LETTERS

Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age

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A diminished capacity to maintain tissue homeostasis is a central physiological characteristic of ageing. As stem cells regulate tissue homeostasis, depletion of stem cell reserves and/or diminished stem cell function have been postulated to contribute to ageing[1]. It has further been suggested that accumulated DNA damage could be a principal mechanism underlying age-dependent stem cell decline[2]. We have tested these hypotheses by examining haematopoietic stem cell reserves and function with age in mice deficient in several genomic maintenance pathways including nucleotide excision repair[3,4], telomere maintenance[5,6] and non-homologous end-joining[7,8]. Here we show that although deficiencies in these pathways did not deplete stem cell reserves with age, stem cell functional capacity was severely affected under conditions of stress, leading to loss of reconstitution and proliferative potential, diminished self-renewal, increased apoptosis and, ultimately, functional exhaustion. Moreover, we provide evidence that endogenous DNA damage accumulates with age in wild-type stem cells. These data are consistent with DNA damage accrual being a physiological mechanism of stem cell ageing that may contribute to the diminished capacity of aged tissues to return to homeostasis after exposure to acute stress or injury.

In the murine haematopoietic system, long-term multilineage differentiation and self-renewal are mediated by long-term reconstituting haematopoietic stem cells (LT-HSCs), which can be isolated from the bone marrow of young and old mice by their unique cell surface phenotype (lineage$^-$c-Kit$^+$Sca-1$^+$flk2$^-$CD34$^-$)[9,10] (Supplementary Fig. 1). To evaluate the effect of deficiencies in nucleotide excision repair (NER), non-homologous end-joining (NHEJ) and telomere maintenance on stem cell reserves during ageing, we quantified the frequency and absolute numbers of LT-HSCs in the bone marrow of young and old XPD^{TTD} (refs 3, 4), $Ku80^{-/-}$ (refs 7, 8), and late-generation $mTR^{-/-}$ (refs 5, 6) mice and controls. These analyses revealed that, regardless of age, neither stem cell frequency (Fig. 1a–d) nor absolute numbers (Supplementary Fig. 2) were appreciably reduced in any of the mutants examined. Indeed, rather than being diminished, the frequency of LT-HSCs in the bone marrow of the mutants increased significantly with age (Fig. 1e–g), which is consistent with the expansion of LT-HSC reserves in BL6 strains of mice ageing naturally (Fig. 1h)[9–11]. Moreover, the degree to which the stem cell pool expanded in each of the mutant strains was closely correlated with age-matched controls (Supplementary Fig. 3). We next evaluated the impact of genomic maintenance and ageing on downstream multipotent progenitor (MPP) and oligopotent progenitor populations (Supplementary Fig. 1)[12]. These analyses revealed that whereas short-term (ST)-HSC reserves were not significantly affected in any of the mutants assayed (Supplementary Fig. 4), downstream MPP^{flk2+}, common myeloid progenitor (CMP) and common lymphoid progenitor (CLP) progenitor populations were frequently diminished in the mutants, although this was not strictly correlated with age (Fig. 1i–k). Taken together, these results indicate that deficiencies in NER, telomere maintenance or NHEJ do not significantly affect the establishment, maintenance or expansion of LT-HSC reserves with age. This suggests that LT-HSCs may be cytoprotected against the accumulation of different types of DNA lesion with ageing, perhaps as a consequence of their largely quiescent state[13]. In contrast, downstream progenitors, which cycle more rapidly[14], were more adversely affected in these mutants, indicating that these populations might be more susceptible to DNA damage responses such as growth arrest or apoptosis, which are characteristically activated in cycling cells at the G1/S and G2/M checkpoints[15].

Although we and others have assayed HSC activity in young telomerase-deficient mice[16,17], the consequence of advancing age and accumulated damage resulting from telomere attrition on LT-HSC function has not been evaluated. We therefore purified LT-HSCs from old (60-week) late-generation (G_3) $mTR^{-/-}$ mutants and controls, and competitively transplanted 50 stem cells against 2×10^5 competitor bone marrow cells with the use of the CD45 congenic system[9]. We reasoned that this strategy would maximize the genomic damage associated with critically short telomeres in LT-HSCs as increased genomic instability[6], and signal-free telomere ends[18] have been shown to accompany the ageing of haematopoietic cells in late-generation $mTR^{-/-}$ mice (Supplementary Fig. 5). Analysis of transplant recipients revealed that short-term reconstitution was modestly reduced in the G_3 $mTR^{-/-}$ LT-HSC-transplanted recipients, yet by 20 weeks after transplantation it had dropped off precipitously (Fig. 2a), with B-cell, T-cell and myeloid lineages all being significantly affected (Fig. 2b). Granulocyte chimaerism was monitored throughout the course of the experiment as a measure of ongoing stem cell function because granulocytes are short-lived and require continued stem cell activity to be generated[19]. These analyses indicated a progressive loss of stem cell function that approached exhaustion by 20 weeks after transplantation (Fig. 2c). Consistent with this, stem cells from primary G_3 $mTR^{-/-}$ transplanted recipients were incapable of serially transplanting secondary recipients, indicating that they had become functionally exhausted (Fig. 2d). Transplantation experiments performed in parallel with LT-HSCs from younger (36-week) G_3 $mTR^{-/-}$ mice revealed that although stem cells from these donors were compromised in comparison with controls (Fig. 2e), the magnitude of the functional decline was not as marked as when stem cells from older G_3 $mTR^{-/-}$ mice were assayed (Fig. 2c).

To test whether diminished self-renewal might underlie the functional exhaustion of aged G_3 $mTR^{-/-}$ LT-HSCs, we assayed the

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capacity of these cells to self-renew in primary transplant recipients[9]. These experiments showed that LT-HSCs from aged G_3 $mTR^{-/-}$ donors were 16-fold less capable than controls of giving rise to phenocopies of themselves (Fig. 2f). We next assayed the intrinsic proliferative capacity of telomerase-deficient stem cells by quantifying the total progeny-cell output of cultured KLSflk2$^-$ cells (LT-HSCs and ST-HSCs combined) from young (19-week), and old (51-week) G_3 mutants and controls. This showed that there was a significant decline in the proliferative capacity of the $mTR^{-/-}$ cells, which was exacerbated with advanced age (Fig. 2g) and underwritten by an increased apoptotic response (Fig. 2h). Cumulatively, these results indicate that telomere attrition limits stem cell function in an age-dependent manner by intrinsically diminishing self-renewal and proliferative capacity, and rendering LT-HSCs susceptible to apoptosis under conditions of stress.

To assay the effect of NER on LT-HSC functional capacity, we competitively transplanted 50 LT-HSCs from 26-week-old XPD^{TTD} and $XPD^{+/+}$ mice. Stem cells from the XPD^{TTD} mice showed a significant diminution in multilineage reconstitution potential (Fig. 3a, b) and a progressive loss of stem activity that approached exhaustion by 16 weeks after transplantation (Fig. 3c). The observation that XPD^{TTD} LT-HSCs were incapable of stably reconstituting secondary hosts during serial transplantation confirmed that stem cell activity had become exhausted (Fig. 3d). Transplantation experiments performed in parallel with stem cells from younger (12-week) XPD^{TTD} and control mice revealed that although stem cells from younger XPD^{TTD} mice were functionally compromised in comparison with controls (Fig. 3e), they performed significantly better than stem cells from older XPD^{TTD} mice (Fig. 3c).

To determine whether diminished self-renewal capacity contributed to the functional decline of XPD^{TTD} stem cells, we assayed the capacity of LT-HSCs to self-renew in primary transplant recipients, which showed that XPD^{TTD} LT-HSCs had a 5.2-fold reduced capacity for self-renewal than controls (Fig. 3f). We next tested the intrinsic proliferative capacity of KLSflk2$^-$ cells from young (16-week) and old (73-week) XPD^{TTD} mutants and controls and found that whereas the cells from young mutants were marginally affected, stem cells from old XPD^{TTD} mice showed significantly reduced proliferative capacity (Fig. 3g), which was associated with increased apoptosis (Fig. 3h). Taken together, these results identify a significant role for xeroderma pigmentosum complementation group D (XPD)-mediated NER in maintaining the functional capacity of LT-HSCs

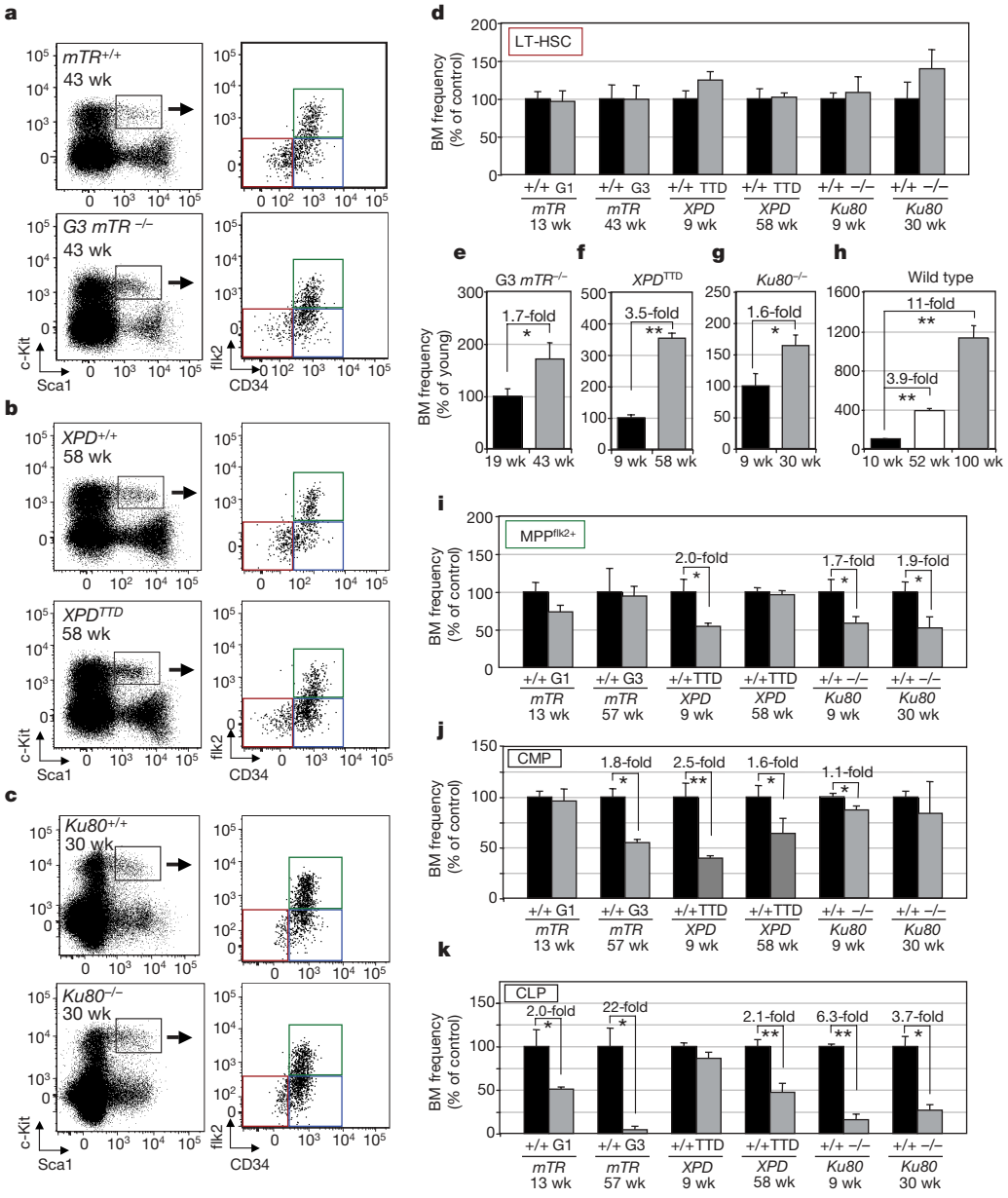

Figure 1 | Stem and progenitor reserves with age in genomic maintenance-deficient mice. **a–c**, Representative FACS profiles pre-gated on live, lineage$^-$ cells showing LT-HSCs, ST-HSCs and MPP^{flk2+} (red, blue and green gates, respectively) in aged G_3 $mTR^{-/-}$ (**a**), XPD^{TTD} (**b**) and $Ku80^{-/-}$ (**c**) mice and controls. **d**, Frequency of LT-HSCs in bone marrow. **e–h**, Expansion of LT-HSCs with age in G_3 $mTR^{-/-}$ (**e**), XPD^{TTD} (**f**), $Ku80^{-/-}$ (**g**) and wild-type (**h**) mice. **i–k**, Frequency of MPP^{flk2+} (**i**), CMP (**j**) and CLP (**k**) cells in bone marrow. Ages are indicated in weeks (wk). Cohorts ranging from $n = 8$ to $n = 13$ mice were used. Significant differences (Student's t-test) are indicated as follows: asterisk, $P < 0.05$; two asterisks, $P < 0.005$. Error bars denote s.e.m.

with age by preserving reconstitution ability, self-renewal potential and proliferative capacity, and by preventing programmed cell death under conditions of stress.

We next assayed the importance of NHEJ on stem cell function by competitive transplantation of LT-HSCs from Ku80-deficient mice and controls. As expected, *Ku80*$^{-/-}$ LT-HSCs were unable to generate mature B and T cells as a result of an inability to undergo V(D)J recombination[7]. Ku80-deficient stem cells were also sharply impaired in their ability to reconstitute myeloid lineages, indicating severely diminished stem cell activity (Fig. 4a, b). Consistent with this was our observation that *Ku80*$^{-/-}$ LT-HSCs were 26-fold less capable of giving rise to phenocopies of themselves than controls in primary transplant recipients, indicative of an attenuated self-renewal capacity (Fig. 4c). Moreover, cultured KLSflk2^{-} cells from *Ku80*$^{-/-}$ mutants had a reduced capacity to proliferate, which was greatly exacerbated with age (Fig. 4d) and was associated with increased apoptosis (Fig. 4e). Cumulatively, these results identify a role for Ku80 and NHEJ in maintaining LT-HSC function by conserving reconstitution potential, self-renewal capacity, proliferative capacity and stem cell viability under conditions of stress.

Our data showing an age-dependent diminution of stem cell function in three different genomic maintenance-deficient settings suggested that accumulated genomic damage might be an important physiological mechanism contributing to stem cell decline with age. To test whether DNA damage accumulation accompanied normal stem cell ageing, we immunostained LT-HSCs from young (10-week) and old (122-week) mice for phosphorylation of histone H2AX (γ-H2AX) as an indicator of DNA damage[20], and quantified the number of γ-H2AX foci in individual stem cells. This analysis revealed that whereas LT-HSCs from young mice were largely devoid of γ-H2AX foci, the vast majority (82%) of the stem cells from old mice stained positively for γ-H2AX, with more than 70% of the cells showing multiple foci (Fig. 4g, h). Similarly, ST-HSCs and MPP^{flk2+} isolated from old mice contained significantly more γ-H2AX foci than their young counterparts (Fig. 4i, j), although the percentage of γ-H2AX-positive old cells decreased as the cells progressed from LT-HSCs through the

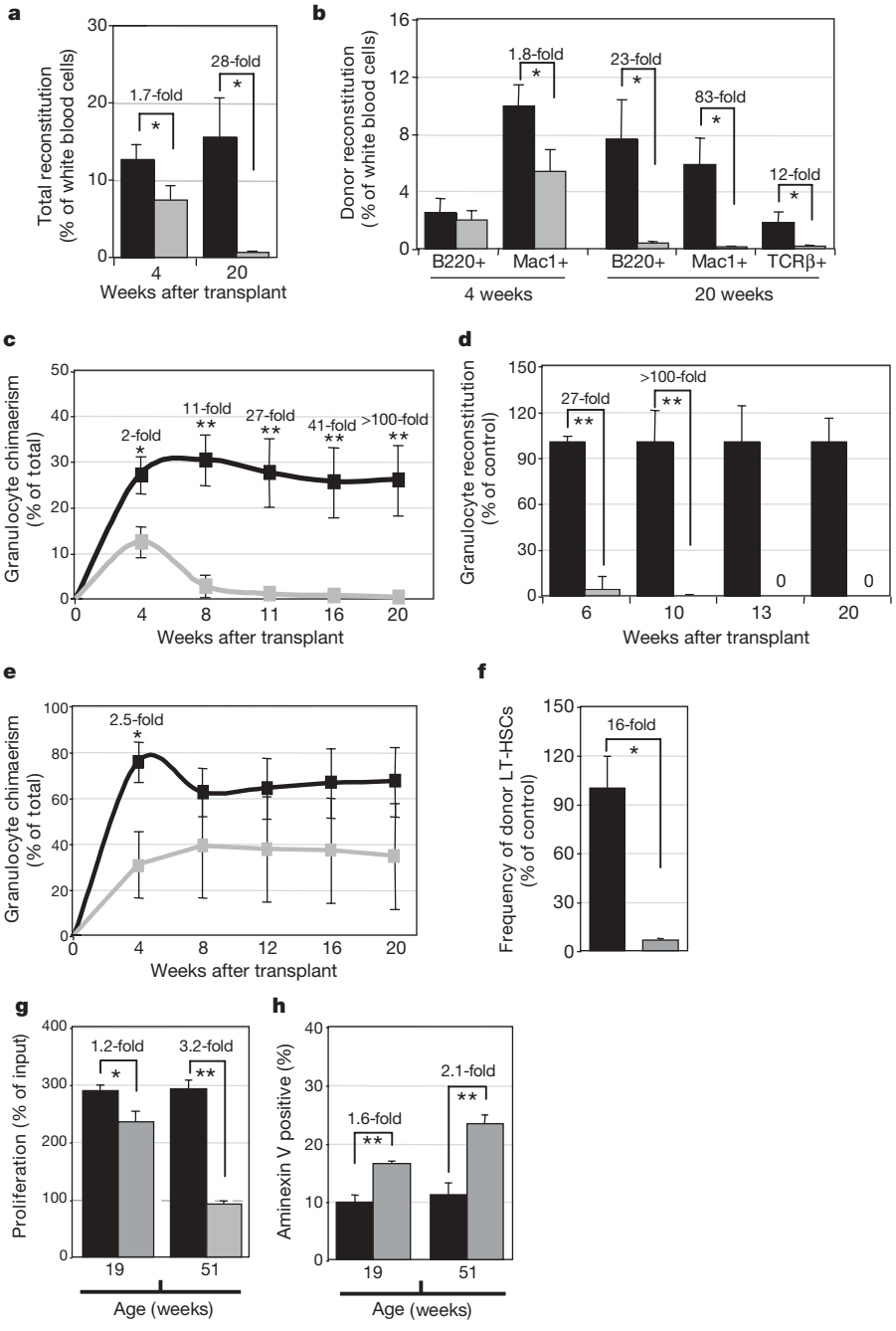

Figure 2 | Telomerase-deficiency limits LT-HSC function with age. a–c, Competitive transplantation of LT-HSCs from 60-week *mTR*$^{+/+}$ (black) or G$_3$ *mTR*$^{-/-}$ (grey) donors ($n = 9$ and $n = 10$ recipients, respectively) showing total reconstitution (**a**), contribution to B cells (B220^{+}), myeloid cells (Mac1^{+}) and T cells (TCR-β^{+}) (**b**), and granulocyte chimaerism (**c**). **d**, Secondary transplantation of *mTR*$^{+/+}$ (black bars) or G$_3$ *mTR*$^{-/-}$ (grey bars) stem cells ($n = 6$ and $n = 5$ recipients, respectively). **e**, Competitive transplantation of LT-HSCs from 36-week *mTR*$^{+/+}$ (black line) or G$_3$ *mTR*$^{-/-}$ (grey line) donors ($n = 6$ and 5 recipients respectively). **f**, Donor LT-HSC frequency from recipients described in **a**. (**g**) Proliferative potential of *mTR*$^{+/+}$, and G$_3$ *mTR*$^{-/-}$ KLSflk2^{-} cells. (**h**) Annexin V positive cells in the experiment described in **g**. Significant differences (Student's *t*-test) are indicated as follows: asterisk, $P < 0.05$; two asterisks, $P < 0.005$. Error bars denote s.e.m.

more committed progenitors (65% in old ST-HSCs; 25% in old MPP^{flk2+}). By the CLP^{flk2+} and CMP stages of differentiation, significant differences in γ-H2AX staining between young and old mice were no longer observed (data not shown). Taken together, these data indicate that DNA damage accumulates in stem cells with age, and suggests that proliferating progenitor cells are either repaired more readily or are eliminated on accumulating damage.

Although studies with purified HSCs have provided great detail about how ageing alters the functional capacity of HSCs[9–11,21], much less is known about the mechanisms driving these changes. Because HSCs are long-lived, age-dependent functional decline could be postulated to result from the accumulation of macromolecular damage in general[22], or DNA damage in particular[2]. In support of this, stem cells from mice with mutations in *Brca2* (ref. 23) or *Msh2* (ref. 24) have reduced repopulating abilities, whereas *Ercc1*-deficient mice have multilineage cytopenias indicating possible stem or progenitor cell dysfunction[25]. Evidence that DNA damage response has a significant bearing on the function of HSCs during ageing was provided in studies demonstrating that reactive oxygen species limit the functional capacity of HSCs from ataxia-telangiectasia mutated (ATM)-deficient mice[26] in a p38-MAPK-dependent manner[27], and in studies on mice bearing a mutated *Rad50* allele, which undergo haematopoietic failure in an ATM-Chk2-dependent fashion[28,29]. The present demonstration that genetic deficiencies in telomere maintenance, NER and NHEJ intrinsically diminish LT-HSC function in an age-dependent manner under conditions of stress indicates that DNA damage accrual may underlie the reduced capacity of stem cells to mediate a return to homeostasis after exposure to injury or stress. Our findings also have implications for stem cell involvement in oncogenesis because they establish that relatively quiescent stem cells can persist in the face of age-dependent DNA damage accrual, and in such a way might serve as a reservoir for the multiple mutagenic events underlying oncogenic transformation.

METHODS SUMMARY

Purification and transplantation of cells. LT-HSCs ($lineage^{-}c\text{-}Kit^{+}Sca1^{+}flk2^{-}CD34^{-}$) were purified and transplanted as described[9]. In brief, bone marrow cells were enriched for c-Kit, stained with fluorescence-conjugated antibodies against Sca1, c-Kit, CD34, flk2 and lineage (CD3, CD4, CD8, Mac-1, B220, Gr-1 and Ter119) and purified by fluorescence-activated cell sorting (FACS). Fifty test cells (CD45.2) were transplanted against 2×10^5 bone marrow competitor cells (CD45.1) into lethally irradiated recipients (CD45.1). Peripheral blood was analysed with simultaneous detection of CD45.1, CD45.2, T-cell antigen receptor (TCR)-β, B220, Mac1 and Ter119.

Proliferation and annexin V analysis. Equivalent cell numbers were sorted and cultured for 3.5–4.5 days in RPMI medium containing 10% fetal calf serum, 10 ng ml^{-1} stem cell factor, thrombopoietin, interleukin (IL)-3, IL-6, IL-11 and Flt3 ligand at 37 °C, 2.5% O_2 and 5% CO_2. Cells were then stained with annexin V and propidium iodide, and analysed by FACS.

γ-H2AX immunostaining. γ-H2AX was revealed by using the SCIPhos (single-cell imaging of phosphorylation) assay[30]. In brief, cells were sorted into droplets of PBS on poly(L-lysine)-coated slides, then fixed, permeabilized and stained with phospho-specific (Ser 139) histone H2AX antibody (Biolegend). After being washed, the cells were stained with a secondary Alexa Fluor 488-conjugated antibody and 4,6-diamidino-2-phenylindole. Quantification of γ-H2AX foci was performed by fluorescence microscopy and analysed statistically with the Mann–Whitney *U*-test.

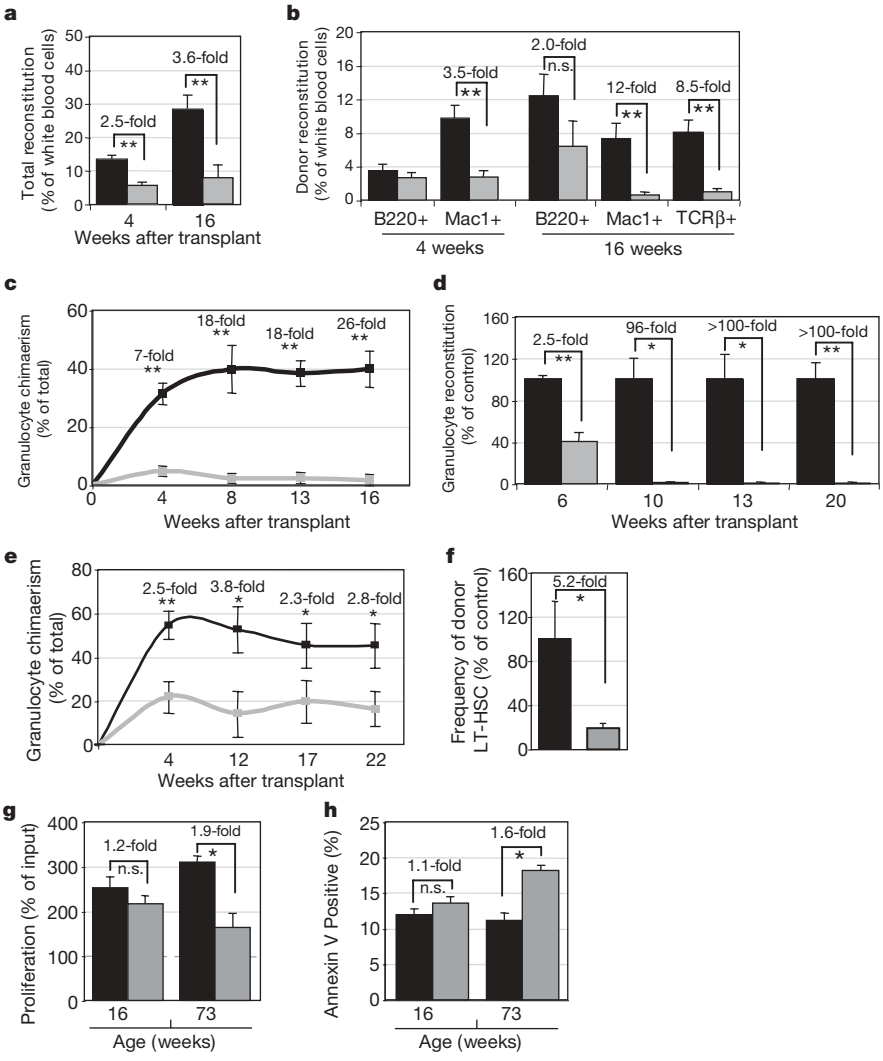

Figure 3 | NER deficiency limits LT-HSC function with age. a–c, Competitive transplantation of LT-HSCs from 26-week $XPD^{+/+}$ (black) or XPD^{TTD} (grey) donors ($n = 9$ recipients for each) showing total reconstitution (**a**), lineage contribution to B cells ($B220^{+}$), myeloid cells ($Mac1^{+}$) and T cells ($TCR\text{-}\beta^{+}$) (**b**), and granulocyte chimaerism (**c**). **d**, Secondary transplantation of LT-HSCs from $XPD^{+/+}$ (black bars) or XPD^{TTD} (grey bars) donors ($n = 5$ recipients for each). **e**, Competitive transplantation of LT-HSCs from 12-week $XPD^{+/+}$ (black line) or XPD^{TTD} (grey line) donors ($n = 8$ and $n = 9$ recipients, respectively). **f**, Donor LT-HSC frequency from recipients described in **a**. **g**, Proliferative potential of $XPD^{+/+}$ (black bars) and XPD^{TTD} (grey bars) $KLSflk2^{-}$ cells. **h**, Annexin V-positive cells in the experiment described in **g**. Significant differences (Student's *t*-test) are indicated as follows: asterisk, $P < 0.05$; two asterisks, $P < 0.005$; n.s., not significant. Error bars denote s.e.m.

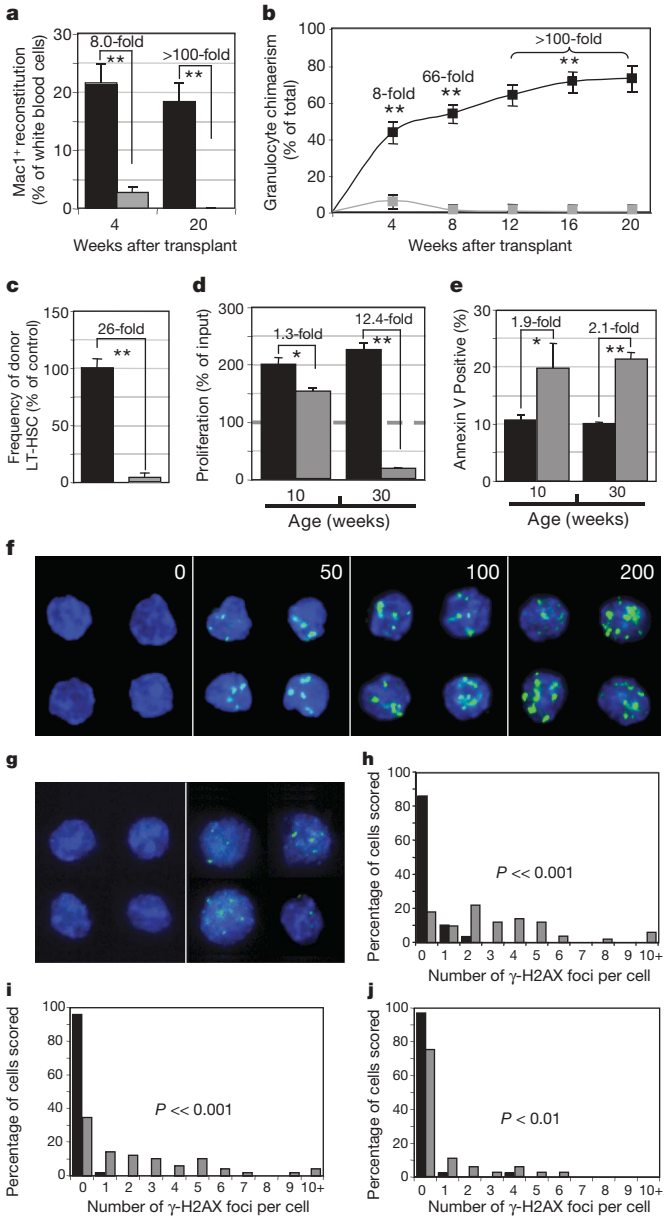

Figure 4 | NHEJ deficiency and endogenous damage accumulation in stem cells with age. a–c, Competitive transplantation of LT-HSCs from 16-week *Ku80*$^{+/+}$ (black) or *Ku80*$^{-/-}$ (grey) donors ($n = 9$ and $n = 7$ recipients, respectively) showing myeloid (Mac1^{+}) reconstitution (**a**) and granulocyte chimaerism (**b**). **c**, Donor LT-HSC frequency from the recipients described in **a**. **d**, Proliferative potential of *Ku80*$^{+/+}$ (black bars) or *Ku80*$^{-/-}$ (grey bars) KLSflk2^{-} cells. **e**, Annexin V-positive cells in the experiment described in **d**. Significant differences (Student's *t*-test) are indicated as follows: asterisk, $P < 0.05$; two asterisks, $P < 0.005$. Error bars denote s.e.m. **f**, Immunostaining of γ-H2AX in c-Kit^{+}Sca1^{+}lin^{-} cells showing irradiation dose (in rads) dependence. **g**, LT-HSCs from 10-week-old (left) and 122-week-old (right) mice immunostained for γ-H2AX. **h–j**, γ-H2AX distribution in LT-HSCs (**h**), ST-HSCs (**i**) and MPP^{flk2+} (**j**). Black bars, 10-week-old mice; grey bars, 122-week-old mice.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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METHODS

Mice. Strains of mice used included XPD^{TTD} mice, which model the human segmental progeroid syndrome trichothiodystrophy (TTD) and have a mutation of the XPD helicase with pleiotropic functional deficits including a partial defect in NER[31,32], $KU80^{-/-}$ mice, which are defective in NHEJ and in double-strand break repair as a result of ablation of KU80 (refs 33–35), and $mTR^{-/-}$ mice, which are defective in telomere maintenance as a result of the targeted disruption of the telomerase RNA component[36,37]. Because mice have long telomeres, the telomerase $mTR^{-/-}$ mutants were backcrossed for several generations to allow telomeres to shorten enough to become debilitating (late generation). When initially reported, $mTR^{-/-}$ mice on a mixed background needed to be backcrossed for four to six generations for telomere dysfunction to be manifested[36,37,38]. However, because mTR is haploinsufficient for telomere maintenance[39], and the BL6 strain to which we backcrossed the mutation have shorter telomeres than other inbred strains[40], the $mTR^{-/-}$ mice could only be interbred through two (G_2) to three generations (G_3) before becoming sterile and showing overt signs of telomere dysfunction such as cachexia and reduced life span (Supplementary Fig. 5) due to critically short telomeres and increased chromosome instability[41]. In all experiments, age-matched wild-type littermate controls were used when possible. For the late-generation telomerase mutants whose breeding scheme did not produce wild-type littermate controls, age-matched controls of the same genetic background were used. All mice were on a C57BL/6 background and were maintained at the Stanford University Laboratory Animal Facility.

Absolute numbers of LT-HSCs. Absolute numbers were calculated from the bone marrow cellularity of the four hindlimb bones, the bone marrow frequency of LT-HSCs and the weight of each mouse, to control for differences in animal size.

Secondary transplantation. Serial transplantation was performed in several ways. Primary recipients were transplanted either competitively with 50 LT-HSCs as described above, or non-competitively with about 5×10^5 bone marrow cells obtained from test or control mice. In the latter case, flow cytometry staining for LT-HSCs was first performed to ensure that the frequency of LT-HSCs in bone marrow from test and control mice was comparable so that we would be transplanting stem cell equivalents. Variations in frequency in bone marrow were adjusted for before transplantation. For secondary transplants, if primary recipients were competitively transplanted we sorted donor-derived (test or control) LT-HSCs from the bone marrow and competitively transplanted 25 or 100 of these cells into the secondary host as described above. If primary recipients had been transplanted non-competitively, 10^6 bone marrow cells were transplanted into lethally irradiated secondary recipients. Peripheral blood analysis of secondary recipients was performed as described above.

Self-renewal determination. Self-renewal was determined as described previously[42]. In brief, primary lethally irradiated recipients (CD45.1) were transplanted non-competitively with stem cell equivalents of test or control bone marrow. Stem cell equivalents were determined by staining donor bone marrow for LT-HSCs to determine frequency before transplantation. At 5–7 months after transplantation, primary recipients were sacrificed and the frequency of donor-derived (CD45.2) LT-HSCs in bone marrow was determined by using an eight-colour flow cytometric protocol with simultaneous detection of CD45.1, CD45.2, lineage, c-Kit, Sca1, CD34 and flk2, along with discrimination of dead cells (propidium iodide). In each experiment three to five recipients transplanted with test or control cells were assayed.

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