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Genomic and functional integrity of the hematopoietic system requires tolerance of oxidative DNA lesions

Short title: **DNA damage tolerance in the hematopoietic system.**

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Key points

- Tolerance of oxidative DNA lesions ensures the genomic and functional integrity of hematopoietic stem and precursor cells.
- Endogenous DNA damage-induced replication stress is associated with mitochondrial dysfunction.

Abstract

Endogenous DNA damage is causally associated with the functional decline and transformation of stem cells that characterize ageing. DNA lesions that have escaped DNA repair can induce replication stress and genomic breaks that induce senescence and apoptosis. It is not clear how stem and proliferating cells cope with accumulating endogenous DNA lesions, and how these ultimately affect the physiology of cells and tissues. Here we have addressed these questions by investigating the hematopoietic system of mice deficient for *Rev1*, a core factor in DNA translesion synthesis (TLS), the post-replicative bypass of damaged nucleotides. *Rev1* hematopoietic stem and progenitor cells displayed compromised proliferation, and replication stress that could be rescued with an antioxidant. The additional disruption of *Xpc*, essential for global-genome nucleotide excision repair (ggNER) of helix-distorting nucleotide lesions, resulted in the perinatal loss of hematopoietic stem cells, progressive loss of bone marrow, and fatal aplastic anemia between 3 and 4 months of age. This was associated with replication stress, genomic breaks, DNA damage signaling, senescence and apoptosis in bone marrow. Surprisingly, the collapse of the *Rev1Xpc* bone marrow was associated with progressive mitochondrial dysfunction and consequent exacerbation of oxidative stress. These data reveal that, to protect its genomic and functional integrity, the hematopoietic system critically depends on the combined activities of repair and replication of helix-distorting oxidative nucleotide lesions by ggNER and Rev1-dependent TLS, respectively. The error-prone nature of TLS may provide mechanistic understanding of the accumulation of mutations in the hematopoietic system upon ageing.

Introduction

The ageing-associated attrition of proliferating tissues is accompanied by mutagenesis and genomic rearrangements, cellular senescence, and mitochondrial dysfunction, possibly in response to the accumulation of endogenous DNA damage [1]. Each cell in the body acquires 10^4-10^5 endogenous DNA lesions per day [2]. Most DNA lesions are repaired by a network of complementary DNA repair systems, each of which deals with a specific class of lesions, as an integral part of the DNA damage response [reviewed in [3-5]]. The dominant, global-genome nucleotide excision repair (ggNER, Figure 1A) pathway specifically recognizes and removes helix-distorting endogenous and exogenous nucleotide lesions [6]. ggNER deficiency, as exemplified by mice with a disruption of the *Xpc* gene, only causes minor phenotypes when the organism is not exposed to exogenous genotoxic agents. This result suggests that unrepaired endogenous helix-distorting DNA lesions can be tolerated at the genome of proliferating cells [6,7]. Unrepaired nucleotide lesions usually arrest processive replication forks, resulting in lesion-containing single-stranded (ss) DNA tracks. Persistent ssDNA tracks can collapse to cytotoxic and recombinogenic double-strand DNA (dsDNA) breaks. To fill such lesion-containing ssDNA tracts and to enable termination of genomic replication, cells have evolved multiple mechanisms, collectively called DNA damage tolerance [8]. Thereby, these mechanisms prevent genomic instability, senescence and apoptosis caused by unreplicated damaged nucleotides. DNA translesion synthesis (TLS) is the major DNA damage tolerance mechanism in mammals. TLS employs specialized DNA polymerases to directly replicate across damaged nucleotides. Since these TLS polymerases frequently misincorporate opposite the lesion, cellular survival by TLS comes at the expense of nucleotide substitution mutagenesis (Figure 1A) [9]. The core TLS polymerase Rev1 inserts cytidines opposite abasic nucleotides and a limited spectrum of nucleotide adducts at the minor groove of the DNA helix [10,11]. Additionally, Rev1 plays an important regulatory role in TLS of helix-distorting nucleotide lesions, of (non-damaged) G-quadruplex structures [12], whereas it also operates in the repair of interstrand DNA crosslinks [9,13].

The long-lived nature of hematopoietic stem cells (HSCs) makes these cells particularly susceptible to endogenous and exogenous genotoxic insults that can limit their functional capacity and that also induce genomic alterations that predispose to hematopoietic malignancies [14]. Therefore, the hematopoietic system provides a paradigm to study the involvement of endogenous DNA damage in development, maintenance, decay and cancer development of proliferating and differentiating tissues [14-18].

Here we investigated the role of Rev1-dependent TLS in the development and maintenance of the hematopoietic system. We reveal the requirement of TLS opposite unrepaired endogenous helix-distorting DNA lesions for the maintenance of HSPCs. We furthermore demonstrate that ggNER and, to a greater extent, TLS provide independent and complementary mechanisms to protect the hematopoietic system against the detrimental phenotypes caused by endogenous DNA lesions.

Methods in brief

Full methods are included in the Supplemental Information.

Mice and cell lines.

All mouse experiments were approved by the ethical review board of the Institute and the, specific pathogen-free, mice were kept according to FELASA guidelines. Wild type, *Rev1*, *Xpc* and *Rev1Xpc* mouse cohorts were obtained by crossing FVB and C57Bl/6 parents. Equal numbers of hybrid males and females were used for most experiments. Transplantation experiments using *Rev1* animals were performed in the C57Bl/6 background. Hairless albino SKH-1 mice were used for measuring sensitivity of the skin to ultraviolet light-induced nucleotide lesions.

Competitive transplantation experiments using Rev1 hematopoietic cells were performed as follows: whole bone marrow or isolated HSCs (alone or, when indicated, together with W41.SJL (c-kit receptor-mutant bone marrow cells), from donors were introduced into lethally irradiated B6.SJL. Secondary transplantation was performed as previously described [20]. Chimaerism of the hematopoietic system was analyzed at different time points after transplantation using multiplex PCR on blood. Reconstitution of the *Rev1Xpc* bone marrow with *Xpc* bone marrow was performed at the age of 1.5 months, by injecting $5x10^6$ *Xpc* bone marrow cells. Unless stated otherwise mice were given an intra-peritoneal injection with Bromodeoxyuridine (BrdU) and ethynyl-2'-deoxyuridine (EdU), one hour before killing with CO_2, to label replicating cells. MEF lines were obtained by spontaneous immortalization of fibroblasts from 13.5-day embryos of the hybrid background. Survival after genotoxin exposure was measured using clonogenic assays.

Whole blood analysis

For whole blood analysis, peripheral blood was manually and automatically counted. For the quantification of white blood cell ratios and Howell-Jolly bodies, blood smears were stained with Giemsa.

Bone marrow and blood preparation for stainings

Bone marrow cells were extracted by flushing femurs and tibia. One intact femur was used to calculate bone marrow cellularities. Cell numbers were normalized to body weight. Paraffin-embedded sections were stained with Hematoxylin and Eosin (HE). Whole blood was extracted from heart. Erythrocytes were lysed before characterization of hematopoietic and blood cells.

Analysis of cultured HSCs

Long-term-HSCs were isolated and cultured for two weeks with or without 100µM N-acetylcysteine. Colony sizes were scored at day 7 and day 14. At day 14, cells were collected for immunofluorescence staining of the DNA damage marker γH2AX.

Cobblestone Area-Forming Cell (CAFC) assay
The CAFC assay assesses the clonogenicity and size *in* vitro of different HSPC populations and was performed as previously described [21,22].

Analysis of HSPC populations
HSPC populations were isolated by fluorescence activated cell sorting (FACS), following labeling of HSPC population-specific surface markers with antibodies, and labeling these with fluorophores. Concentrations and origin of these reagents are depicted in Supplemental Table 1. To identify stromal cells, bone marrow cell suspensions were stained with the stromal cell-specific antibodies followed with fluorophore labeling and FACS. Fetal livers were analyzed after BrdU injection of the mother. Fetal liver cell suspensions were stained with cell surface markers for HSPCs (see above), followed by BrdU staining. For the analysis of proliferation and apoptosis, freshly isolated fetal liver cells were stained with HSPC cell surface markers and then for Ki67 or Annexin V, respectively. 7-Aminoactinomycin D (7-AAD) was added in the cell suspension to stain DNA prior to analysis to exclude dead cells. Data were acquired using flow cytometry.

Immunohistochemistry and immunofluorescence
Whole bone marrow cells were fixated on cytospin adhesion slides and stained for BrdU, Caspase-3, Dec1, γ-H2AX, Ki67, p16, 8OHdG, phospho-p38 or 53BP1. Staining for incorporated EdU was performed according to the manufacturers' instructions. Staining of bone marrow for 4-hydroxynonenal (4-HNE) was performed on deparaffinized sections after antigen retrieval. Sections were counterstained with Mayers hemalum.

Alkaline comet assays
Alkaline single cell electrophoresis ('Comet') assays that enable to detect ssDNA and dsDNA breaks at the genome, and staining for incorporated BrdU to identify S phase nuclei, were performed on bone marrow cell suspensions, essentially as described [23].

TLS assay
The generation of a site-specific single-stranded H-εdC lesion and the determination of the efficiency and mutagenicity of TLS were performed essentially as described for a benzo[a]pyrene-dG adduct [24].

Analysis of mitochondrial function
Mitochondrial membrane potentials were measured by flow cytometry. Measurement of mtDNA was performed by quantitative PCR. The relative mtDNA levels were calculated using formula $2\times2^{\Delta CT}$. Mitochondrial respiration of freshly isolated total bone marrow cells was analyzed using a Seahorse extracellular flux bioanalyzer. For Western blotting of mitochondrial proteins, freshly frozen bone marrow cells were re-suspended in RIPA buffer followed by brief sonication. Electrophoresis, blotting, antibody incubations and visualization of signals using enhanced chemiluminescence was performed using standard protocols.

Results

Rev1 contributes to HSPC maintenance.
Rev1-deficient mice displayed mild cytopenia of the blood, affecting all lineages (Figure 1B, 1C). Analysis of short- and long-term hematopoietic stem cell (HSC) populations revealed a slight, but significant, decrease in the frequency of early hematopoietic progenitors (LSK cells), already at a young age (Figure 1D). Competitive repopulation experiments revealed that long-term *Rev1* HSCs (the LSK-SLAM population) were unable to compete with simultaneously administered W41.SJL

HSCs [20] [that themselves display compromised repopulation capacity] in reconstituting the bone marrow of lethally irradiated wild type mice (Figure 1E). Even in the absence of W41.SJL competitors, *Rev1* HSCs repopulated the recipients' hematopoietic system only inefficiently (Supplemental Figure 1A). This phenotype was further aggravated in serial transplantations, indicating a persistent disadvantage of *Rev1* HSCs (Supplemental Figure 1B). HSCs isolated from 14-day-old fetal livers already displayed reduced repopulation capacity (Supplemental Figure 1C), demonstrating that the attenuation of HSC function occurs early in development and independently of the bone marrow environment. The *in vitro* clonogenicity of *Rev1* HSPCs was significantly reduced, compared with wild type (Figure 1F), and *Rev1* HSC clones were smaller than controls (Figure 1G). The combined defects in transplantability and in *in vitro* growth strongly suggest that *Rev1*-deficient HSPCs display compromised proliferative capacity, which most likely is cell-intrinsic.

ggNER and TLS synergize to protect the hematopoietic system from the genotoxicity of helix-distorting DNA lesions.

We argued that, in case the proliferative defects of *Rev1* HSPCs reflect perturbed TLS of endogenous helix-distorting nucleotide lesions, inactivation of ggNER of this class of nucleotide lesions would exacerbate the hematopoietic phenotypes (Figure 1A). Indeed, disruption of *Xpc* synergistically increased the sensitivity of both *Rev1*-deficient skin and cultured embryonic fibroblasts to helix-distorting photolesions induced by UV light [6] (Supplemental Figures 2A, 2B). Previously we have shown that, upon UV exposure, *Rev1Xpc* fibroblasts display high levels of replication stress, caused by replicons arrested at unrepaired helix-distorting photolesions [25]. Thus, ggNER and Rev1-dependent TLS jointly protect cells from the genotoxic effects of UV light by repairing or tolerating photolesions, respectively (see Figure 1A). *Rev1Xpc* embryos were significantly smaller than wild type or single-deficient littermates, and the double-deficient mice were born at sub-Mendelian ratios (Supplemental Figures 2C, 2D), consistent with enhanced sensitivity to endogenous helix-distorting nucleotide lesions. While *Xpc* mice displayed near-wild type lifespans, *Rev1* single-deficient mice died slightly earlier than their wild type littermates (20 *versus* 23 months of age, on the average; Figure 2A) of various causes unrelated to the hematopoietic defects. This shortened life span suggests that ggNER is not sufficient to repair all of its substrates, making proliferating cells dependent on Rev1-mediated TLS. In contrast, TLS+ggNER double-deficient, *Rev1Xpc* mice died at, on the average, 3.5 months of age (Figure 2A) displaying aplasia of the bone marrow and pancytopenia of bone marrow and blood that most markedly affected neutrophils (Figure 2B, 2C, 2D, Supplemental Table 2, Supplemental Figure 2E-2H). HSC counts were strongly reduced already in the liver of *Rev1Xpc* fetuses, and this reduction aggravated through life (Figure 2E, Supplemental Figure 2I). In conclusion, the hematopoietic phenotypes of *Rev1* deficient mice are exacerbated synergistically by concomitant ggNER deficiency, which provides a strong argument that failure to replicate endogenous helix-distorting nucleotide lesions results in hematopoietic attrition. We then investigated the fate of *Rev1Xpc* embryonic liver HSCs. All *Rev1Xpc* HSC subpopulations, and also long-term progenitor cells, were characterized by a reduced G0 and increased Ki67-positive S/G2/M fraction, suggesting exit from quiescence and enhanced proliferation or delay in progression through S phase (Figure 2F, Supplemental Figure 2J). In support with the latter, the fraction of *Rev1Xpc* LSK cells and short-term HSC that resided in S phase was increased in *Rev1Xpc* embryonic liver HSC (Figure 2G, Supplemental Figure 2K). The concurrent emergence of a BrdU-positive sub-G0 LKS fraction suggested increased death of proliferating *Rev1Xpc* HSC (Figure 2G, Supplemental Figure 2K) although we could not detect increased apoptosis (Supplemental Figure 2L).

To confirm aplastic anemia as the cause of death of *Rev1Xpc* mice we transplanted them with *Xpc* bone marrow cells. This procedure indeed significantly rescued the degenerative hematopoietic phenotypes of these mice and greatly increased their life span (Figure 2H, Supplemental Figure 3A). Importantly these results also suggest that the hematopoietic phenotypes of *Rev1Xpc* mice are cell-autonomous. Consistently, the composition of the *Rev1Xpc* bone marrow stromal compartment appeared to be affected only marginally (Supplemental Figures 3B, 3C).

Rev1-dependent TLS protects the bone marrow against replication stress, senescence, apoptosis and DNA breaks.

We wanted to investigate whether Rev1-mediated TLS of endogenous nucleotide lesions protects the genome of hematopoietic cells against DNA breaks. Indeed, whereas erythrocytes normally are devoid of nuclear DNA, blood-derived erythrocytes of *Rev1* and *Rev1Xpc* mice frequently contained chromosomal fragments that presumably were derived from chromosomes broken during the preceding erythroblast stage [so-called Howell-Jolly bodies [26]], (Figure 3A). This phenotype was absent from the *Xpc*-reconstituted *Rev1Xpc* bone marrow, confirming that the DNA breakage was cell-intrinsic (Supplemental Figure 4A). To assess single- and double-strand DNA breaks also in bone marrow we used alkaline single-cell electrophoresis ('comet') assays [23]. Compared with wild type HSPCs, *Xpc*, *Rev1* and *Rev1Xpc* HSPCs displayed increased comet sizes, and thus increased DNA breaks, during S phase, suggesting arrested replicons, or dsDNA breaks, at endogenous helix-distorting nucleotide lesions (Supplemental Figure 4B). However, only in the absence of Rev1 these breaks persisted beyond S phase (Figure 3B, Supplemental Figure 4C), indicating that the strand discontinuities were protracted due to the persistent inability to complete genomic replication. In agreement, staining for the DNA breaks and replication stress markers γH2AX and 53BP1 [27,28] was increased in bone marrow cells, not only of 3-months old *Rev1Xpc* mice but also in old *Rev1* mice (Figure 3C, 3D, Supplemental Figure 4D, 4E). Beyond 3 months of age, proliferation and replication in *Rev1Xpc* bone marrow ceased (Figure 4A, 4B, Supplemental Figure 5A, 5B), concomitant with the induction of senescence and apoptosis (Figures 4C-4E, Supplemental Figure 5C-5E). Collectively, these data indicate that, in the absence of Rev1, endogenous helix-distorting DNA lesions induce replication stress and genomic breaks that compromise proliferation and viability of HSPCs.

Rev1-dependent TLS provides tolerance of endogenous lipid peroxidation-derived nucleotide adducts.

Oxidative DNA lesions are abundant in proliferating cells [15-18]. Moreover, the notion that the *Rev1Xpc* phenotypes are cell-autonomous, combined with the specific depletion of neutrophils (see above) that produce high levels of reactive oxygen species (ROS) [29], hinted at helix-distorting oxidative DNA lesions as possible culprits for the *Rev1* and *Rev1Xpc* HSPC phenotypes. The scarcity of long-term *Rev1Xpc* HSCs precluded their analysis, and therefore we investigated responses to endogenous oxidative stress in cultured *Rev1* single-deficient HSCs. Consistent with the results described above, these cells displayed enhanced γH2AX staining indicating replication stress (Figure 5A, Supplemental Figure 6A). However, culture in the presence of the reactive oxygen species (ROS) scavenger N-acetylcysteine (NAC) [30] rescued this γH2AX accumulation (Figure 5A, Supplemental Figure 6A, 6B). This important result confirms that the endogenous ROS induce replication stress in HSC, in the absence of *Rev1*-dependent TLS.

Helix-distorting oxidative nucleotide lesions comprise cyclic purines and long-chain hydroxyalkenal (aldehyde) adducts, and these lesions are derived from lipid peroxidation. Notably, these lesions have been associated with human ageing [31,32] and they represent endogenous substrates for ggNER [33,34]. To investigate the involvement of Rev1 in tolerance of 4-Oxo-2(E)-nonenal (4-ONE)-deoxycytidine, a prototypic helix-distorting hydroxyalkenal-nucleotide adduct (Figure 5B), we performed a quantitative *in cellulo* TLS assay [32]. Indeed, mutagenic TLS at the 4-ONE-cytidine adduct largely depended on Rev1 in mouse embryonic fibroblasts (MEFs, Figure 5C). Consistently, *Rev1* or *Xpc* and to a greater extent, *Rev1Xpc* MEFs were hypersensitive to Paraquat that induces oxidative stress by poisoning mitochondria (Figure 5D) whereas *Rev1Xpc* MEFs also were hypersensitive to 4-Hydroxy-2(E)-nonenal (4-HNE), a compound closely related to 4-ONE (Figure 5E). Taken together, these data strongly suggest that a defect in Rev1-dependent TLS of persistent helix-distorting oxidative DNA lesions at the nuclear genome is responsible for the degenerative hematopoietic phenotypes of *Rev1* and *Rev1Xpc* mice.

Although *a priori* one would not expect overall cellular ROS levels to be increased in the absence of ggNER and TLS, we observed significant accumulation of the oxidative stress and ageing marker Lipofuscin [35] in bone marrow of *Rev1Xpc* mice (Figure 5F). Also intracellular levels of 4-HNE, as well as expression of the oxidative stress response marker phospho-p38 [36] were increased in *Rev1Xpc*, compared with *Xpc*, bone marrow cells (Figure 5G, 5H, Supplemental Figure 6C). Consequently, also levels of oxidative DNA lesions at the genome were increased in *Rev1* and, to a greater extent, *in Rev1Xpc*, bone marrow, as demonstrated by staining for genomic 8-hydroxy-2'-deoxyguanosine (8OHdG; Figure 5I). Since 8OHdG is no substrate for ggNER this result emphasizes that a *de novo* source of oxidative stress only indirectly is caused by the ggNER and TLS deficiency.

Progressive mitochondrial dysfunction and oxidative stress in *Rev1Xpc* HSPCs.
The progressive increase of ROS levels in *Rev1Xpc* bone marrow suggested the emergence of a *de novo* source of oxidative stress in response to replication stress at helix-distorting nucleotide lesions. To investigate the origin of this phenomenon we focused our attention to the mitochondrial compartment as the dominant source of intracellular ROS. Since mitochondrial proliferation is an early cellular stress cellular response [37] we first quantified mitochondrial DNA and protein in bone marrow. Indeed, in bone marrow of 3-months old *Rev1Xpc* mice these amounts were significantly increased (Figures 5J, 5K, Supplemental Figure 6D). This suggests that replication stress at endogenous nucleotide lesions may lead to mitochondrial proliferation.

The mitochondrial uncoupling protein UCP2, that is induced by 4-HNE [38] and also the expression of the transcriptional coactivator PGC-1 α, a key controller of mitochondrial biogenesis [39,40], participate in the mitochondrial response to oxidative stress [38,40,41]. In bone marrow of *Rev1Xpc* mice, the expression of both mitochondrial proteins was strongly increased, consistent with the presence of chronic oxidative stress signaling (Figure 5L).

We then investigated mitochondrial function in bone marrow of all four genotypes. Compared with *Xpc* bone marrow, the mitochondrial membrane potential was attenuated in *Rev1Xpc* bone marrow, already at the age of 1 month (Figure 5M). Also in *Rev1* single-deficient mice the mitochondrial membrane potential appeared to be reduced slightly, although this failed to reach significance (Supplemental Figure 6G). In live bone marrow cells of 3 months old *Rev1Xpc* mice the basal, ATP-dependent and reserve respiratory capacities in viable cells had virtually vanished (Figure 5N; Supplemental Figures 6E, 6F); although cell counts were reduced in *Rev1Xpc* bone marrow, the replication and proliferation viable cells was not significantly different between the genotypes (Figures 4A, 4B, 4E, Supplemental Figure 6G). This suggests that the mitochondrial proliferation and concomitant dysfunction may be a corollary of nuclear replication stress at the nuclear genome, originating the exacerbated ROS production in proliferating *Rev1Xpc* bone marrow cells.

Discussion

The attrition of tissues during ageing is associated with the accumulation of oxidative and other endogenous DNA lesions at the nuclear genome of long-term stem and precursor cells [5,17,18]. However, the exact character of these lesions, their biological impact, the mechanistic basis of their cytotoxicity, and the pathways involved in pleiotropic responses to these damages have largely remained unexplored [3]. The effect of ROS on the genomic integrity of long-term HSCs is restrained by a hypoxic environment (the stem cell niche) and by a metabolically quiescent state, employing glycolysis rather than oxidative respiration [36,42,43]. This suggests that the exposure of HSCs to ROS negatively affects their function. Indeed, ageing HSC display DNA damage responses although controversy exists about the nature of the underlying damage, and whether the damage is induced during dormancy [44] or during proliferation [45]. Recently, the ageing-associated decay of HSCs has been attributed to replication stress resulting from the decreasing expression of the minichromosome maintenance helicase [46,47]. Here we use *Rev1* mice to demonstrate that replication stress at helix-distorting oxidative DNA lesions at the nuclear genome is associated with the functional and genomic attrition of the hematopoietic system (Figures 1, 3, 5, Supplemental Figure 4). The hematopoietic phenotypes of *Rev1* mice are synergistically aggravated when, additionally, ggNER is compromised (resulting from the disruption of *Xpc*; Figures 2-4, Supplemental Figures 2-5). These data reveal that ggNER and TLS jointly preserve the genomic and functional integrity of the hematopoietic system by, respectively, repairing endogenous helix-distorting DNA lesions and suppressing replication stress at these lesions (Figure 6). The observation that loss of HSCs occurred during ontogeny, and independent of tissue context (both in the prenatal liver and in postnatal bone), further confirms that these phenotypes are cell autonomous.

Mitochondrial dysfunction causes attrition of the hematopoietic system [48,49], and a genomic DNA damage-dependent communication between the nucleus and mitochondria, leading to mitochondrial attrition, has been associated with ageing-related pathologies [37,51,53]. Although Rev1 is not found in mitochondria [50], viable *Rev1Xpc* and, to some extent, also *Rev1* bone marrow cells develop mitochondrial dysfunction suggesting that nuclear replication stress affects mitochondrial activity. In *Rev1* liver and cultured fibroblasts, mitochondrial dysfunction is associated with elevated activity of poly(ADP) ribose polymerase 1 (PARP1), possibly at ssDNA breaks. This presumably results in depletion of the PARP1 substrate and essential mitochondrial cofactor, NAD^+ (Borhan Fakouri et al., under revision). A similar mechanism causes mitochondrial dysfunction in cells with a defect in the minor, transcription-coupled, NER subpathway [52]. We hypothesize that the increase in genomic replication stress, caused by ROS production by dysfunctional mitochondria accelerates the collapse of the *Rev1Xpc* hematopoietic system (Figure 6F). Nevertheless, direct evidence for this hypothesis is lacking and we cannot formally exclude that the mitochondrial dysfunction reflects, rather than originates, the decay of the *Rev1Xpc* bone marrow.

The decay of the hematopoietic system of *Rev1* and, to a greater extent, *Rev1Xpc* mice may represent dramatically accelerated hematopoietic ageing. The hematopoietic phenotypes of *Rev1* single-deficient mice, and the finding that *Xpc* or *Rev1* single-deficient cells already display moderate sensitivity to UV light (Supplemental Figure 2B), emphasize that ggNER and TLS are unable to either repair or bypass, respectively, all helix-distorting nucleotide lesions. We therefore hypothesize that the phenotypes of *Rev1* and *Rev1Xpc* bone marrow represent exacerbated phenotypes that contribute to the physiological functional attrition of HSC in the ageing bone marrow [1,15]. Also small-chain endogenous aldehydes have been identified as a threat to the integrity of the hematopoietic system [54,55]. Therefore, multiple types of unreplicated endogenous DNA damage in parallel may participate in the ageing-associated functional decline of the hematopoietic system. Nucleotide substitutions and inefficient DNA repair are strongly correlated with ageing-associated hematopoietic and other malignancies [6,16,56-60]. Since Rev1-mediated TLS of damaged nucleotides, including lipid peroxidation-adducted nucleotides, is highly mutagenic (Figure 5B, [9]) we hypothesize that the accumulation of such mutations is the price to pay for the protection of the hematopoietic system against endogenous helix-distorting oxidative nucleotide lesions by error-prone TLS. Finally, future investigations may also address the question whether Rev1-mediated TLS is preserved, and perhaps provides a mechanism of survival, in leukemic cells and therefore represents a potential therapeutic target.

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Figure legends

Figure 1: *Rev1* HSC display competitive and proliferative defects (see also Supplemental Figure 1).

The involvement of TLS in tolerance of endogenous DNA damage in the hematopoietic system was investigated by analyzing *Rev1* blood and bone marrow, by competitive repopulation experiments and by culture of HSPCs *in vitro*.

*p < 0.05. **p <0.01. ***p <0.001. ****p <0.0001. Data are mean ± S.E.M.

A. Helix-distorting nucleotide lesions (blue spheres) can be repaired by global-genome nucleotide excision repair (ggNER), dependent on the *Xpc* gene. In case a lesion escapes timely repair, it arrests processive replication (black rectangle), resulting in replication stress and DNA damage signalling. The lesion can be bypassed post-replicatively by *Rev1*-dependent DNA translesion synthesis (TLS, zig-zag line). Thereby, TLS prevents the induction of replication stress and double-strand (ds) DNA breaks. TLS frequently misincorporates (in red) opposite the damaged nucleotide, which originates nucleotide substitution mutations.
B. Cytopenia in 26-30 months-old *Rev1* (n=11), compared with age-matched wild type (WT), mice (n=6).
C. Relative contribution of myeloid and lymphoid cells in the wild type (WT) and *Rev1* blood at 3 months of age (3m) and when moribund (MB). N=10.Note the low contribution of neutrophils in *Rev1Xpc* blood.
D. Frequencies of LSK, LSK34- and LSK-SLAM cells in bone marrow of 5 months old *Rev1* (n=5) and WT mice (n=5). Frequencies are depicted as % of mononuclear cells.
E. Impaired function of *Rev1*-deficient HSCs as demonstrated by competitive repopulation assays. Top: Scheme of competitive transplantation experiments. Bottom: Competitive transplantation of WT (n=9) and *Rev1* HSC (n=8). (See also Supplemental Figure 1).
F. Impaired proliferative capacity of HSPC as demonstrated by reduced cobblestone area forming cell (CAFC) numbers from 5 months old WT (n=4) and *Rev1* mice (n=4).
G. Sizes of colonies after single-cell sorting of LSK-SLAM cells from 5 months old *Rev1* (n=3) and WT mice (n=3). BM: bone marrow.

Figure 2. Rev1-dependent tolerance of unrepaired endogenous nucleotide lesions protects HSCs (see also Supplemental Figures 2 and 3).

Analysis of the hematopoietic system of wild-type, ggNER (*Xpc*), TLS (*Rev1*) and TLS+ggNER (*Rev1Xpc*) mice reveals that ggNER and TLS jointly protect HSPCs against cell-autonomous genotoxicity of endogenous helix-distorting DNA lesions.

*p < 0.05. **p <0.01. ***p <0.001. ****p <0.0001. Data are mean ± S.E.M.

A. Kaplan-Meier curves depicting survival of mice of all four genotypes: WT (n=64), *Xpc* (n=10), *Rev1* (n=53), *Rev1Xpc* (n=41). Survival of the different genotypes was compared with wild type mice using the Wilcoxon test.
B. Progressive bone marrow aplasia in *Rev1Xpc* mice. Bar size: 50 μm) *Xpc*: 1 m (n=3), 1.5 m (n=3), 3 m (n=3), MB (n=6). *Rev1Xpc*: 1 m (n=3), 1.5 m (n=3), 3 m (n=3), MB (n=3). Right panels: Quantification of bone marrow cells at 1 (*Xpc* n=10, *Rev1Xpc* n=11) and 1.5 months (*Xpc* n=4, *Rev1Xpc* n=4) of age, respectively.
C. *Rev1Xpc* mice develop severe cytopenia. M: months MB: moribund (see panel C for survival data). WT: 1.5 m (n=8), 3 m (n=7), MB (n=12). *Xpc*: 1.5 m (n=5), 3 m (n=12), MB (n=7). *Rev1*: 1.5 m (n=6), 3 m (n=17), MB (n=33). *Rev1Xpc*: 1.5 m, (n=10), 3 m (n=11), MB (n=10).
D. Relative contribution of myeloid and lymphoid cells in the blood of all genotypes. Note the low contribution of neutrophils specifically in *Rev1Xpc* blood.

E. Bone marrow aplasia in *Rev1Xpc* mice is caused by progressive loss of long-term HSCs (LSK-SLAM cells). MNCs: mononuclear cells. Number of mice analysed: foetal liver: n=4 for all genotypes. 2 weeks old: *Xpc* (n=3), *Rev1Xpc* (n=3). 1.5 months old: *Xpc* (n=4), *Rev1Xpc* (n=4).

F. Increased S/G2/M fractions in *Rev1Xpc* LSK cells, long-term and short term HSC from fetal liver, suggesting increased replication, as shown by Ki67 staining (G0 cells are Ki67-negative). N=4 for all genotypes

G. Increased proliferation of *Rev1Xpc* HSPC as demonstrated by in vivo Brdu labeling. *Xpc* (n=3) and *Rev1Xpc* (n=3) mice.

H Rescue of early death, bone marrow aplasia and cytopenia of *Rev1Xpc* mice by transplantation with *Xpc* bone marrow, indicating a hematopoietic cell-intrinsic origin of HSC exhaustion. Survival: *Rev1Xpc* (n=41), transplanted *Rev1Xpc* (n=10); Bone marrow: *Rev1Xpc* (n=3), transplanted *Rev1Xpc* (n=5); Blood cellularity: *Rev1Xpc* (n=10), transplanted *Rev1Xpc* (n=7). Survival of *Rev1Xpc* mice was compared with wild type mice using the Wilcoxon test.

Figure 3. Rev1 protects against replication stress and genomic breaks in the hematopoietic system (see also Supplemental Figure 4).
We investigated the induction of DNA breaks in the absence of Rev1-mediated TLS at endogenous helix-distorting DNA lesions in blood and bone marrow.

*p < 0.05. **p <0.01. ***p <0.001. ****p <0.0001. Data are mean ± S.E.M.

A. Chromosome fragments (Howell-Jolly bodies) in erythrocytes of 3 months old *Rev1* and *Rev1Xpc* (arrowheads) mice. Bar size: 10 µm. Right panel: quantification. WT: 3 m (n=5), MB (n=6). *Xpc*: 3 m (n=5), MB (n=6). *Rev1*: 3 m (n=5), MB (n=9). *Rev1Xpc*: 3 m (n=6), MB (n=8).

B. Chromosome breaks outside of S phase, measured by single-cell alkaline Comet gel electrophoresis of bone marrow of 3 months old mice. WT (n=4), *Xpc* (n=4), *Rev1* (n=4), *Rev1Xpc* (n=4). Tail intensities of Bromodeoxyuridine-negative cells are shown.

C, D. Increased DNA breaks in bone marrow hematopoietic cells of *Rev1* and *Rev1Xpc* mice as demonstrated by γH2AX (C) and 53BP1 (D) immunostaining. The fraction of positive cells shown was normalized relative to 3-months old WT. WT: 3 m (n=8), MB (n=6). *Xpc*: 3 m (n=7), MB (n=6). *Rev1*: 3 m (n=6), MB (n=6). *Rev1Xpc*: 3 m (n=5-6), MB (n=9).

Figure 4. Rev1 protects against endogenous DNA damage-induced senescence and apoptosis (see also Supplemental Figure 5).
Proliferation, replication, senescence and apoptosis were quantified in bone marrow of all four genotypes.

*p < 0.05. **p <0.01. ***p <0.001. ****p <0.0001. Data are mean ± S.E.M.

A, B. Reduced proliferation (Ki67 immunostaining; A) and replication (BrdU and EdU incorporation, B) in the bone marrow of moribund *Rev1Xpc* mice. WT: 3 m (n=7-9), MB (n=5-6). *Xpc*: 3 m (n=6-8), MB (n=6). *Rev1*: 3 m (n=7), MB (n=6). *Rev1Xpc*: 3 m (n=6), MB (n=9).

C-E. Increased senescence and apoptosis in the bone marrow of *Rev1Xpc* mice as demonstrated by immunostaining for Dec1 (C), p16 (D) and Caspase-3 (E). WT: 3 m (n=4-8), MB (n=5-6). *Xpc*: 3 m (n=5-8), MB (n=5-6). *Rev1*: 3 m (n=3-7), MB (n=6). *Rev1Xpc*: 3 m (n=3-6), MB (n=6-8). M: months, MB: moribund (see Figure 2A for survival data). The fraction of positive cells shown was normalized relative to 3-months old wild type bone marrow.

Figure 5. Rev1-dependent TLS and ggNER converge on helix-distorting oxidative DNA lesions resulting from progressive mitochondrial dysfunction (see also Supplemental Figure 6).

We investigated the involvement of Rev1-dependent TLS at helix-distorting lipid peroxidation-derived nucleotide adduct by treating *Rev1* HSC with a radical scavenger, by using an *in cellulo* TLS assay, by investigating the sensitivity of *Rev1* cells to a lipid peroxidation-derived aldehyde, to oxidative stress, and by measuring oxidative stress in bone marrow. We then characterized the quantity and functionality of *Rev1Xpc* mitochondria.

*p < 0.05. **p <0.01. ***p <0.001. ****p <0.0001. Data are mean ± S.E.M.

A. DNA breaks (γH2AX) in cultured HSC (LSK-SLAM), WT (n=3) and *Rev1* (n=3), treated or non-treated with the ROS scavenger N-acetylcysteine (NAC). The fraction of positive cells shown was normalized relative to wild type.
B. The prototypic DNA-reactive lipid-peroxidation-derived aldehyde 4-Oxo-2(E)-Nonenal and its adduction to a Cytosine base (H-εdC).
C. Top: TLS assay at a site-specific H-εdC. MEFs were transfected with the substrate, followed by incubation to allow TLS, and by recovery of covalently-closed progeny plasmids in *E. coli*. The fraction of recovered substrate, compared with an undamaged internal control, is a measure of TLS activity of the MEFs. Bottom: Relative efficiency and mutation spectrum of TLS events at a site-specific H-εdC lesion.
D. Clonal survival of wild type, *Rev1*, *Xpc* and *Rev1Xpc* MEFs in response to the addition of the mitochondrial poison Paraquat to the growth medium.
E. Clonal survival of *Xpc* and *Rev1Xpc* MEFs in response to the addition of 4-HNE to the growth medium.
F-I. Oxidative stress in the bone marrow of *Rev1Xpc* mice as evidenced by: (E) Lipofuscin accumulation (brown inclusions) in bone marrow of moribund mice; (F) 4-HNE-positive cells; (G) Activation of p38 signaling [phospho (γ)p38 staining] and (H) accumulation of free radical-induced oxidative DNA lesions (OHdG-positive cells) in *Rev1Xpc* mice: 1m (n=3-4), 3m (n=3-5), MB (n=5-6). The fraction of positive cells shown was normalized relative to 3-months old wild type bone marrow.
J. Relative mitochondrial DNA (mtDNA) contents, as determined by rtPCR, in bone marrow from *Xpc* (n=5-6) and *Rev1Xpc* (n=5-6) mice. All mtDNA levels were normalized to those in 3 months old *Xpc* mice.
K. Western blot of mitochondrial complexes I-IV in bone marrow from *Xpc* and *Rev1Xpc* mice (4 mice per group). Lamin B1: internal standard.
L. Expression of the mitochondrial stress proteins UCP2 and PGC-1α in WT, *Xpc*, *Rev1* and *Rev1Xpc* bone marrow. Lamin B: internal standard. Wk: weeks, M: months, MB: moribund (see Figure 2C for survival data).
M. Mitochondrial membrane potentials in bone marrow of *Xpc* and *Rev1Xpc* mice. All potentials in *Rev1Xpc* bone marrow were normalized to those in *Xpc* bone marrow of the same age. 1 m (n=4), 1.5 m (n=4), 3 m (n=4-6), MB (n=3-4).
N. Basal oxygen consumption rates in viable cells from bone marrow from *Xpc* and *Rev1Xpc* mice. All oxygen consumption rates in *Rev1Xpc* bone marrow were normalized to those in *Xpc* bone marrow of the same age. 2 w (n=5-6), 3 m (n=3).

Figure 6. Model for the role of mutagenic TLS in maintenance of the hematopoietic system.

A. Genomic nucleotides, damaged by endogenous sources or by chemical decay, form a threat to DNA transactions such as transcription or replication, in case they remain unrepaired.
B. Processive replication is arrested by a nucleotide, damaged by a helix-distorting oxidative adduct.
C. The damaged nucleotide is bypassed by TLS. This prevents replication stress, but at the expense of the frequent incorporation of an incorrect nucleotide opposite the lesion (in red).
D. Subsequent repair of the damaged nucleotide, or replication of the lower DNA strand, fixates the mutation. This contributes to the acquisition of clonal mutations in the ageing hematopoietic system. Mutations in hematopoietic cells acquired during ageing have been associated with the development of myeloid neoplasms in humans.

E. Stalled replicons that are not released by TLS can collapse to double-stranded DNA breaks. DNA damage signalling at single-stranded DNA gaps opposing the lesions and at double-stranded DNA breaks induces senescence or apoptosis, ultimately resulting in collapse of the hematopoietic system.

F. We hypothesize that failure to release arrested replicons may underlie the observed mitochondrial dysfunction, possibly via depletion of NAD+ that is required both at DNA breaks and for mitochondrial respiration. This may lead to increased ROS production and to the induction of additional oxidative DNA lesions. A positive feedback loop between replication stress at the nuclear genome and mitochondrial dysfunction is proposed to further accelerate the collapse of the hematopoietic system.

Figure 1

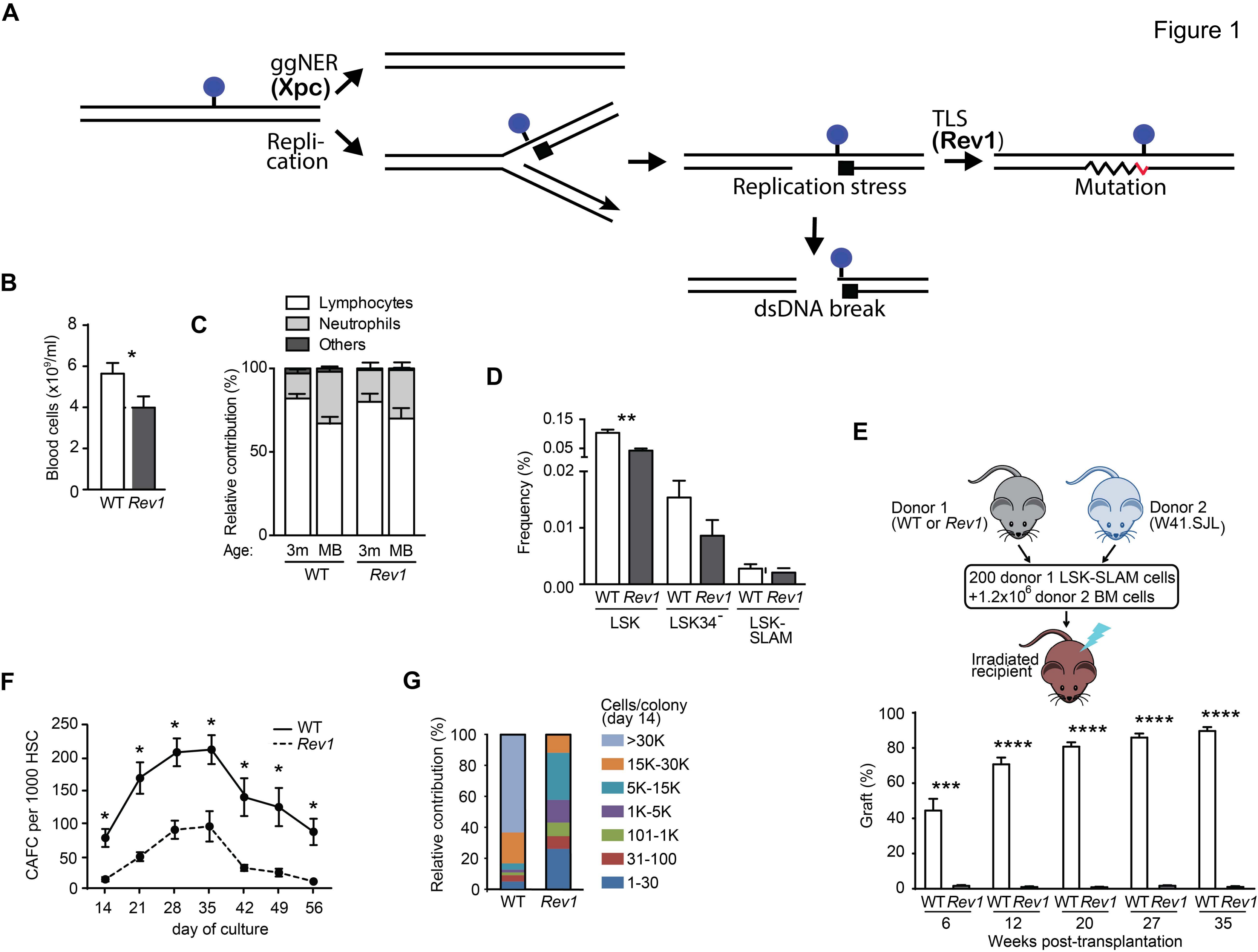
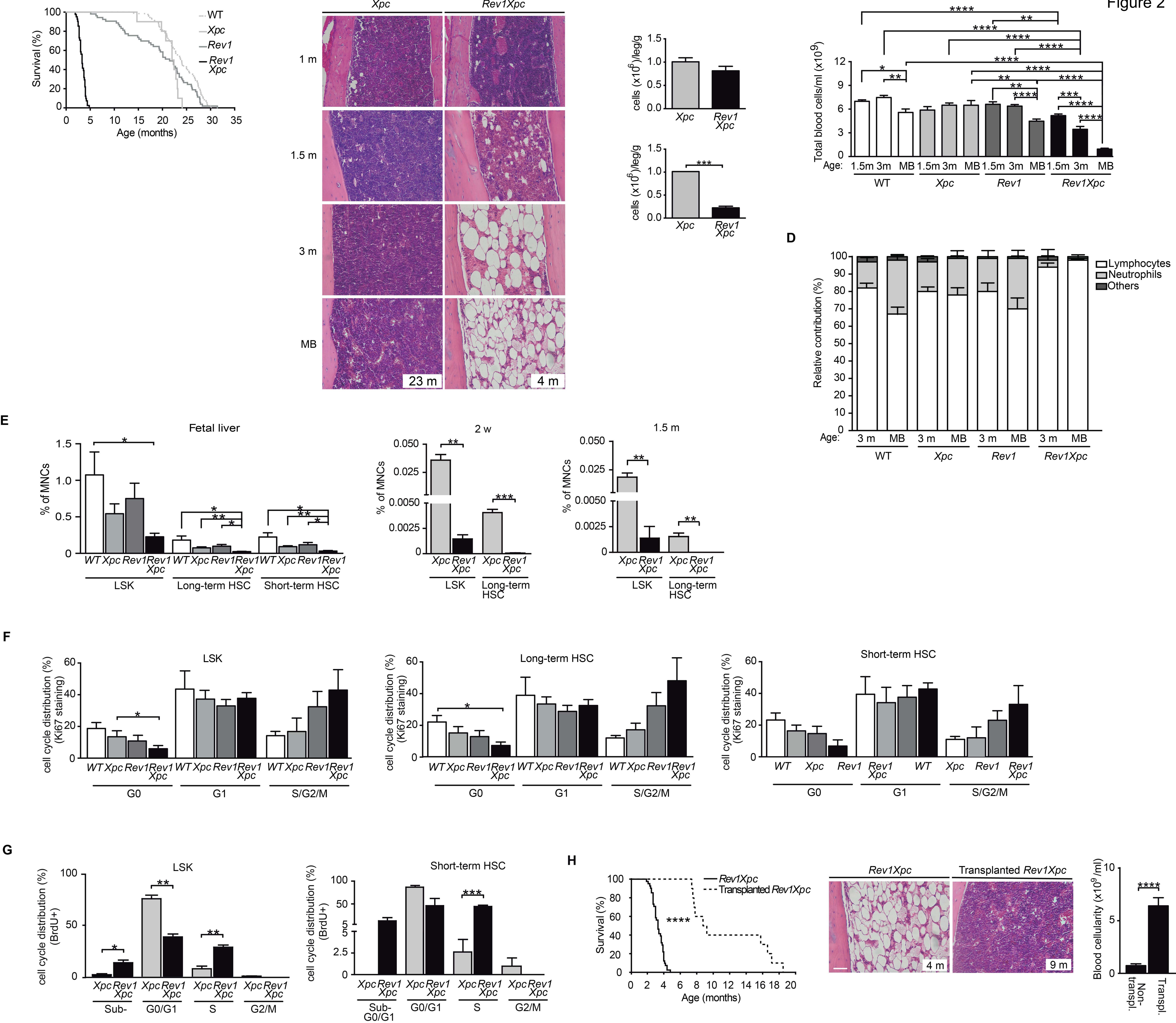

A
ggNER
(Xpc)
Repli-
cation
Replication stress
TLS
(Rev1)
Mutation
dsDNA break
B
Blood cells (x10^9/ml)
WT Rev1
C
Lymphocytes
Neutrophils
Others
Relative contribution (%)
Age: 3m MB 3m MB
WT Rev1
D
Frequency (%)
WT Rev1 WT Rev1 WT Rev1
LSK LSK34- LSK-SLAM
E
Donor 1 (WT or Rev1)
Donor 2 (W41.SJL)
200 donor 1 LSK-SLAM cells +1.2x10^6 donor 2 BM cells
Irradiated recipient
Graft (%)
Weeks post-transplantation
F
CAFC per 1000 HSC
WT
Rev1
day of culture
G
Cells/colony (day 14)
>30K
15K-30K
5K-15K
1K-5K
101-1K
31-100
1-30
Relative contribution (%)

Figure 2


A
B
C
D
E
F
G
H
WT
Xpc
Rev1
Rev1 Xpc
Survival (%)
Age (months)
Rev1Xpc
1 m
1.5 m
3 m
MB
23 m
4 m
cells (x10^6)/leg/g
Total blood cells/ml (x10^9)
Age: 1.5m 3m MB
Relative contribution (%)
Lymphocytes
Neutrophils
Others
Age: 3 m MB
Fetal liver
2 w
1.5 m
% of MNCs
LSK
Long-term HSC
Short-term HSC
cell cycle distribution (%) (Ki67 staining)
G0
G1
S/G2/M
cell cycle distribution (%) (BrdU+)
Sub-G0/G1
G0/G1
S
G2/M
Transplanted Rev1Xpc
9 m
Blood cellularity (x10^9/ml)
Non-transpl.
Transpl.

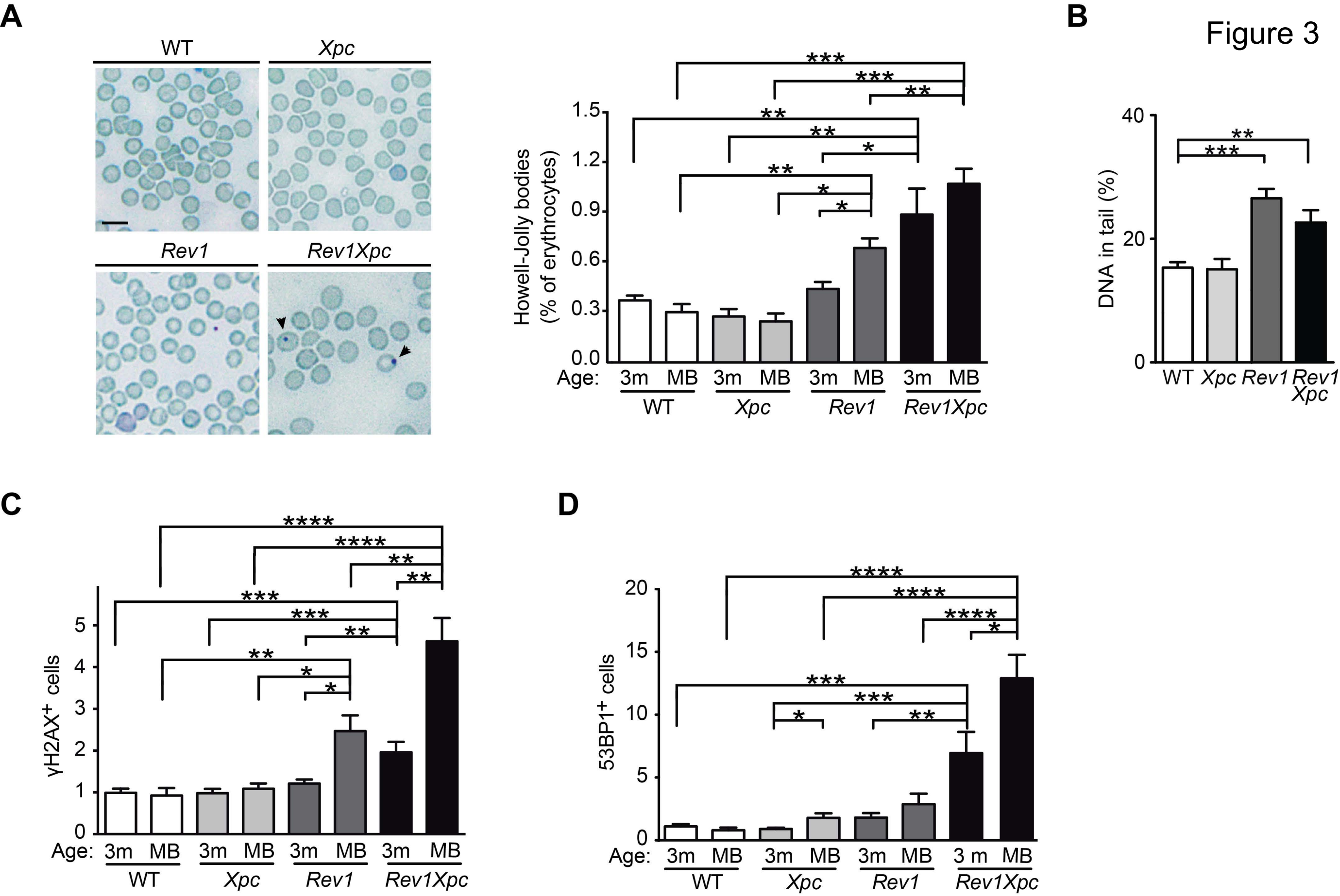
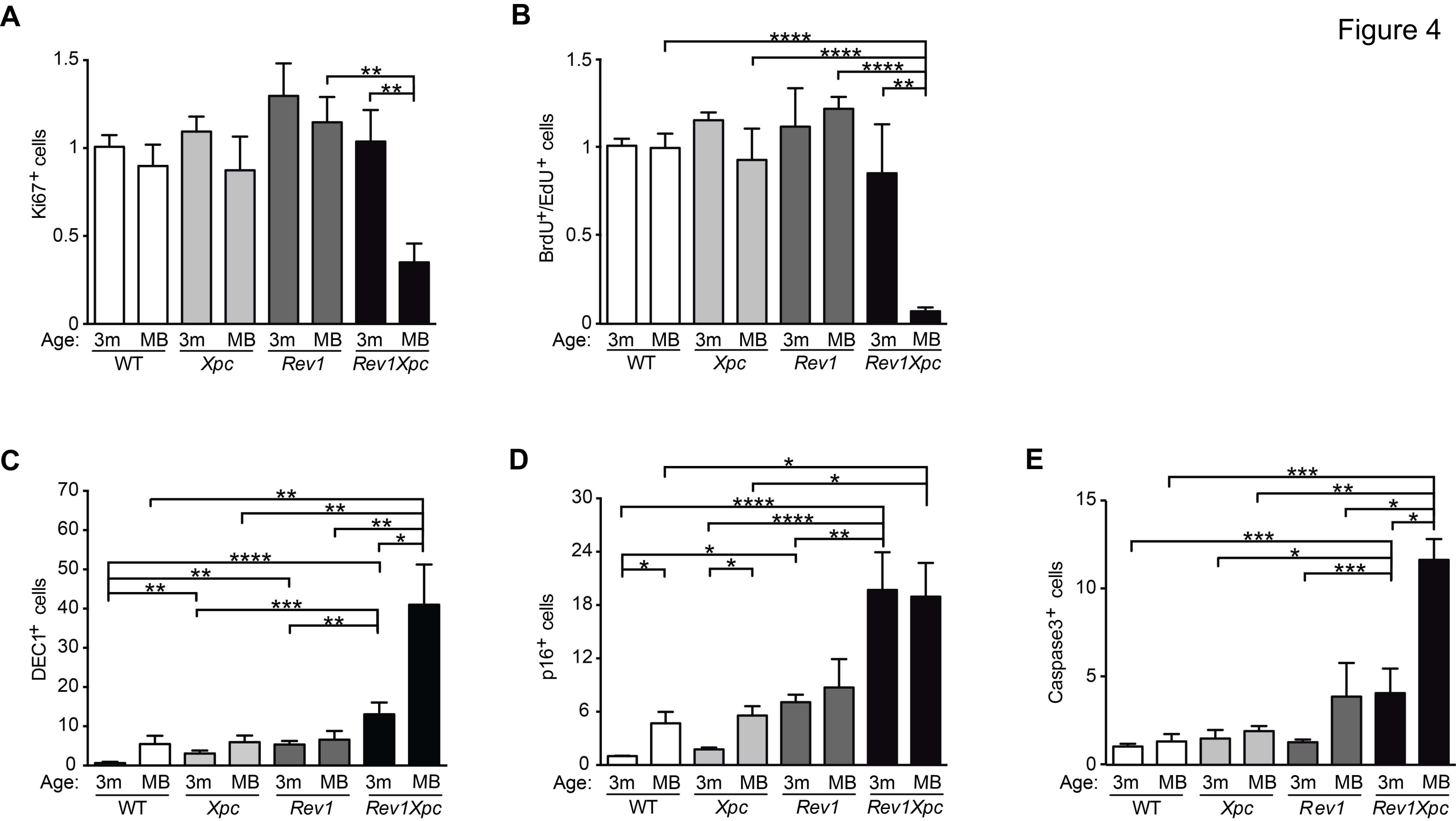


Figure 3
A
WT
Xpc
Rev1
Rev1Xpc
Howell-Jolly bodies (% of erythrocytes)
1.5
1.2
0.9
0.6
0.3
0.0
Age: 3m MB 3m MB 3m MB 3m MB
WT Xpc Rev1 Rev1Xpc
B
DNA in tail (%)
40
20
0
WT Xpc Rev1 Rev1 Xpc
C
γH2AX+ cells
5
4
3
2
1
0
Age: 3m MB 3m MB 3m MB 3m MB
WT Xpc Rev1 Rev1Xpc
D
53BP1+ cells
20
15
10
5
0
Age: 3m MB 3m MB 3m MB 3 m MB
WT Xpc Rev1 Rev1Xpc

Figure 4



A
Ki67⁺ cells
1.5
1
0.5
0
**
**
Age: 3m MB 3m MB 3m MB 3m MB
WT
Xpc
Rev1
Rev1Xpc
B
BrdU⁺/EdU⁺ cells
****
****
****
**
C
DEC1⁺ cells
70
60
50
40
30
20
10
0
**
**
**
*
****
**
**
***
**
D
p16⁺ cells
30
24
18
12
6
0
*
*
****
****
**
*
*
*
E
Caspase3⁺ cells
15
10
5
0
***
**
*
*
***
*
***

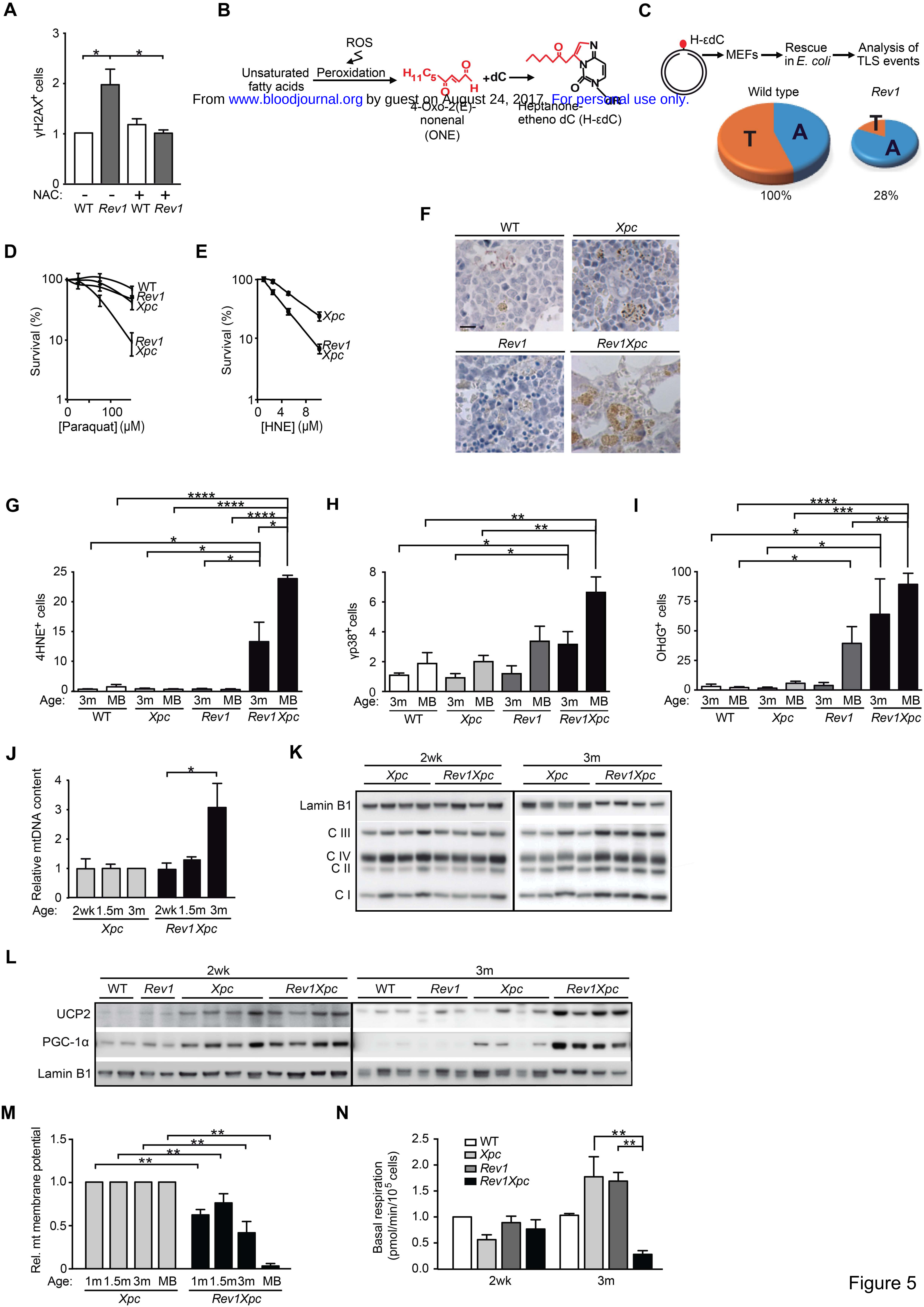

A
γH2AX+ cells
NAC:
WT Rev1 WT Rev1
B
ROS
Unsaturated fatty acids
Peroxidation
4-Oxo-2(E)-nonenal (ONE)
+dC
Heptanone-etheno dC (H-εdC)
C
H-εdC
MEFs
Rescue in E. coli
Analysis of TLS events
Wild type
Rev1
100%
28%
D
Survival (%)
[Paraquat] (μM)
WT
Rev1
Xpc
Rev1 Xpc
E
[HNE] (μM)
F
WT
Xpc
Rev1
Rev1Xpc
G
4HNE+ cells
Age:
3m MB
H
γp38+cells
I
OHdG+ cells
J
Relative mtDNA content
2wk 1.5m 3m
K
2wk
3m
Lamin B1
C III
C IV
C II
C I
L
UCP2
PGC-1α
M
Rel. mt membrane potential
1m 1.5m 3m MB
N
Basal respiration (pmol/min/10^5 cells)
WT
Xpc
Rev1
Rev1Xpc

Figure 5

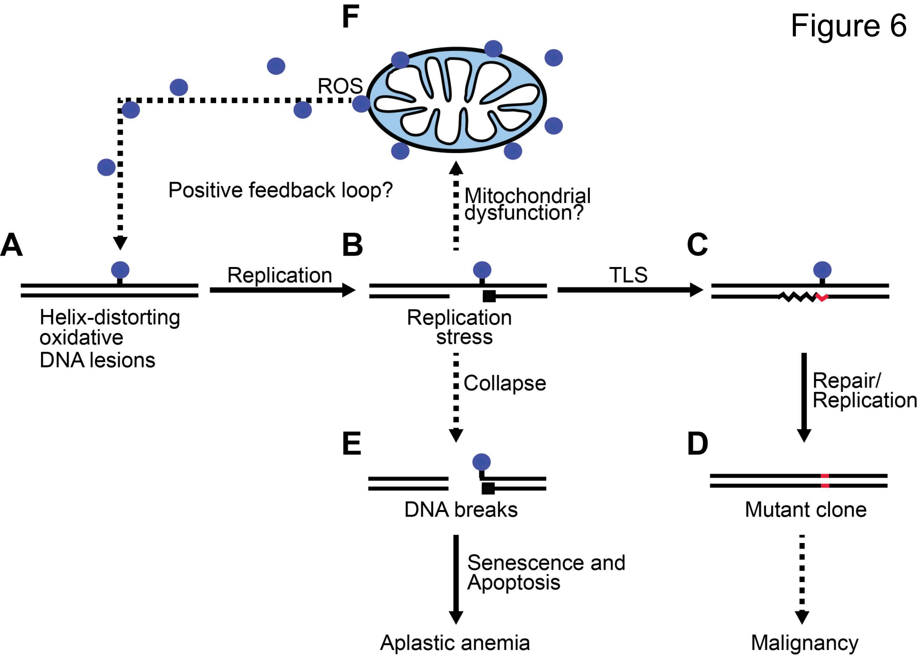


Figure 6
F
ROS
Positive feedback loop?
Mitochondrial dysfunction?
A
Replication
B
TLS
C
Helix-distorting oxidative DNA lesions
Replication stress
Collapse
Repair/ Replication
E
D
DNA breaks
Mutant clone
Senescence and Apoptosis
Aplastic anemia
Malignancy

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Genomic and functional integrity of the hematopoietic system requires tolerance of oxidative DNA lesions

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