid progenitors in developing embryos, we were unable to isolate B cells from Paxx<sup>−/−</sup> Xlf<sup>−/−</sup> mice to examine their radiosensitivity in vitro. However, recent data using Paxx/Xlf single-knockout and double-knockout v-Abl transformed pro-B cells derived from wild-type mice demonstrated that, in this cell lineage as in the developing CNS, a synthetic relationship between Paxx and XLF exists in terms of cellular sensitivity to IR as well as in the process of V(D)J recombination [Kumar et al. 2016; Lescale et al. 2016b]. Thus, there appears to be a tissue-specific requirement for Paxx and XLF: In some cells, both Paxx and XLF are required for NHEJ, but, in others, either one or the other suffices to a major degree. In this regard, we note that tissue-specific requirements for XLF in mice have been demonstrated previously [Li et al. 2008].

Since the generation of XLF-deficient mice was first reported [Li et al. 2008], it has been unclear why the phenotype of these mice is relatively mild compared with mice in which other core NHEJ factors are mutated given that XLF is important for DSβ repair by NHEJ in cells [Ahnesorg et al. 2006; Buck et al. 2006]. To help explain these findings, functional redundancy with other factors was suggested and, in due course, demonstrated by XLF loss being shown to display nonepistatic or synthetic-lethal relationships with loss of components of the ATM–H2AX–53BP1 pathway or with C-terminal mutants of the V(D)J recombinase protein RAG2 [Zha et al. 2011; Liu et al. 2012; Oksenych et al. 2012; Lescale et al. 2016a]. Our data reveal that Paxx and XLF also share
500 cells per embryo were counted.

**Materials and methods**

**Animals**

Care and use of all mice used to generate data for this protocol were carried out in accordance with UK Home Office regulations, UK Animals [Scientific Procedures] Act of 2013. Paxx-deleted (C57BL/6NTac-Paxx<sup>−/−</sup>, mice were generated using CRISPR/Cas9, Kault [B6.129-Xrc4<sup>−/−</sup>]; stock no: 004361, Lig4<sup>B6;129S6-Lig4tm1Fwa/Kvm stock no: 004361], and pan-nuclear γH2AX-positive [B] cells of the representative genotypes. More than 500 cells per embryo were counted. n ≥ 3 per genotype. Statistical analysis was performed using one-way ANOVA. (γ ≥ 3 per genotype. Statistical analysis was performed using Dunnett’s multiple comparisons test.

One or more redundant functions, as, unlike single mutants, mice lacking both proteins display severe growth defects, extensive cell death in the CNS, and an almost complete block to lymphocyte development similar to that of Xrc4<sup>−/−</sup> or Lig4<sup>−/−</sup> mice. Interestingly, unlike the situation when combining XLF and ATM loss, combined loss of PAXX and ATM did not lead to a phenotype more severe than that of mice lacking ATM alone. Considering our findings, it will be of interest to further explore the overlapping and nonoverlapping functions of PAXX and XLF in NHEJ during development, define the basis for the apparent tissue-specific effects of their combined loss, and establish how PAXX might operate in the context of ATM-mediated DSB repair processes.

**Histology, immunohistochemistry, and immunoblotting**

All major organs were isolated following euthanasia and then fixed in 10% formalin overnight. Embryos were dissected on cold PBS and fixed in 4% paraformaldehyde. On the second day, the fixed organs were transferred to 70% ethanol, placed in cassettes, and embedded in paraffin, and serial 5-μm sections were collected on Superfrost Plus slides (Thermo Fisher Scientific) using a Leica microdissection system (LMD7000). For immunohistochemistry apoptosis and DNA damage, quantitation was done using antibodies against cleaved caspase 3 (Abcam, ab13847) or γH2AX (clone JBW501, Millipore), respectively. For Western blotting, antibodies against PAXX [Abcam, ab126353] and β-actin (Sigma, A5316) were used. Histology images were obtained using an Aperio Scanscope (Aperio Technologies). Statistical analysis was performed using Prism 7 [Graphpad]. Student’s t tests were always two-tailed.

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