DNA Damage Tolerance in Hematopoietic Stem and Progenitor Cells in Mice

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Abstract

DNA damage tolerance (DDT) enables bypass of DNA lesions during replication, thereby preventing fork stalling, replication stress, and secondary DNA damage related to fork stalling. Three modes of DDT have been documented specifically translesion synthesis (TLS), template switching (TS), and repriming. TLS and TS depend on site-specific PCNA K164 mono- and polyubiquitination, respectively. To determine the role of DDT in maintaining hematopoietic stem cells (HSC) and progenitors we used \( \text{Pcna}^{\text{K164R/K164R}} \) mice as a unique DDT defective mouse model. Analyzing the composition of HSC and HSC-derived multipotent progenitors (MPPs), HSC numbers were strongly reduced, likely due to increased differentiation of HSC towards myeloid/erythroid associated MPP2s. This skewing came at the expense of lymphoid-primed MPP4s number, which appeared compensated by increased MPP4 proliferation. Furthermore, defective DDT decreased the number of MPP-derived common lymphoid (CLP), common myeloid (CMP), megakaryocyte-erythroid (MEP), and granulocyte-macrophage (GMP) progenitors, accompanied by increased cell cycle arrest in CMPs. The HSC and MPP phenotypes are reminiscent of premature aging and stressed hematopoiesis, and indeed progressed with age and are exacerbated upon cisplatin exposure. Bone marrow transplantations revealed a strong cell intrinsic defect of DDT deficient HSC in reconstituting lethally irradiated mice and a strong competitive disadvantage when co-transplanted with wild-type HSC. These findings indicate a critical role of DDT in maintaining HSC and progenitors, and preventing premature aging.

Significance

Hematopoietic stem cells are the prime source of hematopoietic cells, and mutations in HSCs can kick-start tumorigenesis. Therefore, genome maintenance is key in preventing malignant transformation. Unlike DNA repair, the role of DNA damage tolerance (DDT) as part of the DNA damage response (DDR) network in HSC maintenance remains unknown. Taking advantage of DDT deficient \( \text{Pcna}^{\text{K164R/K164R}} \) mice, we investigated the role of DDT in maintaining stem cells and their longevity. DDT deficient \( \text{Pcna}^{\text{K164R/K164R}} \) mice revealed a progressive impairment of HSC, identifying DDT as a key player in preserving stem cell fitness and preventing premature aging.
Introduction

Hematopoietic stem cells (HSC) are able to maintain a steady HSC population over lengthy periods of time through self-renewal. In addition, HSC are pluripotent and can give rise to most specialized hematopoietic lineages (1, 2). Functionally distinct hematopoietic precursor subsets have been identified based on expression markers and functional transplantation analyses (3-5). These subsets are defined as long term HSC (LT-HSC), short term HSC (ST-HSC), multipotent progenitor 2-4 (MPP2, MPP3, MPP4), common lymphoid progenitor (CLP), common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP), and granulocyte-macrophage progenitor (GMP) (Table 1). Lineage⁻, Sca-1⁺, cKit⁺ (LSK) subset contains LT-HSC, ST-HSC, MPP2, MPP3, and MPP4. The hematopoietic stem and progenitor cell (HSPC) compartment comprises LT-HSC, ST-HSC, MPP2, and MPP3. Lineage⁻, cKit⁺, Sca-1⁻ (LKS⁻) subset includes CMP, MEP, and GMP.

During aging, the HSC potential declines and HSC differentiation is skewed towards the erythroid/myeloid associated MPP2 lineage, seemingly at the expense of lymphoid-associated MPP4 and CLP (6). Consequently, lymphocyte production decreases during age and functionality of the lymphoid system declines. This age-related phenomenon of decreased lymphoid functionality is termed immuno-senescence and thought to be initiated in aging HSCs (7, 8).

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Abbreviations: Int. = intermediary
Defects in DNA damage repair pathways progressively impair the fitness of HSCs and are linked to premature aging (7, 9-12). In addition, replicative stress has been implicated in HSC decline and aging (13). During S phase, DNA is copied by replicative polymerases epsilon and delta on the leading and lagging strand, respectively (14, 15). However, these replicative polymerases can be stalled by replication blocks, like DNA lesions, G4-stacks, ribonucleotide misincorporation, and RNA/DNA hybrids that persisted into or arose during S phase (16-18). To bypass such replication blocking lesions or structures and prevent secondary DNA damage due to prolonged fork stalling, three principle modes of DNA damage tolerance (DDT) are distinguished: translesion synthesis (TLS), template switching (TS), and repriming (19-23). PCNA K164 specific modifications are key in switching efficiently between a replicative and a damage-tolerant mode of DNA replication. In mammals, PCNA K164 can be sumoylated, however sumoylation is not K164-specific (24). In contrast, DNA-damage induced monoubiquitination at lysine 164 of PCNA (PCNA-Ub) is site specific and highly conserved. PCNA-Ub facilitates efficient polymerase switching to a damage tolerant Y-family TLS polymerase that can accommodate non-Watson/Crick base pairs within their enlarged catalytic centers enabling replication to continue across a lesion, albeit more error-prone. PCNA K164 polyubiquitination (PCNA-Ub^n) signals TS where the intact genetic information of the sister chromatid provides the template for an error-free bypass of the fork-stalling lesion. PCNA K164 independent mechanisms of TLS recruitment, e.g. the Y-family TLS polymerase REV1 can recruit other Y-family TLS polymerases to stalled forks (25-28). In S. Cerevisiae a DDT independent role of PCNA K164 modification has been suggested and seems to play a minor role in lagging strand synthesis under unperturbed conditions (29), though this study cannot exclude differential DDT activities on the leading versus lagging strand in response to endogenous DNA damage and replication blocking lesions (19). In mammals, TLS is the predominant pathway and PCNA-Ub mediated TLS can be readily observed upon DNA damage induction, whereas PCNA-Ub^n mediated TS seems less frequent (30, 31). Intense research over the last years highlighted the relevance of PCNA K164-dependent DDT in genome maintenance and its critical activity within the DNA damage response network (32-38). Yet, the overall relevance of these DDT pathways in the rapidly renewing hematopoietic system remained to be defined.

In this study, we investigated the role of DDT in HSC and progenitor cells. The contribution of DDT in the maintenance of HSCs was determined by analyzing the bone marrow (BM) of DDT deficient \( Pcn_{a}^{K164R/K164R} \) mice (34). Detailed analyses of the hematopoietic compartment of DDT defective \( Pcn_{a}^{K164R/K164R} \) mice revealed a critical contribution of DDT in determining the fitness of HSC and their progeny. A selective skewing of hematopoiesis towards the myeloid/erythroid biased MPP2 in the LSK compartment indicated that defective DDT greatly accelerates aging of the hematopoietic compartment in \( Pcn_{a}^{K164R/K164R} \) mice. These findings highlight the relevance and critical contribution of DDT analogous to DNA repair,
within the DNA damage response network and the importance of DDT in safeguarding long-term tissue homeostasis.

Results

DDT is required to maintain HSCs and progenitor cells

To investigate the relevance of DDT in maintaining hematopoietic stem cell and progenitors, we analyzed DDT deficient mice with a *Pcna*<sup>K164R/K164R</sup> mutation. The distinct hematopoietic subsets were quantified using defined gating strategies and markers (Table 1, SI Appendix, Fig. S1A) (3). Following this strategy, the MPP1 subset is included in LT-HSC.

In young adult mice (2 months), the total number of nucleated cells per femur were comparable between WT and *Pcna*<sup>K164R/K164R</sup> mice. However, when analyzing various hematopoietic subsets, major changes were observed. The LSK population in the BM was decreased 2.1-fold, from 41*10<sup>3</sup> in WT as compared to 19*10<sup>3</sup> cells per femur in *Pcna*<sup>K164R/K164R</sup> mice (Fig. 1A-B). In the femora of *Pcna*<sup>K164R/K164R</sup> mice the LT-HSC decreased 1.4-fold, the ST-HSC 5.3-fold, and the MPP4 4.4-fold. In contrast, the MPP2 subset was selectively increased 2.1-fold in the *Pcna*<sup>K164R/K164R</sup> mice (Fig. 1B-C).

The more differentiated LKS progenitor subset was also decreased 2.5 times (Fig. 1D, SI Appendix, gating in Fig. S1A). Compared to WT, the CMP compartment declined 2.1-fold in *Pcna*<sup>K164R/K164R</sup> mice, GMP 1.9-fold, and MEP 4.0-fold. Furthermore, the CLP were affected as well in *Pcna*<sup>K164R/K164R</sup> mice and decreased 2.4-fold (Fig. 1E). MPP4 and CLP contribute primarily to lymphocytes (3). As MPP4 and CLP numbers were deteriorated in *Pcna*<sup>K164R/K164R</sup> mice, we examined B and T lymphocyte development in *Pcna*<sup>K164R/K164R</sup> mice (SI Appendix, Fig. S1B-D). Using well-defined markers to trace lymphocyte differentiation (39), no major effects were observed in B or T cell development. Similarly, splenic B and T cell populations remained largely unaffected, though B cells were found to be slightly reduced.

In order to establish whether the defects of HSC and other progenitor populations are due to increased DNA damage, we assessed the percentage of γH2AX positive cells per subset (SI Appendix, data in Fig. S2A-F, gating in Fig. S3). As γH2AX increases during S/G2, we corrected for cell cycle status, i.e. percentages are calculated as γH2AX positive cells in S/G2 of all cells in S/G2. γH2AX was slightly increased in all populations, though only significant in *Pcna*<sup>K164R/K164R</sup> LSK S/G2 cells as compared to WT.

In summary, these data indicated an important function of DDT in maintaining the HSC and progenitor populations in the BM. Furthermore, the decrease of ST-HSC and MPP4 combined with the increase of MPP2 is highly reminiscent of previous findings of hematopoietic regeneration and premature aging (3, 6).
Figure 1. PCNA K164R mutation leads to a HSC and progenitor defect.  
(A) Gating of hematopoietic precursor and HSC subsets in 2 months old WT and \( \text{Pcna}^{\text{K164R/K164R}} \) mice.  
(B-E) Quantification of hematopoietic subsets in WT and \( \text{Pcna}^{\text{K164R/K164R}} \) femora. Merged data from 2 experiments are shown, with a total of 6 mice per genotype. T-test was applied to calculate p-values. Stars indicate statistical significance (*: p-value > 0.05, **: p-value > 0.01, ***: p-value > 0.001, ****: p-value > 0.0001).
DDT deficiency is associated with increased proliferation and cell cycle arrest in distinct hematopoietic progenitor subsets

As the number of cells in progenitor compartments decreased and the total BM cell numbers were equal, we reasoned that the LSK and LKS- progenitor compartments should increase the proliferation in order to compensate for the decrease of progenitor cells. To examine whether the compromised early hematopoiesis in Pcna\textsuperscript{K164R/K164R} mice leads to a compensatory proliferation or DDT-deficiency related cell cycle arrest at S/G2, we measured the chromatin content in HSC and progenitor cells. In Pcna\textsuperscript{K164R/K164R} BM cells the percentage of cells in S/G2 increased 1.6-fold in the LSK, 1.6-fold in the HSPC, and 1.7-fold in the MPP4 population (Fig. 2A, SI Appendix, gating in Fig. S4). The increased percentage of S/G2 cells in LSK and HSPC of Pcna\textsuperscript{K164R/K164R} is most likely related to a selective increase of MPP2 cells per femur, which had a very high percentage of cells in S/G2. In contrast, within the HSPC subset, no subset differed significantly regarding the percentage of S/G2. In the case of MPP4 the increase in S/G2 cells could be caused by increased proliferation or cell cycle arrest. To distinguish between these possibilities, we injected mice with the thymidine analogue 5-ethynyl-2'-deoxyuridine (EdU) for 16 hours. EdU incorporation into DNA enables a quantification of actively proliferating cells. Following this approach, we observed that the frequency of EdU positive cells in the MPP4 compartment was increased in Pcna\textsuperscript{K164R/K164R} mice, supporting a concept were increased proliferation compensates the lack of MPP4s (Fig. 2B, SI Appendix, Fig. S5).

The increased EdU incorporation in Pcna\textsuperscript{K164R/K164R} LSK and HSPC as compared to WT, likely relates to the presence of highly proliferative MPP2 precursors and their increased cell number in Pcna\textsuperscript{K164R/K164R} mice.

In contrast to LSK, LKS- (1.2-fold) and CMP (1.3-fold) populations had an increased S/G2 percentage in Pcna\textsuperscript{K164R/K164R} mice and a reduced frequency of EdU positive cells in Pcna\textsuperscript{K164R/K164R} mice (Fig. 2A-B, SI Appendix, Fig. S5). These data implicate that the failure to tolerate endogenous DNA damage leads to an increased cell cycle arrest in the LKS- compartment of Pcna\textsuperscript{K164R/K164R} mice.

In summary, these distinct alterations in the percentage of cells in S/G 2 and EdU incorporation in LSK and LKS- subsets caused by the Pcna\textsuperscript{K164R/K164R} mutation suggests that the BM subsets of these mice have either differential in terms of endogenous replication impediments, sensitivity to DNA damage, or response upon the DNA damage signaling.

The hematopoietic system of DDT deficient mice is extraordinarily sensitive to cisplatin.

Primary cell lines derived from Pcna\textsuperscript{K164R/K164R} mice are highly sensitive to cisplatin (CsPt) (30, 35, 40). To quantify the sensitivity of Pcna\textsuperscript{K164R/K164R} BM to inter- and intrastrand crosslinks, mice were injected intravenously (i.v.) with 0.8 mg/kg CsPt or mock treated with PBS and the BM was analyzed after 2 days. The dose used here was relatively low, as the maximal tolerable dose used in C57BL/6J mice is 6 mg/kg CsPt, which is 7.5-fold lower.
In WT mice, the total number of BM cells remained unaffected upon low dose CsPt treatment (Fig. 3A-B). Pathological analysis of BM revealed that administration of low dose CsPt with a concentration of 0.8mg/kg did not visibly affect BM hematopoiesis in WT mice. Likewise, vehicle controls in both WT and \(Pcna^{K164R/K164R}\) mutant mice showed no visible changes when mock treated with PBS. In contrast, the \(Pcna^{K164R/K164R}\) mutant mice showed massively disturbed myelopoiesis, erythropoiesis, and thrombopoiesis (Fig. 3A, SI Appendix, Fig. S6B). Particularly the erythropoietic population was greatly depleted.

**Figure 2.** Defective DDT leads to increased proliferation and cell cycle arrest in different hematopoietic subsets.

(A) Percentage of S/G2 cells in WT and \(Pcna^{K164R/K164R}\) in 2-month-old mice. Combined data from 2 experiments are shown.

(B) Percentage of EdU positive cells in WT and \(Pcna^{K164R/K164R}\) BM. Mice were treated with EdU for 24 hours. Pooled data of 2 experiments are shown. T-test was used to calculate p-values. Stars indicate statistical significance (*: p-value > 0.05, **: p-value > 0.01, ***: p-value > 0.001, ****: p-value > 0.0001).
Flow cytometric analysis of HSC and progenitors revealed that in WT mice only the CMP (1.3-fold decrease) and MEP populations (3-fold decrease) were affected upon CsPt exposure, indicating that especially the myeloid/erythroid lineage is sensitive to CsPt in WT mice (SI Appendix, Fig. S6, Table S2).

In line with the pathological analysis, counting total BM numbers of \( \text{Pcna}^{K164R/K164R} \) mice, CsPt treatment reduced the number of nucleated BM cells per femur from \( 18 \times 10^6 \) in mock treated mice to \( 10 \times 10^6 \) in CsPt treated mice (1.8-fold) (Fig. 3B, SI Appendix, Fig. S6). Most remarkably, HSC and progenitors were greatly affected. The LSK (57-fold), HSPC (41-fold), LT-HSC (52-fold), ST-HSC (335-fold), MPP2 (4-fold), MPP3 (32-fold), and MPP4 (102-fold) populations were severely diminished after CsPt treatment in \( \text{Pcna}^{K164R/K164R} \) mice compared to CsPt treated WT mice (Fig 3C-D, SI Appendix, gating in Fig. S6A). In the LKS progenitors, the CMP (940-fold), GMP (219-fold), and MEP (1755-fold) populations are all tremendously reduced in \( \text{Pcna}^{K164R/K164R} \) mice after treatment as compared to the treated WT control mice (Fig 3E, SI Appendix, gating in Fig. S6A). As expected, subsets most strongly affected by CsPt were those with a higher S/G2 percentage, with the exception of ST-HSC and MPP2. ST-HSCs were decreased most and MPP2 decreased the least, suggesting ST-HSC can differentiate rapidly into MPP2 upon stress to secure erythropoiesis.

In summary, the hematopoietic defect of DDT deficient mice could be exacerbated by replication stress induced by CsPt. The PCNA K164 ubiquitination defect rendered HSCs, MPPs and LKS-subsets highly sensitive to crosslinking agents. However, not all hematopoietic subsets were equally impaired. Especially the number of ST-HSC, CMP, and MEP showed the largest fold change upon CsPt exposure, both in WT and \( \text{Pcna}^{K164R/K164R} \) mice.

**DDT strongly contributes to HSC fitness**

In order to determine the functionality of HSCs in \( \text{Pcna}^{K164R/K164R} \) mice, we performed competitive BM transplantations. BM cells were transplanted by *i.v.* injection of a \( 1 \times 10^6 \) 1:1 mixture of Ly5.1 WT with Ly5.2 WT or \( \text{Pcna}^{K164R/K164R} \) BM cells into irradiated Ly5.1 recipient mice. By applying of the Ly5.1/Ly5.2 discrimination system the relative fitness of DDT proficient and deficient HSC was determined by measuring their contribution to the B lymphocytes, T lymphocytes, and granulocytes. In all 3 subsets, we observed that in the WT vs. WT setting, the contribution of Ly5.2 WT reaches the expected plateau around 50-60% after 10 weeks (Fig. 4A-C, SI Appendix, gating in Fig. S7). However, in the \( \text{Pcna}^{K164R/K164R} \) vs. WT setting the contribution of Ly5.2 \( \text{Pcna}^{K164R/K164R} \) was dramatically reduced. After 2 weeks, there is a small contribution of Ly5.2 \( \text{Pcna}^{K164R/K164R} \) cells to granulocytes and B cells, which may arise from differentiated progenitor populations. Their contribution further decreased below 5% after 5 weeks and remained under 5% up to 20 weeks.
Chapter 7

A

WT

PcnaK164R/K164R

PBS

Cspt

B

Total BM

0.0

5.0 \times 10^6

1.0 \times 10^7

1.5 \times 10^7

2.0 \times 10^7

2.5 \times 10^7

Nucleated cells per femur

C

LSK

HSC

MPP4

D

LT HSC

ST HSC

MPP2

MPP3

Nucleated cells per femur

E

LKS

CMP

GMP

MEP

Nucleated cells per femur

Legend:

- WT PBS
- PcnaK164R/K164R PBS
- WT Cspt
- PcnaK164R/K164R Cspt

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Ly5.2 Pcna<sup>K164R/K164R</sup> T cells however, start from 50% contribution at 2 weeks, likely derived from CLPs that populated the thymus (39). Similar to granulocytes and B cells, after 5 weeks the contribution of Pcna<sup>K164R/K164R</sup> also decreased below 5%.

Twenty weeks after transplantation, we examined the BM and found the population of HSC containing LSK and more differentiated LKS<sup>-</sup> in the Ly5.2 Pcna<sup>K164R/K164R</sup> almost absent, whereas WT Ly5.2 contributed 50%, as expected (Fig. 4D). These results indicated a severe defect in transplantation capacity of Pcna<sup>K164R/K164R</sup> HSCs. These data suggest that Pcna<sup>K164R/K164R</sup> HSCs are rapidly outcompeted by the WT HSCs, alternatively Pcna<sup>K164R/K164R</sup> HSCs have difficulty in reaching the HSC niche.

To discern these hypotheses, we performed non-competitive BM transplantations by i.v. injection of 2*10<sup>6</sup> Ly5.2 WT or Pcna<sup>K164R/K164R</sup> BM cells into irradiated syngeneic Ly5.1 recipient mice. After 2 and 4 weeks, their transplantation capacity was assessed on the basis of Ly5.2 cells at different stages of hematopoiesis. In this non-competitive BM transplantation assay, WT BM cells were much more effective in repopulating the depleted BM. After 2 weeks, the femur of WT reconstituted recipient mice contained 15*10<sup>6</sup> Ly5.2 positive cells, whereas the femur of Pcna<sup>K164R/K164R</sup> reconstituted recipient mice contained only 6*10<sup>6</sup> cells (Fig. 4E). This 2.5-fold reduction in reconstitution activity likely relates to intrinsic differences regarding the transplantation efficiency of Pcna<sup>K164R/K164R</sup> BM cells. This defect was even more pronounced when analyzing HSC containing LSK and HSPC (Figure 4F, SI Appendix, Fig. S8A-F). Also, the LKS<sup>-</sup>, CMP, GMP, and MEP progenitor population numbers were much lower after 2 weeks in mice reconstituted with Pcna<sup>K164R/K164R</sup> BM. Despite the fact, that the BM cellularity of Pcna<sup>K164R/K164R</sup> reconstituted recipients improved substantially after 4 weeks, major differences remained when comparing defined BM precursor subsets. Of note, the observed differences 2 weeks after transplantation are unlikely explained by preexisting differences in the initial subset composition of transplanted BM cells, as these differences exceed the preexisting differences by far.

In summary, these data indicate a severe intrinsic repopulation defect of Pcna<sup>K164R/K164R</sup> HSCs. This defect likely explains our observations made in the competitive BM reconstitution assays.

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**Figure 3. Hematopoietic system strongly depends on DDT for tolerating crosslinking agents.**

(A) H&E stained sternum of WT and Pcna<sup>K164R/K164R</sup> injected with PBS or CsPt. The scale bar indicates 50 μm. The original magnification was 20 times.

(B-E) The number of nucleated cells per femur 2 days after injection of 0.8 mg/kg CsPt or PBS. One representative experiment out of 2 is shown.
Figure 4: PCNA K164R mutation leads to a HSC transplantation defect.
(A-C) Competitive BM transplantation of WT or Pcna\textsuperscript{K164R/K164R} Ly5.2 BM mixed with Ly5.1 BM transplanted into lethally irradiated Ly5.1 recipient mice. Contribution of Ly5.2 BM to the blood for B cells, T cells and Gr-1+ macrophages were measured for 20 weeks. 1 out of 2 representative experiment is shown. One million BM cells were transplanted in irradiated mice (*: p-value > 0.05).
(D) Percentage of Ly5.2 positive cells in the LSK and LKS - subset 20 weeks post transplantation are shown for WT and Pcna\textsuperscript{K164R/K164R} mice, indicating the near absence of Pcna\textsuperscript{K164R/K164R} HSC and precursor cells. One representative experiment out of 2 is shown.
(E-F) Short term non-competitive transplantation of WT or Pcna\textsuperscript{K164R/K164R} BM. Total number of cells per femur (E) and number of HSC containing HSPCs per femur (F) are shown. One million BM cells were transplanted in lethally irradiated mice. BM was isolated 2 and 4 weeks post transplantation and analyzed. One representative experiment out of 2 is shown.

DDT deficiency results in premature aging of the early hematopoietic compartment
During aging, the number of MPP2 cells increase and the number of MPP4 cells progressively decrease in the BM (6). This suggests a priority for producing MPP2 at the expense of MPP4, as MPP2 are important precursors for the essential myeloid/erythrocyte lineage and MPP4s are the precursors of less important lymphoid lineage. In this way oxygen supply is safeguarded. Interestingly already after 2 months, Pcna\textsuperscript{K164R/K164R} mice showed an increased MPP2 and a decrease of MPP4. This phenotype caused by defective DDT is consistent with accelerated aging, presumably due to increased replication stress. If this hypothesis is correct, one expects this phenotype to be further enhanced during aging. We therefore compared the cellularity of the hematopoietic subsets of WT and Pcna\textsuperscript{K164R/K164R} at 9-10
months of age (Fig. 5A-E). When comparing 2 months and 9-10 months old \( Pcnak^{164R/K164R} \) and WT mice the fold change of the ST-HSC, MPP4, and CLP subsets further increased. MPP2 on the other hand remained higher in \( Pcnak^{164R/K164R} \) mice throughout this period (SI Appendix, Fig. S9). In contrast, in the LKS subsets aging did not increase the initial difference. This suggests that during stressed hematopoiesis the maintenance of LKS- cells is a priority, as these contain the essential erythroid progenitors.

Figure 5: Hematopoietic phenotype of DDT impaired mice is progressive with age.
(A) FACS plots of 9-10 months aged WT and \( Pcnak^{164R/K164R} \) mice.
(B-E) Number of nucleated cells per femur of different progenitors and HSC subsets in young 2 month and 9-10 month aged mice.
In summary, defective DDT leads to accelerated aging of the hematopoietic system characterized by an increased selective skewing towards the MPP2 subset in LSK, primarily at the expense of the MPP4 subset.

Discussion

While the role of DNA repair in HSC maintenance has been established, the contribution of DDT to HSC and progenitor maintenance remained to be addressed. We here reported an elegant model to study stressed hematopoiesis and demonstrate a critical cell intrinsic role for DDT in maintaining adult HSCs and early hematopoiesis. \( \text{Pcna}^{K164R/K164R} \) cells are defective in TLS and TS, leading to increased replication stress and sensitivity to fork stalling DNA lesions. In 2-month-old mice, the \( \text{Pcna}^{K164R/K164R} \) mutation causes a reduced cellularity of LSK, LT-HSC, ST-HSC, MPP4, LKS\(^-\), CMP, GMP, MEP, and CLP subsets in the BM of mice. In contrast, the lack of PCNA K164-dependent DDT was associated with a selective increase of the myeloid/erythroid–associated MPP2 in \( \text{Pcna}^{K164R/K164R} \) mice. This increase is likely due to increased stress induced differentiation of HSCs which reduced the number of HSC, towards MPP2 at the cost of the number of MPP4 (3, 41, 42).

Cell cycle and cell proliferation studies revealed an increased percentage of S/G2 cells and EdU incorporation in MPP4, suggestive of compensatory proliferation to counteract the strong loss of these lymphoid-primed progenitor cells. As MPP4 mainly contributes to the lymphoid lineage, the increased proliferation could explain the unaffected B and T cell development in \( \text{Pcna}^{K164R/K164R} \) mice. In contrast, LKS\(^-\) and CMP progenitors had an increased S/G2 proportion combined with lower EdU incorporation in \( \text{Pcna}^{K164R/K164R} \), pointing to a cell cycle arrest caused by increased fork stalling and secondary DNA damage due to defective DDT.

In order to determine whether the BM defect of \( \text{Pcna}^{K164R/K164R} \) mice can be enhanced by exogenous DNA damage, mice were exposed to CsPt. While WT BM subsets were marginally affected, \( \text{Pcna}^{K164R/K164R} \) HSC and progenitor subsets were almost depleted. This marked hypersensitivity to DNA damage in mice deficient for PCNA K164-dependent DDT, further highlights the relevance of DDT in maintaining hematopoiesis and HSCs.

Aging is characterized by a selective skewing of hematopoiesis towards the myeloid/erythroid associated MPP2 in the LSK subsets in aged mice (6). The skewing towards MPP2 in the LSK compartment of \( \text{Pcna}^{K164R/K164R} \) mice therefore likely relates to accelerated aging induced by increased replication stress due to defective DDT. We hypothesize that stress induced regeneration, like replication stress, of the hematopoietic system results in a shifted differentiation towards MPP2. This model is in line with previous reports documenting a shift towards MPP2 in the LSK compartment upon stress induced regeneration of hematopoietic
system (3). This skewing is further increased during aging. While the differentiation bias was already evident in 2 months old Pcna<sup>K164R/K164R</sup> mice, this bias increased further at 9-10 month of age. This finding supports the notion of accelerated aging in the Pcna<sup>K164R/K164R</sup> BM. Apparently, the capacity to tolerate DNA damage and prevent replication stress is critical in maintaining homeostasis in the BM compartment. The failure to tolerate DNA damage strongly accelerates aging of the BM compartment, indicating an important function of PCNA K164-dependent DDT in HSC maintenance. Previously, defects in DNA repair pathways (9) and increased replication stress (13) were identified as potent drivers of premature HSC aging, which is in line with our findings.

In our model of stressed hematopoiesis due to deficient DDT of Pcna<sup>K164R/K164R</sup> mice, HSC shift differentiation from mainly to MPP4 in WT to MPP2 in Pcna<sup>K164R/K164R</sup> (Fig. 6).

**Figure 6: Model for effect of DDT deficiency on HSC and early progenitors.**
Steady state hematopoiesis in WT mice is indicated. In DDT deficient Pcna<sup>K164R/K164R</sup> mice, replication stress induced differentiation of HSC towards myeloid/erythroid associated MPP2 in Pcna<sup>K164R/K164R</sup> is indicated by red arrow. Furthermore, compensatory proliferation for MPP4 is specified, as well as cell cycle arrest in CMP and GMP subsets. Arrows indicate direction of differentiation. Blue dots indicate LSK subsets and green LKS-. Each dot represents 500 nucleated cells per femur.
Of note, Rad18 deficient mice do not show a decrease of LSK cells (Yang Yang, NAR 2016). This likely relates to the existence of alternative E3 ligases targeting PCNA K164 (43, 44), alternatively an unknown PCNA K164 modification may play a role in maintaining HSC and progenitors. An additional phenotype of \( \text{Pcna}^{K164R/K164R} \) mice is infertility due to a complete absence of germ cells (34). The infertility, the BM phenotype and the sensitivity to crosslinking agents are shared phenotypes with Fanconi Anemia (FA) mouse models (45-47). Future research should demonstrate the contribution of PCNA K164 dependent DDT in the FA pathway.

The FA pathway is involved in the repair of interstrand crosslinks (48, 49). DDT pathways on the other hand likely tolerate a myriad of replication blocks. These replication blocks may include base damages like deamination, methylation and oxidation, intra- and interstrand crosslinks induced by endo- and exogenous genotoxins, G4-stacks, RNA-DNA hybrids, and ribonucleotide misincorporation (16-18, 50, 51). Consequently, DDT impaired HSCs are likely to be sensitized to this complex spectrum of lesions. For survival, PCNA K164 deficient DDT HSCs are expected to depend on alternative DDT pathways such as REV1 dependent DDT. Given the impact of a DDT defect on HSC, it is likely that other stem cells, especially those in highly proliferative compartments are also sensitive. Further research will have to reveal the sensitivity of other tissue stem cells and the relevance of DDT for tissue homeostasis.

In conclusion, this study signifies for the first time the relevance of DDT in preventing premature aging in the hematopoietic compartment and safeguarding HSC functionality. These data highlight DDT as important arm of the DNA damage response network.

**Methods**

**Mice and breeding**

\( \text{Pcna}^{K164R/K164R} \) knock in mouse model was described previously (34). All mice were kept on C57BL/J6 background under specific-pathogen-free (SPF) conditions. \( \text{Pcna}^{K164R} \) mice were maintained heterozygous. All experiments were performed according to institutional, national and European guidelines.

**Antibodies**

Antibody specifications are listed in Supplemental Table 1.

**Flow cytometry**

**Hematopoietic precursor subset analysis**

Mice were euthanized at indicated age (2 months or 9-10 months), and BM from femora was
flushed out using 21 Gauge syringes in cold PBEA buffer (1x PBS 0.5% BSA, 2mM EDTA, 0.02% Sodium Azide). The samples were kept on ice. We used 5*10^6 cells per staining. BM cells were first stained with a biotinylated lineage+ antibody mix for 30 minutes and washed in PBEA twice. For quantifying stem cells and MPP populations: cKit-APC, Sca-1-PerCp/Cy5.5, CD48-FITC, CD135-PE, CD150-PE/Cy7, streptavidin-APC/Cy7 were used. For quantifying LKS- progenitor populations: cKit-APC, CD34-FITC, CD16/32-PE/Cy7, streptavidin-APC/Cy7, Sca-1-Pacific Blue. To quantify CLPs: cKit-APC, CD127-PerCp/Cy5.5, CD135-PE, streptavidin-APC/Cy7, Sca-1-Pacific Blue was used. Cells were washed twice using PBEA and resuspended to in 400 μl PBEA. For stem cell and MPP, 4’,6-diamidino-2-phenylindole (DAPI) was used as life/dead staining, while for LKS- and CLP propidium iodide (P.I.) was used. All measurements were performed on Becton Dickinson (BD) Fortessa. For the analysis FlowJo (version 10.0.8r1) was used.

Cell cycle analysis of BM populations
Cell surface staining was performed as described in Hematopoietic precursor subset analysis. Samples were incubated in Cytofix/Cytoperm (BD) for 15 minutes. Cells were washed using Permwash and harvested in PBEA containing 10 μg/ml DAPI. For the analysis FlowJo (version 10.0.8r1) was used.

Assessing γH2AX levels of BM populations
Cell surface staining was performed as described in hematopoietic precursor subset analysis. BM cells were first labeled with biotinylated Lin+ antibody mix. Subsequently, LSK populations were stained with CD135-PE, SCA-1-PerCp/Cy5.5, cKit-APC, streptavidin APC/Cy7. Progenitor populations were stained with SCA-1-PerCpCy5.5, CD16/32-PE/Cy7, cKit-APC, streptavidin-APC/Cy7. Cells were fixed and permeabilized using Cytofix/Cytoperm (BD biosciences). Cells were stained with H2AX antibody for 30 minutes at RT. For the secondary staining, anti-mouse IgG-AF488 was used. DAPI (10 μg/ml) was used for chromatin labeling. A non-specific IgG isotype control was used as negative control. For the analysis FlowJo (version 10.0.8r1) was used.

Spleen/Thymus
According to (19).

In vivo EdU incorporation assay
200 μl 10mM EdU in PBS was injected intraperitoneally. After 16 hours BM was isolated as described in hematopoietic precursor subset analysis. Click-iT Plus EdU Flow Cytometry kit
AF488 (ThermoFisher) protocol was followed. BM was stained using biotinylated Lineage+ antibody mix, cKit-APC, Sca-1-PerCp/Cy5.5, CD135-PE, CD150-PE/Cy7, streptavidin APC/Cy7, followed by Alexa Fluor488 picolyl azide staining. A non-EdU treated mouse was used as negative control. For the analysis FlowJo (version 10.0.8r1) was used.

**Competitive BM reconstitution**

BM was isolated from 3 Ly5.2 WT and 3 Ly5.2 PCNA<sup>K<sup>164R/K164R</sup></sup> 2-month-old mice and mixed 1:1 with the BM isolated from 1 Ly5.1 WT mouse. 1*10<sup>6</sup> Ly5.1/Ly5.2 mixed BM cells were transplanted into lethally irradiated (2x 5.5 Gray, with 3h interval) Ly5.1 recipient mice. Blood (50 μl) was taken after 2, 4, 8, 12, 16, and 20 weeks. Using Ly5.1-PE and Ly5.2-PE/Cy7 antibodies we measured the contribution in the blood of Ly5.1 and Ly5.2 cells. Cells were also stained with Gr1-APC/Cy7, CD3-FITC, CD19-APC, and CD11b-PerCp/Cy5.5 for subset analysis. DAPI was used for life/death staining. Twenty weeks post transplantation, the mice were euthanized and the BM was isolated for analysis, as described earlier. With the use of SCA-1-PerCp/Cy5.5, cKit-APC, streptavidin APC/Cy7, Ly5.1-PE and Ly5.2-PE-Cy7. Irradiated mice were treated with Enrobaclin, for the first 4 weeks after irradiation. For the analysis FlowJo (version 10.0.8r1) was used.

**Non-competitive BM transplantation**

The BM of Ly5.2 WT and Pcna<sup>K164R/K164R</sup> was isolated. Recipient Ly5.1 mice were lethally irradiated (2x 5.5 Gray, with a 3h interval) and transplanted with 1*10<sup>6</sup> WT or Pcna<sup>K164R/K164R</sup> BM cells. Two and four weeks post transplantation BM and blood was isolated in order to assess contribution of the transplanted BM, as described in the competitive BM reconstitution. For the analysis FlowJo (version 10.0.8r1) was used.

**In vivo CsPt sensitivity assay**

Mice were injected intravenously with 0.8 mg/kg Cisplatin (Accord) or PBS. After 2 days, the BM was isolated and analyzed as described in Hematopoietic precursor subset analysis.

**Calculations and statistics**

We determined the number of viable cells per subset by DAPI or PI staining and based on the ratio of subset of interest and viable population times the number of cells per femur. We standardized the number of cells per femur to 20*10<sup>6</sup> when no difference in total BM numbers was found between WT and Pcna<sup>K164R/K164R</sup>. T tests were performed using PRISM 6 (version 6.0), *: p value > 0.05, **: p value > 0.01, ***: p value > 0.001, ****: p value > 0.0001.
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Authorship Contributions

HJ, EC, OAB, PvdB, and BP designed experiments. PvdB, OAB, BP, and CL performed the experiments. BP and PvdB analyzed the data. BP and HJ wrote the manuscript. OAB, PvdB, CL, and EC commented on the manuscript.

Conflict of Interest Disclosures

Authors do not have any conflict of interest.

References

Chapter 7


Supplemental Information
Figure S1. WT and *Pcna*\(^{K164R/K164R}\) hematopoiesis
(A) Gating strategy of BM subsets of WT BM cells.
(B) B and T cell subsets in the spleen
(C) Thymocyte development in WT and *Pcna*\(^{K164R/K164R}\) mice
(D) B cell development in the BM of in WT and *Pcna*\(^{K164R/K164R}\) mice.
Figure S2. Percentage of γH2AX positive cells per subset

A B
C D
E F

WT PcnaK164R/K164R

G1 S/G2
0 20 40 60 80 100
% γH2AX positive cells per indicated cell cycle status

LSK MPP4 HSPC LKS-
CMP GMP

Figure S2. Percentage of γH2AX positive cells per subset
Figure S3. Gating strategy for γH2AX positive cells
Figure S4. Gating strategy for cell cycle
Figure S5. Gating strategy for EdU incorporation assay
Figure S6. CsPt sensitivity in nucleated cells per femur
(A) Representative FACS plots of BM populations after 2 days of CsPt treatment in WT and \( Pcna^{K164R/K164R} \).
(B) Representative image of H&E staining of sternum WT and \( Pcna^{K164R/K164R} \) injected with PBS or CsPt. The scale bar indicates 500 µm. The original magnification was 2.5 times.
Figure S7. Gating strategy for BM transplantation assays
Figure S8. Short term non-competitive BM transplantation

(A-F) Number of nucleated cells per indicated subset 2 and 4 weeks after transplantation of Ly5.2 BM of WT or PcnaK164R/K164R into irradiated Ly5.1 recipient mice. 1 out of 2 representative experiment is shown.
Figure S9. Hematopoietic precursor subsets in aged mice
(A) Fold changes ($\text{Pcn}_{\text{K164R/K164R}}$ / WT) comparing young adult 2-month-old and 9-10 month aged mice.
### Table S1. List of antibodies used in this study

<table>
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<tr>
<th>Antigen-conjugate(s)</th>
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<th>Dilution</th>
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<td>Lineage Cell Detection Cocktail-Biotin, mouse</td>
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