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## Genotoxic stresses promote clonal expansion of hematopoietic stem cells expressing mutant p53

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Clonal hematopoiesis increases with age, where a single mutant hematopoietic stem or progenitor cell contributes to a significant, measurable clonal proportion of mature blood lineages.<sup>1–5</sup> Evolution of mutant clonal hematopoiesis with age predisposes the elderly to myelodysplastic syndromes (MDS), acute myeloid leukemia (AML), and other aging-associated diseases, suggesting that mutations identified in hematopoietic stem cells contribute to disease development.<sup>1–5</sup> However, the mechanisms by which age-induced stem cell mutations cause clonal expansion of hematopoietic stem cell are largely unknown.

Recently, acquired somatic mutations in *TP53* gene were identified in the blood of aged healthy individuals and the frequency of *TP53* mutations increases with age.<sup>3–5</sup> *TP53* mutations rank top five among mutations identified in aged healthy individuals.<sup>3–5</sup> The *TP53* gene, which encodes the tumor suppressor p53, is the most frequent target for mutation in human cancer, including hematological malignancies.<sup>6</sup> *TP53* mutations are present in 10% of MDS cases and 20 to 30% in secondary MDS arising after exposure to radiation or alkylating agents.<sup>1, 7</sup> The frequency of *TP53* mutations in AML is approximately 10%. However, in AML with complex karyotype, the frequency of p53

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### CONFLICT OF INTEREST

The authors declared that no conflict interest exists.

### AUTHOR CONTRIBUTIONS

SC and YL designed the research. SC, RG, CY, MK, and SZL performed the research. SC and YL analyzed the data and performed the statistical analysis. MCY, HB, RK, HSB, and LDM provided reagents and/or input to the study. SC and YL wrote the manuscript. All authors read, comment on, and approved the manuscript.

mutations and/or deletions is almost 70%.<sup>8</sup> Further, *TP53* mutations are associated with poor prognosis and decreased survival in MDS and AML.<sup>7-9</sup> However, the role of *TP53* mutations in clonal hematopoiesis and the pathogenesis of hematological malignancies is largely unknown.

To date, no studies have investigated the *in vivo* effect of *TP53* mutations expressed from the endogenous locus in hematopoietic cells, which might allow delineation of how these mutations contribute to the pathogenesis of hematological malignancies.<sup>10-13</sup> The vast majority of missense *TP53* mutations are mapped to the DNA-binding domain (DBD) of p53 protein, and usually abrogate its sequence-specific DNA-binding activity.<sup>6</sup> Previous studies show that codon 248 of the p53 protein is most frequently mutated in MDS and AML.<sup>7-8</sup> p53<sup>R248W</sup> mutant has also been identified in aged healthy individuals.<sup>3-5</sup> To better understand the role of mutant p53 in hematopoiesis when expressed physiologically from its endogenous promoter, we utilized the humanized p53<sup>R248W</sup> knock-in mice, expressing human p53 mutant protein from the endogenous murine *Trp53* promoter.<sup>14</sup> Given that most *TP53* mutations in clonal hematopoiesis and hematological malignancies are monoallelic missense mutations,<sup>3-9</sup> we examined HSC function in the heterozygous p53<sup>R248W/+</sup> mice to recapitulate clinical conditions. We first analyzed the peripheral blood and bone marrow of p53<sup>+/+</sup> and p53<sup>R248W/+</sup> mice (8 to 12 week-old). Peripheral blood cell counts and bone marrow cellularity were comparable among these mice (Figures S1a, S1b, S1c, S1d, S1e, and S1f). In p53 knockout mice, there is a dramatic increase of LT-HSCs;<sup>11</sup> however, we found that p53<sup>+/+</sup> and p53<sup>R248W/+</sup> mice have a similar number of LT-HSCs (Figures 1a and S1g). In addition, expression of p53<sup>R248W</sup> did not affect the frequency of myeloid progenitors (Figures 1a, 1b, and S1g). To determine the role of p53<sup>R248W</sup> in hematopoiesis *in vivo*, we performed competitive bone marrow transplantation assays as shown in Figure S1h. We transplanted 5 × 10<sup>5</sup> donor bone marrow cells (p53<sup>+/+</sup> or p53<sup>R248W/+</sup>, CD45.2<sup>+</sup>) into lethally irradiated (9.5 Gy) recipient mice (CD45.1<sup>+</sup>CD45.2<sup>+</sup>) along with 5 × 10<sup>5</sup> competitor marrow cells (CD45.1<sup>+</sup>). At 16 weeks post transplantation, the repopulating ability of p53<sup>R248W/+</sup> cells was significantly higher than that of the wild type cells (Figure 1c). We also observed increased frequency of donor-derived hematopoietic stem and progenitor cells in the bone marrow of recipient mice repopulated with p53<sup>R248W/+</sup> cells (Figures 1d, 1e, and S1i). p53<sup>R248W</sup> did not affect myeloid and lymphoid differentiation in the peripheral blood and the bone marrow of the primary recipient mice (Figures 1f and 1g). We then transplanted 3 × 10<sup>6</sup> bone marrow cells isolated from the primary recipient mice into lethally irradiated secondary recipients. Sixteen weeks after transplantation, p53<sup>R248W/+</sup> cells continued to show increased repopulating ability (Figure 1h). p53<sup>R248W</sup> did not affect myeloid and lymphoid differentiation in the peripheral blood of secondary recipients (Figure 1i). These findings suggest that expression of mutant p53 in normal HSCs does not cause leukemic transformation, but rather generates a premalignant state.<sup>15</sup>

HSCs in aged mice have decreased per-cell repopulating activity, self-renewal and homing abilities, myeloid skewing of differentiation, and increased apoptosis with stress.<sup>1,16</sup> To determine the role of mutant p53 in hematopoiesis during aging, we maintained the secondary transplantation recipient mice shown in Figure 1h for more than 12 months. We observed enhanced repopulating potential of p53<sup>R248W/+</sup> bone marrow cells compared to p53<sup>+/+</sup> cells 48 weeks after transplantation (Figure 1j). We found decreased frequency of

donor-derived myeloid cells and increased frequency of lymphoid cells in the PB of the recipients repopulated with  $p53^{R248W/+}$  cells (Figure 1k).

Tumor suppressor regulates HSC quiescence and response to genotoxic stress.<sup>11</sup> Given that  $p53^{-/-}$  mice are resistant to 5-FU treatment,<sup>10</sup> we hypothesized that  $p53^{R248W/+}$  mice may be resistant to chemotherapy treatment. We treated  $p53^{+/+}$  and  $p53^{R248W/+}$  mice with 5-FU weekly and then monitored their survival. While most wild-type mice were died 4 weeks following 5-FU treatment, all  $p53^{R248W/+}$  mice were still alive (Figure 2a). To measure the kinetics of hematopoietic recovery, we administered a single dose of 5-FU (200mg/kg) and serially followed peripheral blood cell counts. 5-FU-treated  $p53^{R248W/+}$  mice had less severe leukopenia than  $p53^{+/+}$  mice, with more rapid recovery (Figures 2b, S2a, S2b, S2c, and S2d). We treated  $p53^{+/+}$  and  $p53^{R248W/+}$  mice with one dose of 5-FU (200mg/kg) and then examined bone marrow cellularity, HSC frequency, and apoptosis one week later. We found that  $p53^{R248W/+}$  mice exhibited higher bone marrow cellularity compared to  $p53^{+/+}$  mice following 5-FU treatment (Figure S2e). There were more hematopoietic stem and progenitor cells in the bone marrow of 5-FU treated  $p53^{R248W/+}$  mice compared to that of the  $p53^{+/+}$  mice (Figures 2c, S2f, and S2g). Moreover,  $p53^{R248W/+}$  hematopoietic stem and progenitor cells were less apoptotic compared to  $p53^{+/+}$  HSPCs following 5-FU treatment (Figures 2d and S2h).

To determine the impact of 5-FU treatment on  $p53^{R248W/+}$  hematopoietic cells *in vivo*, we performed serial competitive transplantation assays using live bone marrow cells isolated from  $p53^{+/+}$  and  $p53^{R248W/+}$  mice treated with one dose of 5-FU (Figure S2i). We found that 5-FU treated  $p53^{R248W/+}$  bone marrow cells showed enhanced repopulation potential compared to 5-FU treated  $p53^{+/+}$  cells at 16 weeks following primary transplantation (Figure 2e). Multi-lineage differentiation of hematopoietic stem and progenitor cells in the peripheral blood was comparable in  $p53^{+/+}$  and  $p53^{R248W/+}$  mice (Figure 2f). We then transplanted  $3 \times 10^6$  BM cells isolated from primary recipient mice into lethally irradiated secondary recipient mice.  $p53^{R248W/+}$  bone marrow cells continued to show enhanced engraftment compared to  $p53^{+/+}$  cells at 16 weeks following secondary transplantation (Figure 2g). Mutant p53 did not affect differentiation in the peripheral blood and the bone marrow of secondary recipient mice (Figures 2h and S2j). The frequency of donor-derived hematopoietic stem and progenitor cells was significantly increased in the BM of secondary recipient mice repopulated with mutant BM cells (Figure 2i). To determine the impact of mutant p53 on DNA damage response, we treated wild type and mutant p53 mice with DMSO or 5-FU and then examined the expression of p53 target gene *p21* in HSPCs. While the mRNA levels of *p21* were significantly increased in wild-type cells following 5-FU treatment, the expression of *p21* in mutant cells was not induced by 5-FU treatment (Figure 2j).

To determine whether functional *TP53* mutations promote clonal expansion of HSCs following chemotherapy treatment, we generated mixed bone marrow chimaeras containing both  $p53^{+/+}$  and  $p53^{R248W/+}$  cells (the ratio of  $p53^{+/+}$  to  $p53^{R248W/+}$  cells is 10:1). 8 weeks following transplantation, recipient mice were treated with DMSO or chemotherapy drug *N*-ethyl-*N*-nitrosourea (ENU), respectively (Figure S3a). While DMSO treatment did not affect the repopulating ability of  $p53^{R248W/+}$  HSCs,  $p53^{R248W/+}$  HSCs outcompeted  $p53^{+/+}$  cells

and become clonal dominance upon ENU treatment (Figure 2k). ENU treatment did not affect the differentiation of  $p53^{+/+}$  and  $p53^{R248W/+}$  HSCs in the peripheral blood of recipient mice (Figure S3b). The frequency of mutant LSKs was significantly increased in the BM of recipient mice at 16 weeks following ENU treatment (Figures 2i and S3c). We sacrificed the recipient mice at 16 weeks following DMSO or ENU treatment and then performed pathological analysis to detect tumor formation. While there were no tumors in recipient mice treated with DMSO, 4 out of 7 recipient mice treated with ENU developed lymphoma and thymoma (data not shown).

Given p53 null hematopoietic stem and progenitor cells are less sensitive to irradiation,<sup>11–13</sup> we examined the impact of mutant p53 on HSC function following irradiation. We found that HSCs expressing mutant p53 show decreased apoptosis and increased repopulating potential compared to wild type HSCs following irradiation. Furthermore,  $p53^{R248W/+}$  HSCs show competitive advantage over  $p53^{+/+}$  cells and underwent clonal expansion following total body irradiation (SC and YL, unpublished data).

Thus, we have identified a critical role for mutant p53 in regulating the response of HSCs to genotoxic stresses. Further, we discovered that chemotherapy and radiotherapy cause the expansion of HSCs expressing mutant p53. While mutant p53 proteins accumulate in cancer cells, the levels of mutant p53 proteins in normal cells are very low.<sup>14, 17</sup> Genotoxic stresses stabilize mutant p53 in hematopoietic cells.<sup>14, 17–18</sup> Given that the  $p53^{R248W}$  mutant has both dominant-negative (DN) and gain-of-function (GOF) roles in human cancer,<sup>6, 14</sup> stabilized mutant p53 may inhibit the wild type p53 function or gain new oncogenic functions through protein-protein interactions to promote leukemic transformation. In addition, the expansion of mutant HSCs following genotoxic stresses may increase the possibility of acquiring additional genetic and/or epigenetic changes that facilitate leukemic transformation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

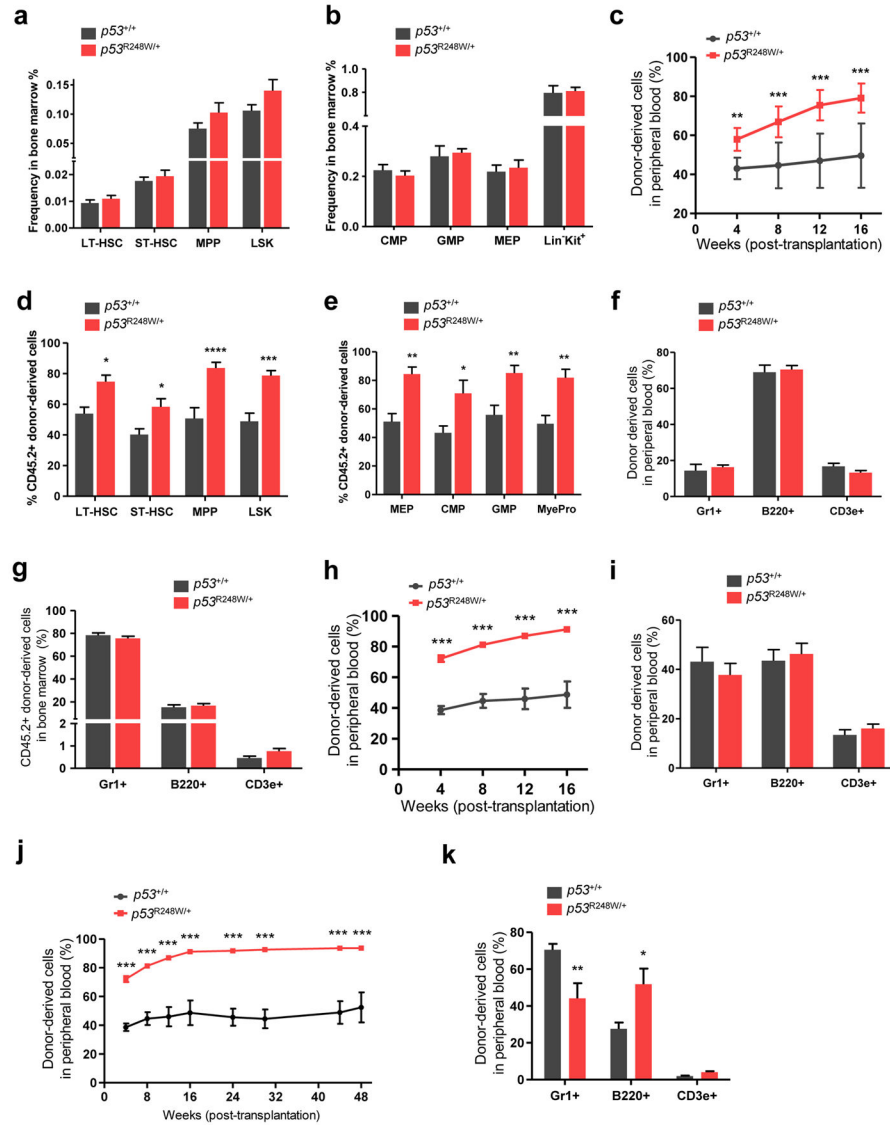
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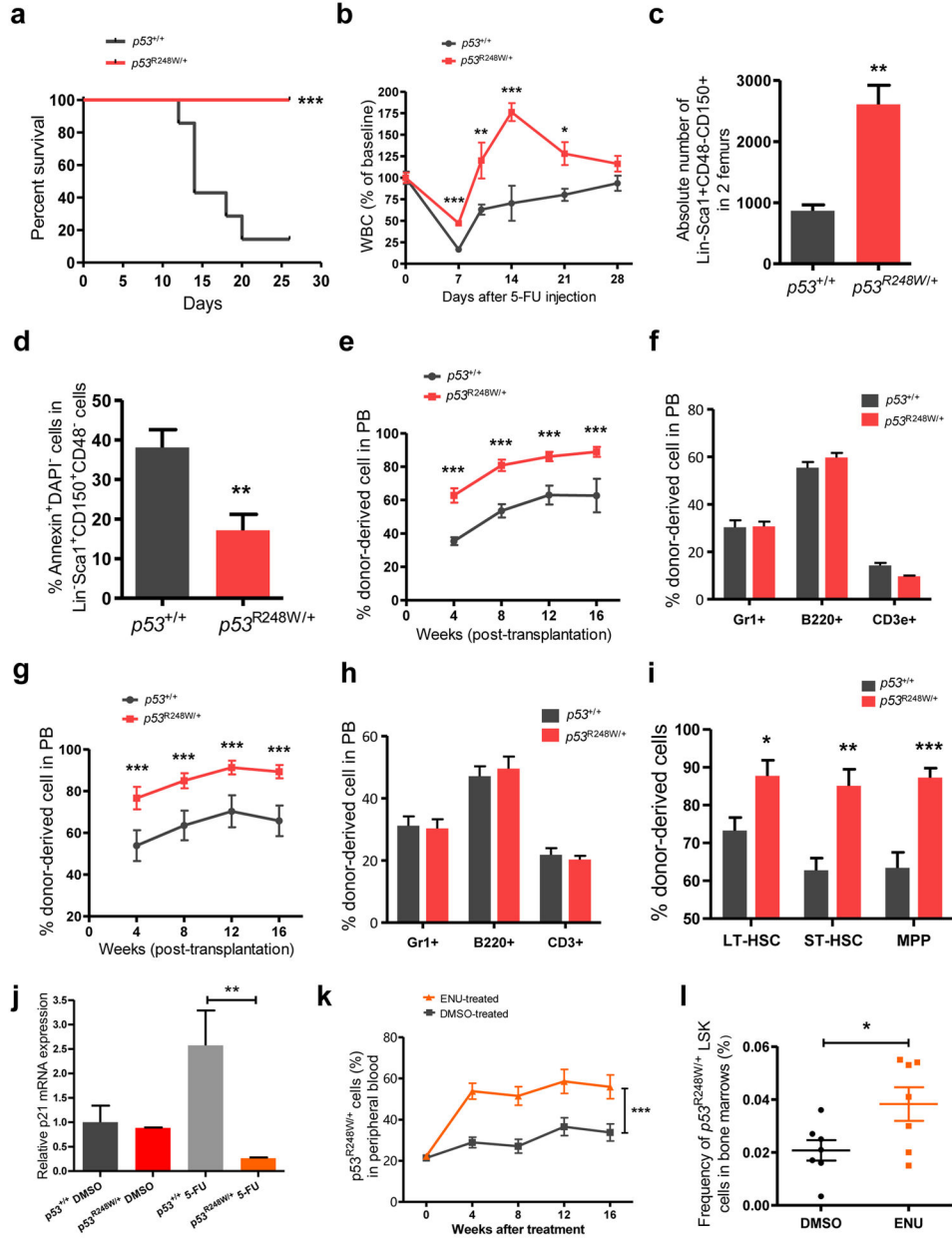
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**Figure 1.**

Mutant p53 enhances the repopulating potential of bone marrow cells. **(a)** The frequency of LT-HSCs, ST-HSCs, MPPs, and LSKs in the BM of young  $p53^{+/+}$  and  $p53^{R248W/+}$  mice.  $n=10$  mice per genotype. **(b)** The frequency of CMPs, MEPs, GMPs, and Lin<sup>-</sup>Kit<sup>+</sup> cells in the bone marrow of young  $p53^{+/+}$  and  $p53^{R248W/+}$  mice.  $n=10$  mice per genotype. **(c)** Percentage of donor-derived (CD45.2<sup>+</sup>) cells in the peripheral blood of primary recipient mice post-transplantation, measured at 4-week intervals. \*\* $p<0.01$ , \*\*\* $p<0.001$ ,  $n=7$  mice per group. **(d)** The frequency of donor-derived LT-HSCs, ST-HSCs, MPPs, and LSKs in the bone marrow of primary recipient mice 16 weeks following transplantation. \* $p<0.05$ , \*\*\* $p<0.001$ ,  $n=7$  mice per group. **(e)** The frequency of donor-derived MEPs, CMPs, GMPs, and myeloid progenitors (Lin<sup>-</sup>Kit<sup>+</sup>) in the bone marrow of primary recipient mice 16 weeks following transplantation. \* $p<0.05$ , \*\* $p<0.01$ ,  $n=7$  mice per group. **(f)** The percentage of donor-derived myeloid cells (Gr1<sup>+</sup>), B cells (B220<sup>+</sup>), and T cells (CD3e<sup>+</sup>) in the peripheral blood of primary recipient mice 16 weeks following transplantation.  $n=7$  mice

per group. **(g)** The percentage of donor-derived myeloid cells, B cells, and T cells in the bone marrow of primary recipient mice 16 weeks following transplantation.  $n = 7$  mice per group. **(h)** The percentage of donor-derived cells in the peripheral blood of secondary recipient mice.  $***p < 0.001$ ,  $n = 7$  mice per group. **(i)** The percentage of donor-derived myeloid cells, B cells, and T cells in the peripheral blood of secondary recipient mice 16 weeks following transplantation.  $n = 7$  mice per group. **(j)** The percentage of donor-derived cells in the peripheral blood of secondary recipients 48 weeks after transplantation.  $***p < 0.001$ ,  $n = 7$  mice per group. **(k)** The percentage of donor-derived myeloid cells, B cells, and T cells in the peripheral blood of secondary recipient mice 48 weeks after transplantation.  $*p < 0.05$ ,  $**p < 0.01$ ,  $n = 7$  mice per group.





**Figure 2.** Chemotherapy treatment promotes clonal expansion of HSCs expressing mutant p53. **(a)** Kaplan-Meier survival curve of  $p53^{+/+}$  and  $p53^{R248W/+}$  mice following weekly 5-FU treatment. 5-FU was administered intraperitoneally weekly (the initial dose was 125 mg/kg, with subsequent doses of 90 mg/kg) for 3 weeks and the survival rates of 5-FU treated mice were measured. Results were analyzed with a log-rank nonparametric test and expressed as Kaplan-Meier survival curves (\*\*\* $p$ <0.001,  $n$  = 10 mice per group). **(b)** Hematopoietic recovery of  $p53^{+/+}$  and  $p53^{R248W/+}$  mice following a single dose of 5-FU treatment (200 mg/kg intraperitoneally). WBC counts are shown at each point after 5-FU administration as a percentage of the initial values for each group of mice. Mean  $\pm$  SEM values are shown (\* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001,  $n$  = 5 mice per group for each time point). **(c)** Absolute



number of HSCs ( $\text{Lin}^- \text{Sca1}^+ \text{CD48}^- \text{CD150}^+$  cells) in the bone marrow of  $p53^{+/+}$  and  $p53^{\text{R248W}/+}$  mice seven days after a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Mean  $\pm$  SEM values are shown (\*\* $p < 0.01$ ,  $n = 5$  mice per group). **(d)** The percentage of early apoptotic (AnnexinV<sup>+</sup>DAPI<sup>-</sup>) HSCs ( $\text{Lin}^- \text{Sca1}^+ \text{CD48}^- \text{CD150}^+$  cells) in the bone marrow of  $p53^{+/+}$  and  $p53^{\text{R248W}/+}$  mice seven days after a single dose of 5-FU treatment (200 mg/kg intraperitoneally). Mean  $\pm$  SEM values are shown (\*\* $p < 0.01$ ,  $n = 5$  mice per group). **(e)** The percentage of donor-derived cells in the peripheral blood of primary recipient mice reconstituted with bone marrow cells from 5-FU treated  $p53^{+/+}$  and  $p53^{\text{R248W}/+}$  mice. Mean  $\pm$  SEM values are shown (\*\* $p < 0.001$ ,  $n = 7-8$  mice per group). **(f)** The percentage of donor-derived myeloid cells (Gr1<sup>+</sup>), B cells (B220<sup>+</sup>), and T cells (CD3e<sup>+</sup>) in the peripheral blood of secondary recipient mice 16-weeks after transplantation. Mean  $\pm$  SEM values are shown (n.s,  $p > 0.05$ ,  $n = 7-8$  mice per group). **(g)** The percentage of donor-derived cells in the peripheral blood of secondary recipient mice. Mean  $\pm$  SEM values are shown (\*\* $p < 0.001$ ,  $n = 7-8$  mice per group). **(h)** The percentage of donor-derived myeloid cells, B cells, and T cells in the peripheral blood of secondary recipient mice 16-weeks after transplantation. Mean  $\pm$  SEM values are shown (n.s,  $p > 0.05$ ,  $n = 7-8$  mice per group). **(i)** The chimerism of donor-derived LT-HSCs, ST-HSCs, and MPPs in the bone marrow of secondary recipient mice at 16 weeks following transplantation. Mean  $\pm$  SEM values are shown (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ,  $n = 7-8$  mice per group). **(j)** The mRNA levels of p53 target gene *p21* in hematopoietic stem and progenitor cells isolated from  $p53^{+/+}$  and  $p53^{\text{R248W}/+}$  mice treated with DMSO or 5-FU were determined by quantitative real-time PCR analysis. \*\* $p < 0.01$ ,  $n = 3$ . **(k)** Mutant p53 promotes clonal expansion of HSCs following chemotherapy treatment. The percentage of  $p53^{\text{R248W}/+}$  cells (CD45.2<sup>+</sup>) in the peripheral blood of recipient mice following ENU or DMSO treatment was determined by flow cytometry analysis at monthly intervals. Mean  $\pm$  SEM values are shown (\*\* $p < 0.001$ ,  $n = 7$  mice per group). **(l)** The frequency of  $p53^{\text{R248W}/+}$  LSK cells (CD45.2<sup>+</sup>) in the bone marrow of recipient mice at 16 weeks following ENU or DMSO treatment. Mean  $\pm$  SEM values are shown (\* $p < 0.05$ ,  $n = 7$  mice per group).