Congenital bone marrow failure in DNA-PKcs mutant mice associated with deficiencies in DNA repair

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Abstract

The nonhomologous end-joining (NHEJ) pathway is essential for radioresistance and lymphocyte-specific V(D)J (variable [diversity] joining) recombination. Defects in NHEJ also impair hematopoietic stem cell (HSC) activity with age but do not affect the initial establishment of HSC reserves. In this paper, we report that, in contrast to deoxyribonucleic acid (DNA)–dependent protein kinase catalytic subunit (DNA-PKcs)–null mice, knockin mice with the DNA-PKcs3A/3A allele, which codes for three alanine substitutions at the mouse Thr2605 phosphorylation cluster, die prematurely because of congenital bone marrow failure. Impaired proliferation of DNA-PKcs3A/3A HSCs is caused by excessive DNA damage and p53-dependent apoptosis. In addition, increased apoptosis in the intestinal crypt and epidermal hyperpigmentation indicate the presence of elevated genotoxic stress and p53 activation. Analysis of embryonic fibroblasts further reveals that DNA-PKcs3A/3A cells are hypersensitive to DNA cross-linking agents and are defective in both homologous recombination and the Fanconi anemia DNA damage response pathways. We conclude that phosphorylation of DNA-PKcs is essential for the normal activation of multiple DNA repair pathways, which in turn is critical for the maintenance of diverse populations of tissue stem cells in mice.

Introduction

DNA double-stranded breaks (DSBs) are among the most dangerous forms of DNA damage. Unrepaired DSBs signal cells to die, whereas misprocessing of DSBs can lead to genomic instability and oncogenic transformation. DSBs are primarily repaired by the nonhomologous end-joining (NHEJ) and homologous recombination (HR) repair pathways. The NHEJ pathway joins the two ends of a DSB directly with little or no requirement for homology, whereas HR uses the intact identical copy of the broken chromosome as a template. Although both NHEJ and HR proteins are recruited to DSBs, pathway choice is regulated in part by the cell cycle phase, with HR being active in S/G2 and NHEJ contributing to DSB repair predominantly in G0/G1. For example, V(D)J (variable [diversity] joining) recombination-associated breaks are generated in G0/G1 and resolved by NHEJ proteins, whereas meiotic DSBs are processed by HR proteins. Nevertheless, a recent study indicates competition between HR and NHEJ for replication- and ionizing radiation (IR)–induced DSBs during the S and G2 phase of the cell cycle (Sonoda et al., 2006).

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Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3 related; BMT, bone marrow transplantation; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; DX, direct repeat; DSB, double-stranded break; ES, embryonic stem; FA, Fanconi anemia; GR, glucocorticoid receptor; HR, homologous recombination; HSC, hematopoietic stem cell; IR, ionizing radiation; MEF, mouse embryonic fibroblast; MMC, mitomycin C; NHEJ, nonhomologous end joining; SCID, severe combined immunodeficiency; TACE, testes-specific angiotensin-converting enzyme.
The DNA-dependent protein kinase (DNA-PK) consists of the large catalytic subunit (DNA-PK catalytic subunit [DNA-PKcs]) and the DNA-binding Ku70/80 heterodimer. The DSB is initially recognized by DNA-PK. The ends are then synapsed and processed by other NHEJ proteins, including Artemis, XLF, and XRCC4, before they are ligated together by ligase IV (Wetters and Chen, 2008). DNA-PKcs belongs to a serine/threonine protein kinase family known as the phosphatidylinositol-3 kinase–like protein kinases. Family members include ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) kinases (Shiloh, 2003). DNA-PKcs, itself, is essential for NHEJ-mediated DSB repair, and loss of the DNA-PKcs protein leads to radiation hypersensitivity as well as severe combined immunodeficiency (SCID) caused by a block in V(D)J recombination. In addition, DNA-PKcs deficiency results in a defect in immunoglobulin class switch recombination (Manis et al., 2002; Cook et al., 2003; Callén et al., 2009). DNA-PKcs is phosphorylated in several regions in vitro upon activation and in vivo upon treatment with IR (Chan et al., 2002; Douglas et al., 2002; Chen et al., 2005; Ma et al., 2005; Meek et al., 2007). IR-induced Ser2056 phosphorylation is mediated by DNA-PKcs autophosphorylation, as it is significantly reduced in a kinase-dead mutant DNA-PKcs and is inhibited by the DNA-PKcs kinase inhibitor (Chen et al., 2005). In contrast, DNA-PKcs phosphorylation at the Thr2609 cluster is dependent on ATM and ATR kinases in response to IR and UV radiation, respectively (Yajima et al., 2006; Chen et al., 2007). DNA-PKcs phosphorylation at either region attenuates DSB repair and leads to increased radiosensitivity (Chan et al., 2002; Douglas et al., 2002; Chen et al., 2005; Meek et al., 2007). Ser2056 phosphorylation is a prerequisite for the pathway switch favoring HR/FA.

### Results

**Generation of a DNA-PKcs^3A/3A knockin mouse model**

Our knockin mouse model of DNA-PKcs harbors three alanine substitutions at Thr2605, Thr2634, and Thr2643 (3A mutation, corresponding to human Thr2609, Thr2638, and Thr2647) of the endogenous mouse DNA-PKcs or \( \text{PRKDC} \) gene locus (Fig. 1 A). The design of the 3A knockin targeting vector was based on a previously described self-excision ACN (testes-specific angiotensin-converting enzyme [tACE]–Cre/neomycin [Neo]) cassette strategy (Bunting et al., 1999). The ACN cassette, including a tACE promoter-driven Cre recombinase, a Neo-selectable marker, and two flanking loxP sites, was fused with two genomic fragments with the longer fragment carrying mutated exon 58 (covering the entire mouse Thr2605 cluster) in close proximity to the ACN cassette (Fig. 1 B). Upon successful gene targeting and subsequent germline transmission in chimeric mice, expression of Cre recombinase during spermatogenesis self-excises the ACN cassette. This process leaves behind one remaining loxP site and allows the expression of DNA-PKcs, Ku70/80, and β-actin. (D) DNA-PKcs^3A/3A MEFs are hypersensitive to radiation [representative of three independent assays]. Error bars represent SD. (E) Survival curves generated by Kaplan–Meier analysis (curves were compared using log-rank test).

![Figure 1](image)

- **A** The alignment of the human Thr2609 and the mouse Thr2605 phosphorylation cluster of DNA-PKcs. Six phosphorylation sites were identified in the human Thr2609 cluster [highlighted with asterisks], which are highly conserved in the mouse except for Ser2624. Boxes indicate the positions of knockin mutations or alanine substitutions. **B** Strategy to introduce knockin mutations into a mouse ES cell. 3A+, exon 58 with T2605A, T2634A, and T2643A mutations; tACE-Cre, Cre recombinase driven by a tACE promoter. **C** 3A mutation does not alter the expression of DNA-PKcs. Whole-cell lysates from different MEFs were analyzed for the expression of DNA-PKcs, Ku70/80, and β-actin. **D** DNA-PKcs^3A/3A MEFS are hypersensitive to radiation [representative of three independent assays]. Error bars represent SD. **E** Survival curves generated by Kaplan–Meier analysis (curves were compared using log-rank test).
clonogenic survival analysis revealed that DNA-PKcs3A/3A MEFs reported (Ding et al., 2003; Chen et al., 2007). Nonetheless, the kinase activity of DNA-PKcs (Fig. S1 D) as previously all genotypes (Fig. 1 C). The 3A substitution also did not affect detected in mouse embryonic fibroblasts (MEFs) derived from targeted allele, as similar levels of the DNA-PKcs protein were not affect the expression of the 3A mutant DNA-PKcs from the one remaining loxP site between exon 58 and exon 59 did not affect the expression of the 3A mutant DNA-PKcs from the targeted allele, as similar levels of the DNA-PKcs protein were detected in mouse embryonic fibroblasts (MEFs) derived from all genotypes (Fig. 1 C). The 3A substitution also did not affect the kinase activity of DNA-PKcs (Fig. S1 D) as previously reported (Ding et al., 2003; Chen et al., 2007). Nonetheless, clonogenic survival analysis revealed that DNA-PKcs3A/3A MEFs were hypersensitive to IR to a similar degree as DNA-PKcs–null MEFs (Fig. 1 D). Complete disruption of the mouse DNA-PKcs gene results in an SCID phenotype (Gao et al., 1998; Taccioli et al., 1998; Kurimasa et al., 1999), but in contrast to Ku-deficient mice, there is no apparent growth retardation, infertility, or life shortening in DNA-PKcs−/− mice (Nussenzweig et al., 1996; Gu et al., 1997; Ouyang et al., 1997). Homozygous DNA-PKcs3A/3A mice were born at the expected Mendelian frequency with similar body weight to littermates, but they became apparently smaller within 2–3 wk of age (Fig. S1 C). In striking contrast to the normal life span of control, heterozygous littermates, and DNA-PKcs−/− mice, the great majority of DNA-PKcs3A/3A mice died shortly after birth (Fig. 1 E). In a cohort of 56 DNA-PKcs3A/3A mice, 75% died within 4 wk of birth, and only one mouse survived >12 wk.

**Lymphocyte hypoplasia and the V(D)J recombination defect in DNA-PKcs3A/3A mice**

Lymphocyte development in DNA-PKcs–null mice is severely compromised because of a block in V(D)J recombination (Gao et al., 1998; Taccioli et al., 1998; Kurimasa et al., 1999). Like DNA-PKcs−/− mice, the number of lymphocytes and the size of lymphoid organs were drastically attenuated in 2-wk-old DNA-PKcs3A/3A mice (Fig. 2 A and Fig. S2 A). In contrast to the early T cell development block at the CD4+CD8− double-negative stage in DNA-PKcs−/− mice (Gao et al., 1998; Kurimasa et al., 1999), DNA-PKcs3A/3A mice have CD4+CD8−, CD4−CD8+, and CD4−CD8− thymocytes, although at significantly reduced numbers (Fig. 2, A and B). Analyses of thymocyte subsets also reveal a severe reduction of DNA-PKcs3A/3A double-negative thymocytes at different developmental stages (Fig. S2 B). Moreover, there is a significant reduction in the numbers of T cells expressing TCR-γ/δ (Fig. S2 C) and TCR-β (Fig. S2 D), which is observed in mice with mutations that cause a subtotal block in V(D)J recombination (Difilippantonio et al., 2008). Nevertheless, single-positive splenic T cells accumulated in the DNA-PKcs3A/3A bone marrow, not only in the lymphoid (L) gate but also in the granulocyte and macrophage (G/M) gate, cell number dropped to a very low level compared with wild-type and knockout (KO) mice.
Congenital bone marrow failure and HSC/progenitor cell depletion in DNA-PKcs<sup>3A/3A</sup> mice

Although lymphocyte development is compromised in DNA-PKcs<sup>3A/3A</sup> mice, this would not be expected to cause early postnatal lethality in DNA-PKcs<sup>3A/3A</sup> mice, as SCID mice live a normal life span. Examination of forward angle versus side angle light scatter plots in bone marrow revealed not only a depletion of cells in the lymphocyte gate (as in SCID mice) but also a lack of cells in the granulocyte and macrophage gate in DNA-PKcs<sup>3A/3A</sup> bone marrow with a characteristically high level of side scatter, suggesting a more general loss of leukocytes (Fig. 2 F).

During our investigation, we noticed that the ears, paws, and internal organs of DNA-PKcs<sup>3A/3A</sup> mice were pale, suggesting the development of anemia. A complete peripheral blood count in 2-wk-old DNA-PKcs<sup>3A/3A</sup> mice. In newborn mice (day 1), myeloid progenitor cells were detected. Boxes indicate the LSK cell gate (Lin<sup>−</sup> c-Kit<sup>−</sup> Sca-1<sup>−</sup>). Error bars represent SD. (D) Flow cytometric analysis of HSC-enriched LSK cells. Bone marrow cells were stained with a lineage marker cocktail, c-Kit, and Sca-1. LSK cells (Lin<sup>−</sup> Sca-1<sup>−</sup> c-Kit<sup>−</sup>) and myeloid progenitor cells (Lin<sup>−</sup> Sca-1<sup>−</sup> c-Kit<sup>−</sup>) were ablated in 2-wk-old (day 14) DNA-PKcs<sup>3A/3A</sup> mice. In newborn mice (day 1), myeloid progenitor cells were detected. Boxes indicate the LSK cell gate (Lin<sup>−</sup> Sca-1<sup>−</sup> c-Kit<sup>−</sup>).

Figure 3. Congenital bone marrow failure and HSC/progenitor cell depletion in DNA-PKcs<sup>3A/3A</sup> mice. (A) The averages of peripheral blood WBC counts at day 1 (D14) and day 14 (D14) after birth (n = 4 for each group). (B) The averages of RBC counts reveal the development of anemia in DNA-PKcs<sup>3A/3A</sup> mice after birth (n = 4 for each group). (C) Bone marrow was depleted in DNA-PKcs<sup>3A/3A</sup> mice. Bone marrow was taken from one femur and one tibia of 2-wk-old mice (n = 3 for each group). (D) Flow cytometric analysis of HSC-enriched LSK cells. Bone marrow cells were stained with a lineage marker cocktail, c-Kit, and Sca-1. LSK cells (Lin<sup>−</sup> Sca-1<sup>−</sup> c-Kit<sup>−</sup>) and myeloid progenitor cells (Lin<sup>−</sup> Sca-1<sup>−</sup> c-Kit<sup>−</sup>) were ablated in 2-wk-old (day 14) DNA-PKcs<sup>3A/3A</sup> mice. In newborn mice (day 1), myeloid progenitor cells were detected. Boxes indicate the LSK cell gate (Lin<sup>−</sup> Sca-1<sup>−</sup> c-Kit<sup>−</sup>), (E) Quantification of flow cytometric analysis of HSCs (n ≥ 3 for each group). Lines indicate the means for each group.

Peripheral blood cells are continuously replenished from HSC pools in hematopoietic organs, particularly in postnatal bone marrow (Kondo et al., 2003). To examine whether a defect in HSC homeostasis was the cause of bone marrow depletion in DNA-PKcs<sup>3A/3A</sup> mice, a population of bone marrow HSCs (Lin<sup>−</sup> Sca-1<sup>−</sup> c-Kit<sup>−</sup>, hereafter referred to as LSK cells) was evaluated by flow cytometric analysis (Morrison et al., 1995). The experiments revealed that bone marrow LSK cells and their derivative Lin<sup>−</sup> Sca-1<sup>−</sup> c-Kit<sup>−</sup> progenitor cells were almost ablated in 2-wk-old DNA-PKcs<sup>3A/3A</sup> mice and were 100 times less than wild type even at day 1 (Fig. 3, D and E), whereas no significant difference was found in wild type, DNA-PKcs<sup>+/3A</sup> mice (Fig. 3 E), or in DNA-PKcs<sup>−/−</sup> mice (Fig. 3 D). These results demonstrate that there is an impaired maintenance or self-renewal of bone marrow HSCs in DNA-PKcs<sup>3A/3A</sup> mice and that the initiation of HSC pathogenesis occurs during embryonic development. Importantly, this dramatic early HSC failure has not been observed in other NHEJ-deficient mouse strains, in which HSC reserves are generally preserved in young mice (Nijnik et al., 2007; Rossi et al., 2007).

Fetal liver HSC dysfunction and loss in DNA-PKcs<sup>3A/3A</sup> mice

The fetal liver is known to be the main source of hematopoiesis during embryonic development, as it provides supportive niches for HSC expansion and maturation (Mikkola and Orkin, 2006). To determine whether HSC homeostasis dysfunction occurred in DNA-PKcs<sup>3A/3A</sup> embryos, fetal liver samples from different developmental stages were harvested and analyzed. Flow cytometric analysis revealed a decreased number of fetal liver LSK cells in DNA-PKcs<sup>3A/3A</sup> embryos at embryonic day 14.5 (0.08 ± 0.04% vs. wild type 0.19 ± 0.06%) and E.16.5 (0.05 ± 0.02% vs. wild type 0.14 ± 0.02%). The LSK cell population in DNA-PKcs<sup>3A/3A</sup> embryos was slightly reduced (0.23 ± 0.10%) but remained within the normal range of wild-type embryos (0.26 ± 0.11%; Fig. 4 A and B). At E12.5, the LSK cell population in DNA-PKcs<sup>3A/3A</sup> embryos was sharply reduced (0.23 ± 0.10%) but remained within the normal range of wild-type embryos (0.26 ± 0.11%; Fig. 4 A and B). At E12.5, the LSK cell population in DNA-PKcs<sup>3A/3A</sup> embryos was sharply reduced (0.23 ± 0.10%) but remained within the normal range of wild-type embryos (0.26 ± 0.11%; Fig. 4 A and B). Significantly, the depletion of HSC reserves in the DNA-PKcs<sup>3A/3A</sup> fetal liver occurs when HSCs are highly proliferative and undergo rapid expansion (Mikkola and Orkin, 2006). To examine whether the HSC loss in DNA-PKcs<sup>3A/3A</sup> mice was a result of defective cell proliferation, freshly isolated fetal liver LSK cells were cultured for ex vivo expansion. Wild-type and DNA-PKcs<sup>3A/3A</sup> LSK cells proliferated exponentially and continuously during the course of ex vivo expansion, whereas DNA-PKcs<sup>3A/3A</sup> LSK cells proliferated normally only in the first 24 h (Fig. 4 C). The expansion of DNA-PKcs<sup>3A/3A</sup> LSK cells slowed down at 48 h and then collapsed at 72 h as a result of massive cell death (unpublished data).
Bone marrow transplantation rescues DNA-PKcs<sup>3A/3A</sup> mice

Defective HSC expansion in fetal liver and depletion of nucleated cells in postnatal bone marrow suggest that HSC failure might be the cause of early lethality in DNA-PKcs<sup>3A/3A</sup> mice. To test this hypothesis, bone marrow transplantation (BMT) was performed in 2-wk-old DNA-PKcs<sup>3A/3A</sup>-recipient mice using wild-type littermates as donors. BMT was performed without preconditioning procedures because DNA-PKcs<sup>3A/3A</sup> mice are highly sensitive to radiation treatment (unpublished data) and are immunoincompetent. After transplantation with wild-type bone marrow cells, DNA-PKcs<sup>3A/3A</sup> recipients had an 80% survival rate at 2 mo and 60% at 1 yr (Fig. 4 D). BMT-rescued DNA-PKcs<sup>3A/3A</sup> mice gained weight normally compared with their heterozygotic littermates (Fig. S3 B). 8 wk after transplantation, peripheral blood cell counts in recipient mice showed a complete recovery from pancytopenia and the restoration of the bone marrow HSC pool (Fig. S3 A). Importantly, PCR genotyping revealed that hematopoietic organs in DNA-PKcs<sup>3A/3A</sup> recipients were reconstituted primarily by wild-type donor cells (Fig. 4 E), indicating a successful hematopoietic reconstitution.

The rescue of DNA-PKcs<sup>3A/3A</sup> mice could also be achieved by transplantation with bone marrow LSK cells from wild-type littermates with a reduced efficiency (Fig. 4 D), as the LSK fraction primarily contains the upstream HSCs. In contrast, bone marrow contains HSCs, progenitors, and partially differentiated progenitors for different hematopoietic lineages and is able to achieve a significant rescue within a shorter window of opportunity than LSKs.

The success of BMT rescue confirmed that HSC failure is the main cause of early lethality in DNA-PKcs<sup>3A/3A</sup> mice. To determine whether there is an intrinsic defect of DNA-PKcs<sup>3A/3A</sup> HSC proliferation in vivo, competitive repopulating assays were performed in irradiated CD45.1-recipient mice transplanted with fetal liver LSK cells from wild-type or DNA-PKcs<sup>3A/3A</sup> embryos (CD45.2 background) as well as the competing wild-type CD45.1 bone marrow cells. As shown in Fig. 4 F, DNA-PKcs<sup>3A/3A</sup> fetal liver LSKs were completely unable to repopulate in vivo, demonstrating the severity of hematopoietic impairment in DNA-PKcs<sup>3A/3A</sup> mice. In contrast, bone marrow cells from 8-mo-old DNA-PKcs<sup>3A/3A</sup> mice retained the reconstitution ability, though with a reduced potential compared with age-matched wild-type bone marrow cells (Fig. 4 G), suggesting a possible age-associated defect in hematopoiesis in DNA-PKcs<sup>3A/3A</sup> mice as also observed in other NHEJ-defective mice (Nijnik et al., 2007; Rossi et al., 2007).

**Summary**

- Bone marrow transplantation rescues DNA-PKcs<sup>3A/3A</sup> mice by reconstituting HSCs.
- Competitive repopulation assays confirm the intrinsic defect in DNA-PKcs<sup>3A/3A</sup> HSCs.
- Age-related loss in hematopoietic potential is observed in DNA-PKcs<sup>3A/3A</sup> mice.

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**Figure 4. Loss of fetal liver HSCs in DNA-PKcs<sup>3A/3A</sup> embryos.** (A) Flow cytometric analysis of HSC-enriched LSK cells in the E16.5 fetal liver. Percentages of LSKs are indicated. Boxes indicate the LSK cell gate (Lin <sup>-</sup>Sca-1<sup>-</sup>c-Kit<sup>+</sup>). (B) Quantitative analysis of fetal liver LSKs. Data show progressive LSK loss in DNA-PKcs<sup>3A/3A</sup> embryos (one dot represents one sample; at least four samples from one time point were analyzed for each genotype). Lines indicate the means for each group. (C) Ex vivo expansion of E13.5 fetal liver LSK cells. DNA-PKcs<sup>3A/3A</sup> LSKs showed limited proliferation capacity compared with wild-type and DNA-PKcs<sup>+/3A</sup> LSKs. (n = 3 for each group). (D) Kaplan-Meier survival curves for untreated DNA-PKcs<sup>3A/3A</sup> mice, bone marrow–transplanted (BMT) DNA-PKcs<sup>3A/3A</sup> mice, and LSK cell–transplanted DNA-PKcs<sup>3A/3A</sup> mice. (*, P < 0.05; **, P < 0.001; log-rank test). (E) Hematopoietic reconstitution in DNA-PKcs<sup>3A/3A</sup> BMT mice by donor cells. DNA samples from the thymus (T), spleen (S), and bone marrow (B) of DNA-PKcs<sup>3A/3A</sup> BMT mice were PCR genotyped. Wild-type PCR products were predominantly presented in all three tissues. The asterisk denotes a 517-bp band of the 1-kb DNA ladder (Invitrogen). (F) Competitive repopulation assay using E13.5 fetal liver cells. DNA-PKcs<sup>3A/3A</sup> HSCs (CD45.1<sup>+</sup>) failed to repopulate in lethally irradiated B6-Ly5.2/Cr host mice (CD45.1<sup>+</sup>CD45.2<sup>+</sup>), n = 4. (G) Competitive repopulation was performed using bone marrow cells from 8-mo-old DNA-PKcs<sup>3A/3A</sup> and age-matched wild-type mice. DNA-PKcs<sup>−/−</sup> HSCs (CD45.1<sup>+</sup>CD45.2<sup>+</sup>) showed reduced repopulating potential. n = 4. Error bars represent SD.
increase of Annexin V staining was also confirmed in freshly isolated fetal liver LSK cells from DNA-PKcs3A/3A embryos (Fig. 5 B), suggesting that HSC loss in DNA-PKcs3A/3A mice is likely caused by excessive apoptotic cell death.

Loss of p53 mitigates the stem cell failure and promotes survival in DNA-PKcs3A/3A mice

To examine whether HSC loss is caused by p53-mediated apoptotic response, DNA-PKcs3A mice were crossed with p53-null mutant mice. Loss of both p53 alleles doubled the life span of homozygotic DNA-PKcs3A/3A mice (abbreviated as 3A; Fig. 5 C) and restored long-term HSCs (Lin−,Sca-1−,c-Kit+,Flt3−,CD34−) in DNA-PKcs3A/3A mice (Fig. 5 D). Strikingly, loss of a single copy of p53 also prolonged the survival of 3A/p53−/− mice. In a cohort of 22 3A/p53−/− mice, 50% survived beyond 8 wk, which is significantly longer (P < 0.001) than the survival of 3A mice. Despite the enhanced survival, there was still a major depletion of bone marrow stem cells in 3A/p53−/− mice. We estimate from two femur bones that there are ~4.2 × 10^{3} LSK cells in 3A/p53−/− mice compared with 2.9 × 10^{6} LSK cells in wild-type mice.

In addition to life-span extension, a p53-null mutation also alleviated the developmental defect of lymphocytes in DNA-PKcs3A/3A mice. Thymocyte cell numbers were partially rescued (60% of wild-type mice) in 3A/p53−/− mice but not in 3A/p53−/− mice (Fig. 5 E), and the distribution of CD4 versus CD8 cells was normalized in 3A/p53−/− thymocytes (Fig. S4 B). The rescue of B cell development was also obvious, as the numbers of B cells in 3A/p53−/− mice were completely normal in bone marrow (Fig. 5 F) but restored to a lesser degree in the spleen (Fig. 5 G). Furthermore, even with the loss of a single p53 gene, the numbers of B cells were partially rescued. There were very few B220+IgM+ mature B cells in the 3A/p53−/+ bone marrow, but these cells were clearly detectable in 3A/p53−/− mice (Fig. S4 C). Thus, in contrast to DNA-PKcs–deficient SCID mice in which loss of p53 does not rescue B cell development (Nacht et al., 1996), there is partial rescue of B lymphocytes in 3A/p53−/− mice. This also suggests that the V(D)J recombination defect in DNA-PKcs3A/3A mice is less severe than in the complete absence of DNA-PKcs.

Increase of genotoxic stress in the intestinal crypt and epidermis

In addition to HSC loss, elevation of the p53-dependent genotoxic stress response in DNA-PKcs3A/3A mice was also evident in the intestinal crypt and epidermis. The intestinal crypt is the proliferation compartment and contains intestine stem cells, which give rise to different intestine epithelial cells for intestinal homeostasis maintenance. Immunohistological analysis revealed that a significant increase of TUNEL-positive cells was found in the intestinal crypt of DNA-PKcs3A/3A mice and that the p53-null mutation alleviated the defect completely (Fig. 6 A). Skin hyperpigmentation is another example of a p53-dependent stress response (McGowan et al., 2008) and can be found in the footpad and tail of DNA-PKcs3A/3A mice (Fig. 6, B and C). As expected, the hyperpigmentation phenotype is completely reversed in 3A/p53−/− mice. Footpad hyperpigmentation became apparent in DNA-PKcs3A/3A mice as early as 1 wk after birth and...
A

B

C

Figure 6. Activation of p53 in the intestinal crypt and epidermis in DNA-PKcs3A/3A mice. [A] Representative figures of a TUNEL assay. Increase of TUNEL-positive apoptotic cells (red) in the DNA-PKcs3A/3A intestinal crypt (outlined with dashed lines). Nuclei were counterstained with DAPI (blue). Bar, 50 µm. (B) Skin hyperpigmentation is clearly distinguishable in the DNA-PKcs3A/3A footpad and tail. (C) Hematoxylin and eosin staining of tail skin section. Massive melanin accumulated in the DNA-PKcs3A/3A epidermis and was abolished in a p53-null background. Bar, 20 µm.

was further darkened with age. In contrast, there was no footpad pigmentation in wild-type and heterozygotic pups, whereas mild footpad pigmentation could also be found in older (>2 mo) heterozygotic mice (unpublished data).

Hypersensitivity of DNA-PKcs3A/3A MEFs to DNA cross-linking agents
DNA-PKcs3A/3A MEFs exhibit a similar radiosensitivity to DNA-PKcs−/− MEFs and defective V(D)J recombination, yet only in DNA-PKcs3A/3A mice is there a block in hematopoiesis and an activation of the p53 pathway. Thus, there are likely to be defects in addition to impaired NHEJ. To test this possibility, DNA-PKcs3A/3A MEFs were subjected to different DNA damage agents (Fig. 7 A and Fig. S5 A). Clonogenic survival analyses revealed that DNA-PKcs3A/3A MEFs displayed a greater sensitivity toward replication stress inducers, including UV irradiation, camptothecin, and mitomycin C (MMC; Fig. 7 A). The most striking difference came from treatment with the DNA cross-linking agent MMC, as clonogenic survival of DNA-PKcs3A/3A MEFs was dramatically reduced, whereas heterozygotic MEFs and, importantly, DNA-PKcs−/− MEFs were not affected. Furthermore, MMC treatment induced a significant increase in chromosome aberrations in DNA-PKcs3A/3A MEFs (Fig. 7 B), among which radial structures were frequently observed (Fig. S5 B).

Hypersensitivity toward DNA cross-linking agents and bone marrow failure are the hallmarks of the inherited human disease FA. The repair of DNA interstrand cross-links is a complex process that involves the FA complex and the HR pathway (Mirchandani and D’Andrea, 2006). To determine whether the FA pathway was affected, we analyzed the induction of FANC2D monoubiquitination in response to DNA damage. Immunoblotting revealed that FANC2D monoubiquitination was attenuated in DNA-PKcs3A/3A MEFs in response to IR and MMC (Fig. 7 C and Fig. S5 D) but was not ablated as observed in FANCA−/− MEFs (Fig. S5 E). This attenuation is not likely caused by a reduced S-phase population in DNA-PKcs3A/3A MEFs, as no clear difference exists in cell cycle redistribution between wild-type and DNA-PKcs3A/3A MEFs (Fig. S5 C). Activated FANC2D is recruited to chromatin and forms nuclear foci where it facilitates HR and interstrand cross-link repair (Garcia-Higuera et al., 2001).

In response to IR treatment, FANC2D foci formation was detected in wild-type cells as early as 20 min after irradiation (unpublished data), and the percentage of cells with FANC2D foci increases with time (Fig. 7 D). In contrast, FANC2D foci in DNA-PKcs3A/3A MEFs appeared with delayed kinetics, and the percentage of cells with foci was ~50% of wild-type MEFs at various time points after IR. A similar defect was detected in the formation of Rad51 nuclear foci (Fig. S5 F), a prerequisite of HR-mediated DNA repair (Haaf et al., 1995). Formation of Rad51 foci was attenuated in DNA-PKcs3A/3A MEFs but was enhanced in DNA-PKcs−/− MEFs, which is likely because of the elevation of HR activity in the absence of NHEJ (Allen et al., 2002). Finally, we analyzed HR activity using the direct repeat (DR)–GFP reporter approach (Pierce et al., 1999). MEFs with a single integration of the DR-GFP reporter were transfected with an ISceI-glucocorticoid receptor (GR)–RFP construct to induce DSB formation and HR reaction (Soutoglou et al., 2007). Our result revealed that HR activity was attenuated in DNA-PKcs3A/3A MEFs, and a significant portion of ISceI-GR-RFP–expressing (RFP+) DNA-PKcs3A/3A cells was GFP− (no recombination) as compared with wild-type MEFs (Fig. 7 E). In contrast, HR activity was further enhanced in DNA-PKcs−/− MEFs because of a lack of NHEJ activity. The negative impact of the DNA-PKcs3A mutant on the HR/FA responses thus provides an explanation for the phenotypical difference between DNA-PKcs3A/3A mice and DNA-PKcs–null mice.

Discussion
The self-renewal property of HSCs and their ability to sustain long-term hematopoiesis require that genome integrity is strictly maintained (Kenyon and Gerson, 2007; Niedernhofer, 2008). It is well characterized that hematopoietic cells are highly sensitive to radiation-induced DNA damage, and bone marrow aplasia is one of main causes for radiation-induced lethality. A recent study showed that HSCs, but not progenitor cells, are preferentially killed by radiation, suggesting that HSCs are among the most sensitive cell types to DNA damage (Wang et al., 2006). HSCs in DNA repair-deficient individuals are highly susceptible to various kinds of genotoxic stresses. Genetically modified mouse models also revealed that defects in the DNA damage response result in the dysfunction of HSC maintenance. Hematopoietic failure with an early development of bone marrow hypocellularity is manifested in mice with a hypomorphic mutation of Rad50 (Rad5085; Bender et al., 2002) and mice knockout of ERCC1, a key factor in nucleotide excision repair and interstrand cross-link repair (Niedernhofer et al., 2004; Prasher et al., 2005). Deficiency of ATM leads to elevated levels of oxidative...
DSBs are among the most dangerous types of DNA lesions and pose a great challenge to cellular survival and genome integrity maintenance. Multiple repair mechanisms, including NHEJ, HR, and additional backup pathways, have evolved to ensure that DSBs are promptly and properly eliminated from the genome. Outside V(D)J recombination events, endogenous DSBs occur primarily in the cycling cells through the conversion of single-strand lesions during replication (Vilenchik and Knudson, 2003). From the standpoint of endogenous DSB production, we could assume that DNA-PK and its downstream NHEJ pathway, which is the predominant DSB repair mechanism in mammalian cells and is operational in all cell cycle phases, have a greater involvement in the replication-associated DNA damage response than we previously thought. However, it is not clear how DNA-PKcs and other factors of DSB repair are coordinated in response to replicative stresses, particularly in the highly proliferative cell population. Our unexpected findings from the DNA-PKcs 3A mouse model provide strong evidence that the impact of DNA-PKcs, particularly its phosphorylation at the mouse Thr2605 cluster (human Thr2609), has a role beyond the NHEJ mechanism.

It recently became clear that the competition between the NHEJ and the HR/FA pathway has important biological significance. For example, loss of the NHEJ factor 53BP1 alleviates stress and a progressive loss of the HSC pool with age (Ito et al., 2004). Similarly, NHEJ deficiency in Ku80 knockout mice diminishes HSC self-renewal capacity, whereas hypomorphic Lig4<sup>−/−</sup> mice display an age-dependent decrease in HSC numbers and bone marrow cellularity (Nijnik et al., 2007; Rossi et al., 2007). In contrast, HSC loss in DNA-PKcs<sup>3A/3A</sup> mice occurs significantly earlier during embryonic development, which resembles Rad50<sup>−/−</sup> and ERCC1<sup>−/−</sup> models, though it is not clear whether there is a functional overlap between the DNA-PKcs<sup>3A</sup> mutant protein and these two DNA repair factors in terms of HSC maintenance. On the other hand, complete DNA-PKcs deficiency leads to a decrease in HSC number (unpublished data) and repopulating ability in aged DNA-PKcs knockout mice. The distinct phenotypes in HSC maintenance between DNA-PKcs knockout and knockin mice is likely a result of their difference in DSB repair capacity, as DNA-PKcs<sup>3A/3A</sup> cells are also defective in the HR/FA responses in addition to the diminished NHEJ activity. Thus, the combined deficiency in DSB repair renders DNA-PKcs<sup>3A/3A</sup> cells hypersensitive to MMC and other replication stress agents. It is conceivable that the HR/FA mechanism, but not the NHEJ, is essential for replication-associated DNA damage repair and is critical for the rapid expansion of HSC populations in developing fetal liver.
the hypersensitivity of HR-defective Brca1 mutant cells to DNA-damaging agents and restores DSB repair by HR (Bouwman et al., 2010; Bunting et al., 2010). In addition, the FA pathway plays a critical role in eliminating toxic NHEJ; suppression of NHEJ alleviates the sensitivity of FA mutant cells to DNA cross-linking agents and facilitates error-free repair by HR (Adamo et al., 2010; Pace et al., 2010). Similarly, our data suggest that the DNA-PKcs3A/3A mutant protein interferes with the HR pathway, which could explain the sensitivity to replication damage and the severity of hematopoietic failure.

We have previously reported that the human Thr2609 cluster is phosphorylated directly by the ATR kinase upon replication stress (Yajima et al., 2006). This ATR-dependent DNA-PKcs phosphorylation virtually facilitates processing of replication-associated DNA lesions through the HR/FA machinery. It is likely that, in such a repair process, the shift from error-prone end joining to faithful repair by the HR/FA is partially achieved by ATR-dependent DNA-PKcs phosphorylation. In support of this, a similar stem cell defect was observed in conditional ATR knockout mice (Ruzankina et al., 2007). It is not clear yet how DNA-PKcs phosphorylation at the Thr2609 cluster promotes HR/FA activities. One possible explanation is that Thr2609 cluster phosphorylation changes the conformation of DSB-bound DNA-PKcs and releases DSB ends to the HR/FA machineries (Meek et al., 2008).

The loss of HSCs in DNA-PKcs3A/3A mice is primarily caused by p53-mediated apoptotic response, as the p53-null mutation reinstates HSC reserves in DNA-PKcs3A/3A mice. In addition to HSC loss, elevation of genotoxic stress and p53 activation was found in other proliferating tissues as evident from intestinal crypt cell apoptosis and epidermal hyperpigmentation. These results reiterate that DNA-PKcs phosphorylation at the human Thr2609/mouse Thr2605 cluster is critical for replication stress response and the maintenance of tissue homeostasis. The tissue-specific outcome of genotoxic stress imposed by the DNA-PKcs3A mutant protein or DNA-PKcs dysfunction may depend on cell type–specific apoptosis/senescence and the compensation capacity from varied sizes of stem cell pools in different tissues and organs.

Gene mutations in the FA protein complex and HR components have been identified in human FA syndrome characterized by congenital bone marrow failure, short stature, abnormal skin pigmentation, reduced fertility, hypersensitivity toward DNA cross-link damages, and increased genomic instability (Moldovan and D’Andrea, 2009). The mechanism underlying the clinical findings of FA remains unclear, given the fact that none of the current mouse models for HR and FA systematically recapitulate these pathophysiological changes. It is interesting to note that the DNA-PKcs3A/3A mouse resembles human FA syndromes, which is caused by telomerase mutations, also displays bone marrow failure and hyper skin pigmentation characters. Conversely, mice with combined mutations on telomerase and Pot1b, a component of the telomere-protecting shelterin complex, exhibit dyskeratosis congenita-like characteristics, including bone marrow failure and footpad hypertpigmentation (Hockemeyer et al., 2008; He et al., 2009). This similarity to DNA-PKcs3A/3A mice suggests a common stress response among different congenital bone marrow failure diseases and warrants further investigation of DNA-PKcs phosphorylation on telomere maintenance.

Materials and methods

Construction of the targeting vector and generation of DNA-PKcs3A/3A knockin mice

The 3A knockin targeting vector was constructed using a previously described self-excision gene-targeting approach (Bunting et al., 1999). The DNA-PKcs3A mice were maintained in the mixed genetic background of 129x C57BL/6 and were bred with p53-null mice in an FVB background. ES cell targeting and generation of chimeric mice were performed at the transgenic core facility at the University of Texas Southwestern Medical Center. All animal experimental protocols were approved by the Animal Care Committee of the University of Texas Southwestern Medical Center.

Clonogenic survival and chromosomal aberrations

Primary and SV40-transformed MEFs from E13.5 embryos were cultured in minimum essential medium supplemented with 10% fetal calf serum and penicillin/streptomycin. MEFs were maintained in a 37°C humidified atmosphere with 3% CO2 and 10% O2. For clonogenic survival, SV40-transformed MEFs were treated with different doses of mutagens and were trypan-blue/reseeded in appropriate numbers in 10-cm tissue-culture dishes for 7 d. For chromosomal aberrations, primary MEFs (passages 2–5) were incubated with 40 nM MMC for 24 h and 0.1 mg/ml colcemid in the last 2 h.

Fluorescence-activated cell sorting and ex vivo expansion of HSCs

Thymus, bone marrow, or fetal liver single-cell suspensions were stained with the indicated antibodies and analyzed using either a FACSCalibur (BD) or LSRII (BD) flow cytometer (Huynh et al., 2008). HSCs were sorted using a flow cytometer (FACSaria; BD) essentially as previously described (Huynh et al., 2008). For apoptosis analysis, cell suspensions were stained with phycoerythrin-anti–Annexin V (BD). Ex vivo expansion of fetal liver HSCs was performed using a previously published protocol (Zhang and Lodish, 2004).

V(D)J recombination assay

Di2.Jb1 coding joint junctions were PCR amplified from total thymocyte DNA using a nested amplification protocol. The PCR conditions and primer design are detailed in Fig. S2.

Bone marrow/HSC transplantation and competitive repopulating assay

Total bone marrow cells (5 × 10^5) or Lin-Scic1-cKit+ cells (1,500–5,000) from wild-type littermates were injected into the retro-orbital plexus of 2- or 3-week-old DNA-PKcs3A/3A mice without preconditioning. Competitive repopulation was performed using wild-type, DNA-PKcs3A/3A, or DNA-PKcs+/- cells (CD45.2 background), mixed with competitive bone marrow cells from B6.Ly5.2/Cr mice (CD45.1 background), and injected into lethally irradiated (5 Gy × 2) B6-Ly5.2/Cr-recipient mice. 2 mo after injection, populations of CD45.1+ and CD45.2+ peripheral blood cells were analyzed by flow cytometry.

DR-GFP HR assay

SV40-transformed MEFs with single-copy incorporation of the DR-GFP reporter (Pierce et al., 1999) were transfected with the IScel-GR-RFP plasmid (Addgene) and treated with 10 nM triamcinolone acetonide for 24 h followed by flow cytometry analysis. HR activities were determined by the percentage of GFP/RFP* double-positive cells among all IScel-GR-RFP* expressing (RFP*) cells.
Online supplemental material

Fig. S1 shows a DNA-PKcs Δ1 mouse model with three alanine substitutions at the Thr2605 cluster of the endogenous DNA-PKcs gene. Fig. S2 shows compromised lymphocyte development in DNA-PKcs Δ1/Δ1 mice. Fig. S3 shows BM T rescue of hematopoietic defects in DNA-PKcs Δ1/Δ1 mice. Fig. S4 shows spontaneous DNA damage and a p53 signaling pathway in DNA-PKcs Δ1/Δ1 cells. Table S1 shows Δ62/61j coding joint junctions cloned and sequenced from wild-type mice. Table S2 shows Δ62/61j coding joint junctions cloned and sequenced from DNA-PKcs Δ1/Δ1 mice. Table S3 shows Δ62/61j coding joint junctions cloned and sequenced from DNA-PKcs Δ1/Δ1 mice. Table S4 shows the peripheral blood cell count of 2-wk-old mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201009074/DC1.

We thank Drs. K.R. Thomas and M.R. Capecchi for providing the ACN mice. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201009074/DC1.

Submitted: 14 September 2010
Accepted: 22 March 2011

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