# Kit Regulates Maintenance of Quiescent Hematopoietic Stem Cells[1]

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**Hematopoietic stem cell (HSC) numbers are tightly regulated and maintained in postnatal hematopoiesis. Extensive studies have supported a role of the cytokine tyrosine kinase receptor Kit in sustaining cycling HSCs when competing with wild-type HSCs posttransplantation, but not in maintenance of quiescent HSCs in steady state adult bone marrow. In this study, we investigated HSC regulation in White Spotting 41 ($Kit^{W41/W41}$) mice, with a partial loss of function of Kit. Although the extensive fetal HSC expansion was Kit-independent, adult $Kit^{W41/W41}$ mice had an almost 2-fold reduction in long-term HSCs, reflecting a loss of roughly 10,000 $Lin^-Sca\text{-}1^+Kit^{high}$ (LSK)$CD34^-Flt3^-$ long-term HSCs by 12 wk of age, whereas $LSKCD34^+Flt3^-$ short-term HSCs and $LSKCD34^+Flt3^+$ multipotent progenitors were less affected. Whereas homing and initial reconstitution of $Kit^{W41/W41}$ bone marrow cells in myeloablated recipients were close to normal, self-renewing $Kit^{W41/W41}$ HSCs were progressively depleted in not only competitive but also noncompetitive transplantation assays. Overexpression of the anti-apoptotic regulator BCL-2 partially rescued the posttransplantation $Kit^{W41/W41}$ HSC deficiency, suggesting that Kit might at least in the posttransplantation setting in part sustain HSC numbers by promoting HSC survival. Most notably, accelerated in vivo BrdU incorporation and cell cycle kinetics implicated a previously unrecognized role of Kit in maintaining quiescent HSCs in steady state adult hematopoiesis.** *The Journal of Immunology,* **2008, 180: 2045–2053.**

With millions of blood cells being produced per second in humans, the hematopoietic system is strictly dependent on the constant replenishment of committed progenitor cells of all blood cell lineages from a rare population of pluripotent hematopoietic stem cells (HSCs)[4]. A defining HSC property is self-renewal, a requisite not only for sustained production of all mature blood cells and a normal HSC pool size, but also for the extensive HSC expansion that takes place during fetal development or following bone marrow (BM) transplantation (1, 2).

Although extensive efforts have been devoted toward the identification and clinical exploitation of HSC maintenance and expansion factors, knowledge about physiological regulators of HSC numbers remains limited (3). A number of hematopoietic cytokines have been demonstrated to potently promote the in vitro survival and growth of HSCs (4), but with the exception of thrombopoietin (5–7) efforts to confirm their proposed roles as physiological regulators of HSC numbers and expansion have been disappointing (3, 8), as have attempts to use cytokines to expand HSCs ex vivo (8).

The cytokine tyrosine kinase receptor Kit (also called c-Kit) is highly expressed on HSCs in steady state adult BM (9) in which HSCs, in contrast to rapidly expanding HSCs in fetal development and posttransplantation, are quiescent or slowly cycling (10–13). Furthermore, Kit ligand is a potent in vitro HSC viability factor (14, 15) that also promotes HSC proliferation when acting in synergy with other cytokines (16).

Studies in mice with either partial (17, 18) or complete (19) loss of function Kit mutations, have revealed severe hematopoietic deficiencies of multiple lineages (20, 21), as well as an important role of Kit in sustaining rapidly cycling HSCs posttransplantation when competing with wild-type (WT) HSCs (18, 22). However, they have failed to support a nonredundant role of Kit in maintaining the size of the HSC compartment in steady state adult hematopoiesis (17–19).

In this study, we sought to obtain a better understanding of the distinct roles and mechanisms of action of Kit in regulating HSC maintenance in steady state adult hematopoiesis. Toward this aim, we applied more advanced and recently described approaches to directly identify phenotypically and functionally distinct subsets of HSCs and multipotent progenitors (MPPs) (23, 24) in white Spotting 41 ($Kit^{W41/W41}$) mice, having a partial loss of Kit function (25). Furthermore, we assessed the potential role of Kit in regulation of HSC apoptosis and cell cycle.

## Materials and Methods

### *Mice*

WT CD45.1, CD45.2, or CD45.1/2 mice and $Kit^{W41/W41}$ (26) CD45.2 mice, all on a pure C57BL/6 background were used in this study. $Kit^{W41/W41}$ mice imported from The Jackson Laboratory and *H2K-BCL-2* mice (27) had been backcrossed to a C57BL/6 background for over 10 generations. All

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[4] Abbreviations used in this paper: HSC, hematopoietic stem cell; BM, bone marrow; FL, fetal liver; LSK, $lineage^-Sca\text{-}1^+Kit^{high}$; MPP, multipotent progenitor; WT, wild type.

animal protocols were approved by the local ethical committee at Lund University.

### *Abs used for phenotyping of BM and fetal liver (FL)*

All Abs were from BD Pharmingen unless otherwise indicated. Abs used for cell surface staining were rat and mouse anti-CD16/32 purified (clone 2.4G2), purified Gr-1 (RB6-8C5), purified CD11b/Mac-1 (M1/70), purified CD4 (H129.9), purified CD8$\alpha$ (53-6.7), purified Ter119 (LY-76), Sca-1-biotin (E13-161.7), Kit-allophycocyanin (2B8), CD34-FITC (RAM34), Flt3-PE (AZF10.1), streptavidin-PE Cy7. Polyclonal goat anti-rat Tricolor and streptavidin-PE Texas Red were from Caltag Laboratories.

### *FACS analysis of Lin$^-$Sca-1$^+$Kit$^{high}$ subpopulations in BM and FL*

FL cells were obtained from time-matched pregnant WT and *Kit$^{W41/W41}$* mice at 14.5 days postcoitum. Modifications of previously described procedures (23, 24) were used to evaluate the distribution of cells within the Lin$^-$Sca-1$^+$Kit$^{high}$ (LSK) compartment with exception of the lineage mixture used for staining FL cells, which did not include CD4 or Mac-1 (28). Analysis was performed on FACSCalibur (BD Biosciences) or FACSDiva (BD Biosciences) and data analysis was conducted using FlowJo software (Tree Star).

### *Cell cycle and caspase-3 analysis by FACS*

Age-matched WT and *Kit$^{W41/W41}$* mice were either allowed to freely drink water containing BrdU (1 mg/ml; Sigma-Aldrich) for 2 wk or were i.p. injected (1 mg/200 $\mu$l) twice (morning and evening) 1 day before isolation of BM cells for analysis. Cells were stained for Lin-purified, Sca-1-PE Cy5.5 (D7; eBioscience), Kit-allophycocyanin, CD34-biotin visualized by streptavidin-PE Cy7, Flt3-PE, and with a BrdU staining kit (FITC) (BD Pharmingen).

Cell cycle and active caspase-3 analysis was performed by staining for Lin-purified, Sca-1-PE Cy5.5 (D7; eBioscience), Kit-allophycocyanin Alexa Fluor 750 (eBioscience), Flt3-PE, and CD34-biotin, followed by fixation with Cytofix/Cytoperm kit (BD Biosciences) and staining for Ki67-FITC (B56; BD Pharmingen) and DAPI (Serva Eletrophoresis) or active caspase-3-FITC (C92-605; BD Pharmingen), respectively. Analysis was performed on a FACS LSR II (BD Biosciences) or FACSDiva (BD Biosciences), and data analysis was conducted using FlowJo software.

### *In vivo repopulation assay in congenic mouse model*

Competitive reconstitution, limiting dose and noncompetitive reconstitution assays were performed as previously reported (23). Lethally irradiated (900 cGy) recipient mice (CD45.1) were transplanted with different numbers of BM cells, and peripheral blood analyzed at various time points posttransplantation for donor reconstitution by FACS. The multilineage peripheral blood analysis was a modification of a previously reported protocol (23, 29). Briefly, peripheral blood leukocytes were stained with donor-specific and lineage-specific CD45.1-PE (A20), CD45.2-FITC (A104), Mac-1-allophycocyanin (M1/70), B220-PE Cy5 and allophycocyanin (RA3-6B2), CD4-PE Cy5 (H129.9), and CD8$\alpha$-PE Cy5 (53-6.7) Abs. Positively reconstituted mice were defined as having a minimum of 0.1% total and 0.02% of each of the myeloid, B and T cell lineages derived from the test population. To determine self-renewal potentials of test cells, secondary ($2^0$) and tertiary ($3^0$) transplantations were performed as previously described (29). When limiting transplantation doses were used, the HSC frequency was calculated by applying Poisson statistics to the proportion of positive recipients using L-calc (StemCell Technologies) (7).

### *Quantitative PCR*

RNA extraction and quantitative PCR of adult BM LSKCD34$^-$Flt3$^-$ of WT and *Kit$^{W41/W4}$* cells were performed as previously described (24). TaqMan Assays-on-Demand probes used were caspase-3 (ABI Assay ID no. Mm00438045_m1) and HPRT (ABI Assay ID no. Mm00446968_m1). Each experiment was performed in triplicates, and differences in cDNA input were compensated by normalizing against HPRT expression levels.

### *Statistics*

For parametric data distribution an unpaired *t* test was used. For nonparametric data distribution a Mann-Whitney test or Van Elteren's test was used. Statistical software used was SAS 8.2, SPSS 15.0, or Prism 4. Data consists of four different strains of mice that we denote by $x_{ij}$, in which $i = 1, 2, 3, 4$ and $j = 1, 2, 3, 4$, and so on, and $n_i$ is the sample size of the $i$-th series. Data are assumed to be independent samples and drawn from a common distribution within each series. By denoting the population mean of series $i$ using $\mu_i$, we would ideally like to test the null hypothesis $H_0$: $\mu_1/\mu_2/ \leq \mu_3/\mu_4$ vs the alternative $H_1$: $\mu_1/\mu_2 > \mu_3/\mu_4$, and a natural test statistic for this would be $\bar{x}_1/\bar{x}_2 - \bar{x}_3/\bar{x}_4$ where $\bar{x}_i$ is the sample mean of series $i$. Estimators involving ratios of sample means are generally difficult to analyze, however, in particular for small sample sizes, and we therefore chose to work with differences of logarithmic data.

Thus let $z_{ij} = \log x_{ij}$, and denote by $\mu'_i$ the population mean of the $i$ transformed series. We tested the null hypothesis $H'_0$: $\mu'_1 - \mu'_2 \leq \mu'_3 - \mu'_4$ vs the alternative $H'_1$: $\mu'_1 - \mu'_2 > \mu'_3 - \mu'_4$. Here $H'_0$ is similar to $H_0$ but not identical, as the operations "population mean" and "logarithm" cannot be interchanged; indeed, for any random variable $X$ it holds that $\mathrm{E}[\log X] \leq \log \mathrm{E}[X]$, where E denotes population mean (*expectation*). This follows from a result known as Jensen's inequality. Nevertheless a first-order Taylor expansion (linearisation) of the logarithm function about the mean $\mu = \mathrm{E}[X]$ of $X$ yields the approximation $\mathrm{E}[\log X] \approx \log \mathrm{E}[X]$. It is also clear that the difference $\mu'_1 - \mu'_2$ measures the difference of the locations of populations 1 and 2, but on a logarithmic scale rather than the original one. To test $H'_0$ we constructed the *t*-type statistic

$$T = \frac{(\bar{z}_1 - \bar{z}_2) - (\bar{z}_3 - \bar{z}_4)}{\sqrt{s_1^2/n_1 + s_2^2/n_2 + s_3^2/n_3 + s_4^2/n_4}}$$

where $\bar{z}_i$ is the sample mean of the $i$-th transformed series and

$$s_i^2 = (n_i - 1)^{-1} \sum_{j=1}^{4} (z_{ij} - \bar{z}_i)^2$$

is the corresponding sample variance. The estimator in the numerator has variance

$$\sum_{ii=1}^{4} \sigma_i^2 / n_i$$

where $\sigma_i^2$ is the population variance of the $i$-th transformed series, and the expression inside the square-root in the denominator of $T$ is an estimate of this quantity. Notice that we did not use a pooled variance estimator because the data did not support an assumption of equal variances, i.e., $\sigma_i^2$ $i$ being equal for all series $i$.

With an equal variance assumption and a pooled variance estimator, $T$ would have had a $t$ distribution for normally distributed data; with unequal variances, the situation is more complex. Thus, we used an extension of the so-called Aspin-Welch approximation to the distribution of the two-sample $t$ test with unequal variances (30), namely approximating the distribution of $T$ with a $t$ distribution with

$$\nu = \frac{\left( \sum_{i=1}^{4} s_i^2/n_i \right)^2}{\sum_{i=1}^{4} (s_i^2/n_i^2)/(n_i - 1)}$$

degrees of freedom. Simulations with means and variances as in the actual data indicated that this approximation works well. The final $p$ value for testing $H'_0$ vs $H'_1$ was computed for a one-sided test, i.e., it was the probability that a sample from a $t$ distribution with $\nu$ degrees of freedom exceeds the observed $T$. The number of mice included in each statistical analysis is specified in each experiment.

## Results

### *Role of Kit in maintenance of HSCs in adult steady state BM*

We first investigated the distribution of different HSC subsets (23, 24) in 14.5 days postcoitum FL of *Kit$^{W41/W41}$* mice. The cellularity of *Kit$^{W41/W41}$* FL was reduced by 27% (Fig. 1*A*). However the frequency (Fig. 1*B*) and the total number of cells (Fig. 1*C*) of LSKFlt3$^-$ and LSKFlt3$^+$ subsets, containing HSCs and MPPs, respectively, were not significantly different in *Kit$^{W41/W41}$* and WT mice (Fig. 1, *B* and *C*).

We next examined whether Kit might be involved in regulating the maintenance of LSKCD34$^-$Flt3$^-$ long-term HSCs, LSKCD34$^+$Flt3$^-$ short-term HSCs, or LSKCD34$^+$Flt3$^+$ MPPs in adult steady state BM (23, 24). Notably, in 10- to 12-wk-old mice, a selective and 1.7-fold reduction in the minor LSKCD34$^-$Flt3$^-$ long-term HSC compartment was observed ($p < 0.05$), whereas the LSKCD34$^+$Flt3$^-$ short-term HSC and LSKCD34$^+$Flt3$^+$ MPP compartments were not significantly affected (Fig. 1, *D–F*). At 28 wk of age, the LSKCD34$^-$Flt3$^-$ long-term HSC compartment was

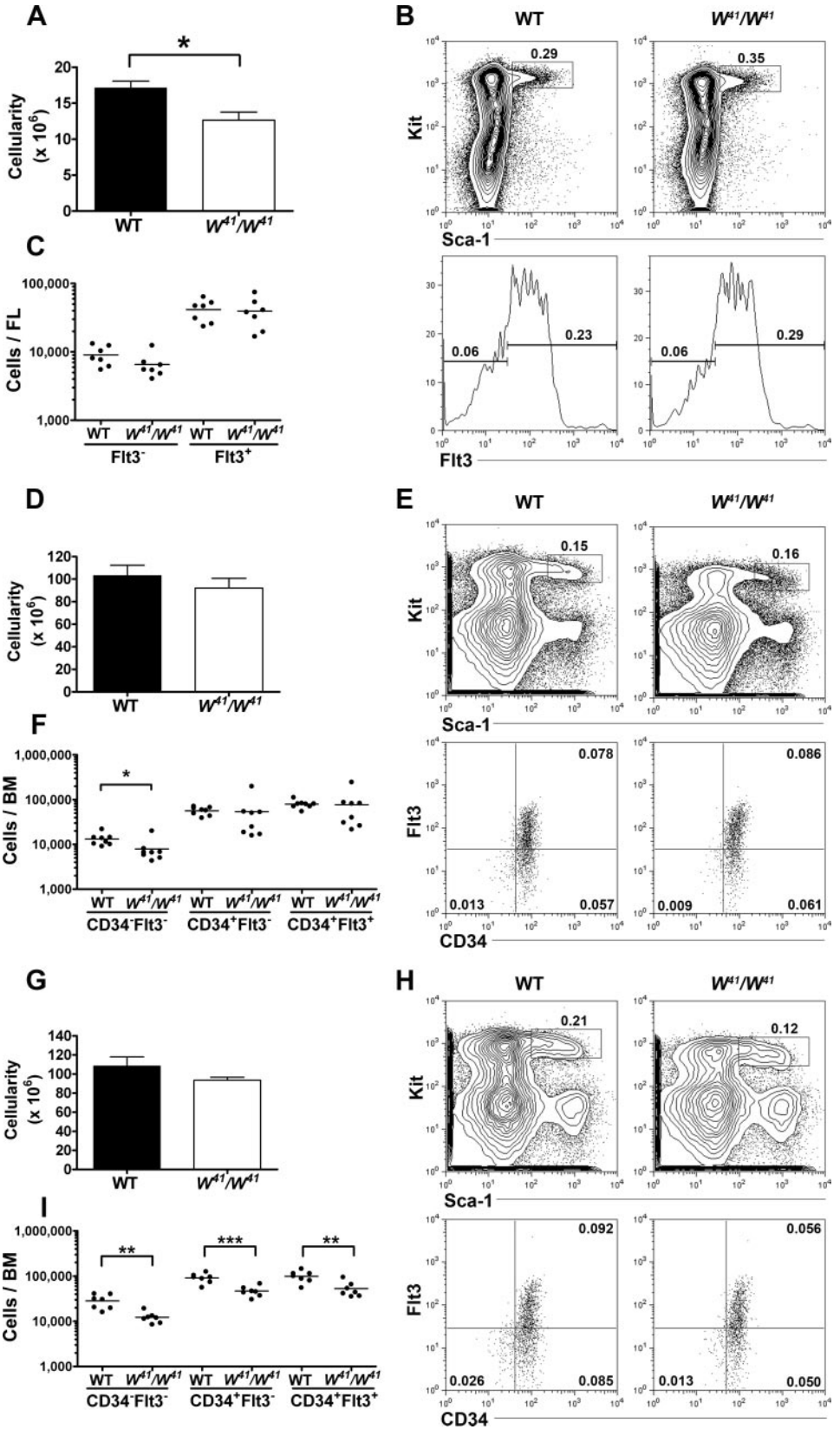


**FIGURE 1.** Adult $Kit^{W41/W41}$ mice have a selective and progressive reduction in LSKCD34$^-$Flt3$^-$ long-term HSCs. *A*, Mean cellularity of FL 14.5 days postcoitum ($n = 7$ mice for each genotype). *B*, Representative FACS plots of LSK analysis in FL from WT and $Kit^{W41/W41}$ mice. Representative histograms of Flt3 expression within the gated LSK compartments are shown (*bottom*). *C*, Absolute numbers of LSKFlt3$^-$ and LSKFlt3$^+$ cells in WT and $Kit^{W41/W41}$ FL. Data are for $n = 7$ mice for each genotype. *D*, Mean BM cellularity of 10- to 12-wk-old WT and $Kit^{W41/W41}$ mice. Data are for $n = 8$ mice for each genotype. *E*, Representative FACS plots of LSK analysis in 10- to 12-wk-old WT and $Kit^{W41/W41}$ BM. The CD34 and Flt3 expression profiles of gated LSK cells are shown (*bottom*). *F*, Absolute numbers of LSK subsets per two femurs and two tibias. *G–I*, Mean BM cellularity, LSK analysis, and absolute LSK subset numbers, as analyzed in *D–F*, except analysis is of 28-wk-old mice ($n = 7$ for each genotype). All data including FACS plots and histograms show mean percentage of total cells for all investigated mice, and error bars represent SD. Horizontal bar indicates mean value in *C*, *F*, and *I*. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

further reduced (2.3-fold, $p < 0.01$) (Fig. 1, *G–I*), and at this time also the LSKCD34$^+$Flt3$^-$ short-term HSC and LSKCD34$^+$Flt3$^+$ MPP compartments were significantly reduced (2.0- and 1.9-fold, respectively; $p < 0.001$ and $p < 0.01$, respectively). Thus, although intact Kit function is not essential for generation and fetal expansion of HSCs, this kinetic analysis points to an important role of Kit in maintaining the long-term HSC compartment in adult steady state BM.

Unlike in steady state BM, where HSCs are mostly quiescent (10, 11), HSCs transplanted into lethally irradiated recipients are activated to expand and rapidly regenerate all levels of hematopoiesis, including regeneration of the HSC compartment itself (2).

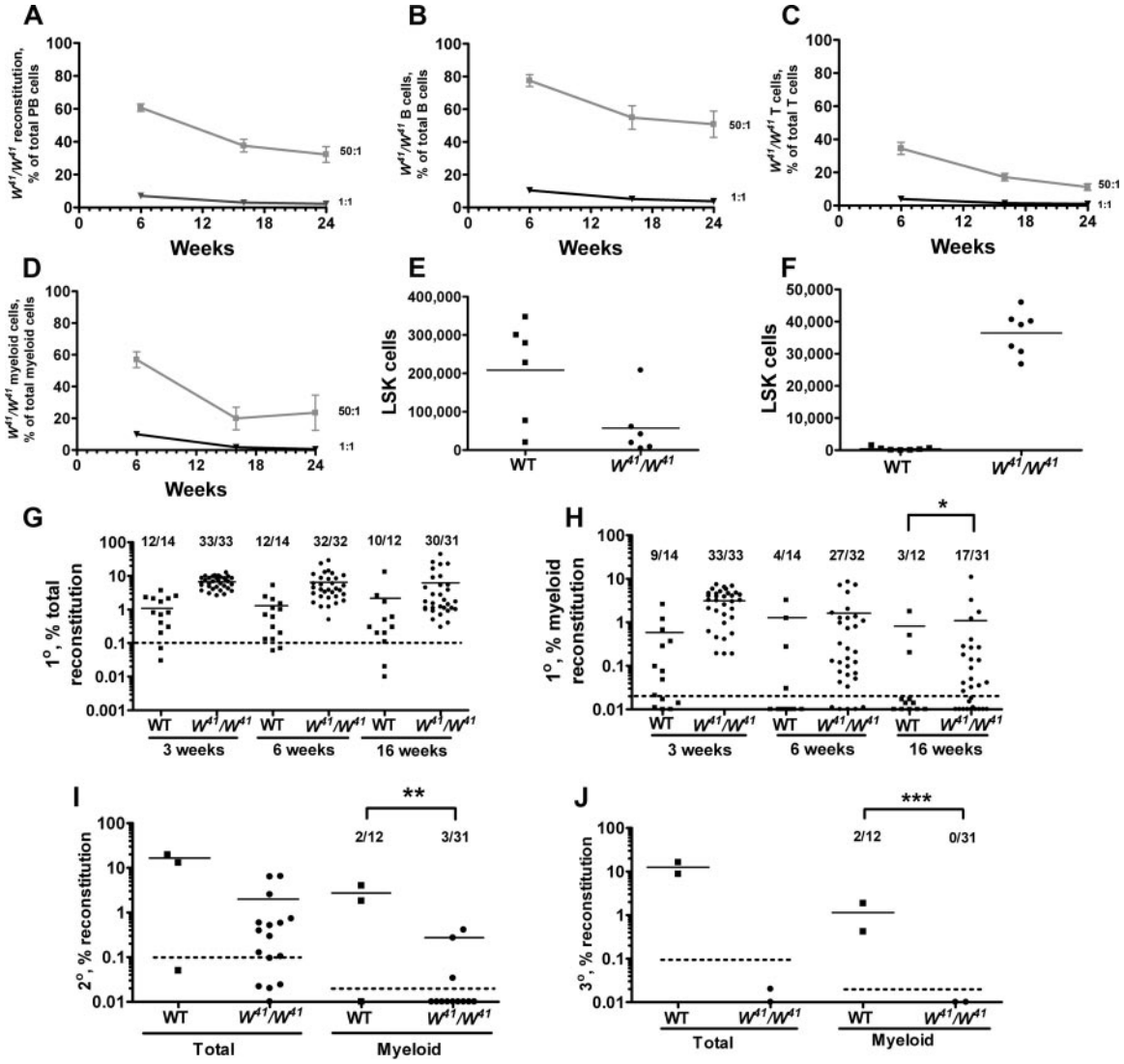


**FIGURE 2.** Kit is critical for sustaining HSC numbers posttransplantation. Lethally irradiated WT mice (CD45.1) were transplanted with $Kit^{W41/W41}$ (CD45.2) BM cells in competition with WT (CD45.1/2 or CD45.1) BM cells from 10- to 12-wk-old mice at ratios 1:1 ($2 \times 10^6$ WT and $2 \times 10^6$ $Kit^{W41/W41}$) and 50:1 ($1 \times 10^5$ WT and $5 \times 10^6$ $Kit^{W41/W41}$). Peripheral blood was analyzed 6, 16, and 24 wk posttransplantation for $Kit^{W41/W41}$ contribution toward total (*A*), B cell (*B*), T cell (*C*), and myeloid (*D*) reconstitution. Mean and SEM are shown for $n = 7$ mice in each group. *E* and *F*, Reconstitution of LSK cells in BM of mice transplanted with a 50-fold excess $Kit^{W41/W41}$ cells, as compared with competing WT BM cells, at 17 wk ($n = 6$ mice) (*E*) and 2 wk ($n = 7$) (*F*) after transplantation, presented as LSK cells per two femurs and two tibias. *G* and *H*, 100,000 $Kit^{W41/W41}$ (CD45.2) or 10,000 WT (CD45.2) cells were transplanted into lethally irradiated WT mice (CD45.1) together with 200,000 WT BM support (CD45.1) cells. Peripheral blood was analyzed at 3, 6, and 16 wk posttransplantation. Data are the percentage of CD45.2 contribution to total reconstitution (*G*) and myeloid reconstitution (*H*) in primary ($1^0$) recipients. After 16 wk, all positive mice (>0.1% total and 0.02% multilineage donor reconstitution) were serially transplanted (0.5 femur equivalent) into secondary ($2^0$) (*I*) and subsequently tertiary ($3^0$) (*J*) lethally irradiated WT (CD45.1) recipients and peripheral blood was analyzed 12 wk posttransplantation. Horizontal bars (*E–J*) indicate mean values of positive mice. Frequency indicates fraction of total transplanted mice positive for multilineage (combined myeloid, B cell, and T cell) reconstitution. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

This self-renewal activation from a relatively small HSC transplantation dose (2), puts considerably more demand on each individual HSC than in steady state hematopoiesis. Thus, we next transplanted lethally irradiated WT mice with whole BM cells, in either 1:1 or 50:1 ratios of $Kit^{W41/W41}$ and WT cells, respectively (Fig. 2, *A–F*). At 24 wk posttransplantation, contribution of $Kit^{W41/W41}$ cells toward total peripheral blood cells was only 2% and 32% in the 1:1 and 50:1 recipients, respectively (Fig. 2, *A–D*), with just 1% and 24% respective contribution to the short-lived myeloid lineage (Fig. 2*D*) in agreement with previous studies (18). Furthermore, although transplanted at a 50-fold excess, $Kit^{W41/W41}$ BM cell contribution to the LSK compartment at 25 wk posttransplantation was only 22% (Fig. 2*E*), which translates into an ~200-fold reduction in the ability of $Kit^{W41/W41}$ BM cells to reconstitute the LSK compartment. In contrast, the $Kit^{W41/W41}$ contribution to the LSK compartment was much higher at 2 wk posttransplantation analysis of 50:1 ratio recipients (Fig. 2*F*), where $Kit^{W41/W41}$ cells contributed to 80-fold more of the LSK compartment than WT cells, suggesting that homing and initial engraftment of HSCs is not significantly affected by Kit deficiency. The inability of Kit-deficient HSCs to sustain the HSC pool in a competitive transplantation setting was confirmed in primary ($1^0$), secondary ($2^0$), and tertiary ($3^0$) recipients using limiting doses of $Kit^{W41/W41}$ BM cells together with competitor BM cells (Fig. 2, *G–J*). Based on the ability to sustain multilineage reconstitution in primary recipients, in particular of short-lived myeloid cells, a significant reduction in

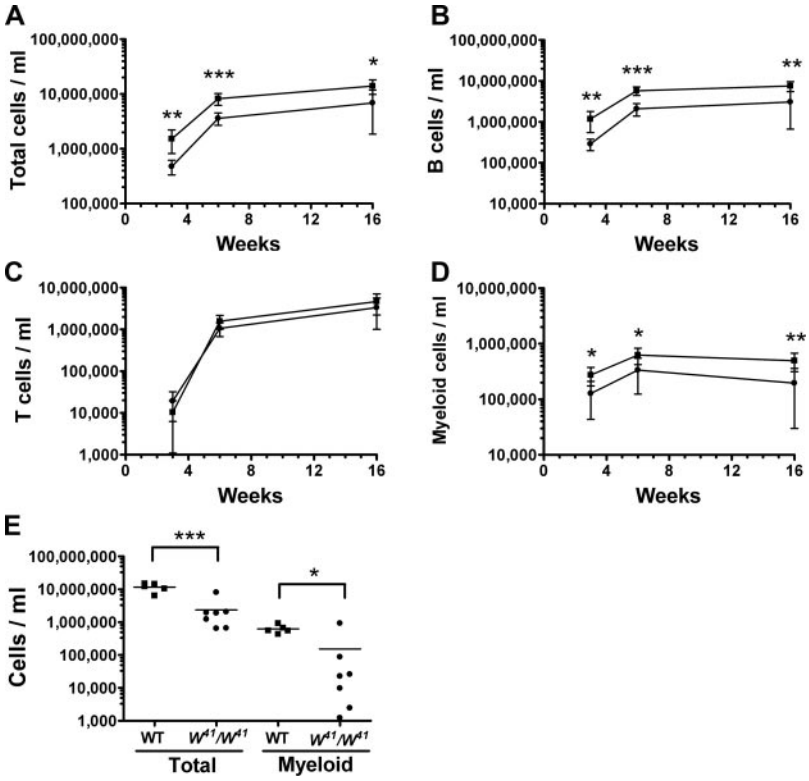


**FIGURE 3.** Reduced repopulation and self-renewal of Kit-deficient HSCs in a noncompetitive transplantation assay. Lethally irradiated WT recipients (CD45.1) were transplanted with $5 \times 10^5$ $Kit^{W41/W41}$ or WT (CD45.2) BM cells, from 10- to 12-wk-old mice, in a noncompetitive setting. At 3, 6, and 16 wk after transplantation, contribution toward donor-derived (CD45.2) total (*A*), B cell (*B*), T cell (*C*), and myeloid (*D*) reconstitution was determined. Data are for $n = 7$ mice of each genotype. Symbols represent WT (squares) and $Kit^{W41/W41}$ (circles) mice. Error bars indicate SD. *E*, Half a femur equivalent from positive primary ($1^0$) recipients was serially transplanted into secondary ($2^0$) recipients, and peripheral blood was analyzed at 12 wk, ($n = 5$ WT mice and $n = 7$ $Kit^{W41/W41}$ mice). Horizontal bar indicates mean value. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

the frequency of long-term repopulating cells was found in the $Kit^{W41/W41}$ mice (1/35,000 and 1/125,000 for WT and $Kit^{W41/W41}$ BM cells, respectively, $p < 0.05$) (Fig. 2, *G* and *H*). This difference was further exacerbated when comparing the ability of WT and BM cells to sustain multilineage reconstitution in the secondary ($p < 0.01$) and tertiary ($p < 0.001$) recipients (Fig. 2, *I* and *J*).

These experiments and previous findings (17, 18, 22), demonstrating reduced maintenance and regeneration of Kit-deficient long-term HSCs were exclusively observed in settings in which Kit-deficient HSCs were cotransplanted with WT HSCs. Thus, it remained possible that the role of Kit in regulating posttransplantation HSC expansion might not be relevant in a noncompetitive BM transplantation. Thus, we next transplanted lethally irradiated mice with a moderate number ($5 \times 10^5$) of WT or $Kit^{W41/W41}$ BM cells. At 16 wk posttransplantation, total and myeloid reconstitution levels were significantly reduced (2- and 2.5-fold, respectively) in recipients of $Kit^{W41/W41}$ compared with WT BM cells (Fig. 3, *A–D*). This reduction was further exacerbated in secondary recipients, wherein total and myeloid reconstitution levels were reduced 5- and 4-fold, respectively, at 12 wk posttransplantation (Fig. 3*E*), demonstrating an important role of Kit in HSC expansion and maintenance also in a noncompetitive setting.

### *Role of Kit in survival and maintenance of quiescent adult HSCs*

We next sought to establish whether the physiological role of Kit in regulating HSCs might primarily reflect the reported ability of Kit ligand to potently promote HSC survival in vitro (14, 15). Quantitative PCR analysis demonstrated that transcriptional expression of caspase-3 was not altered in $Kit^{W41/W41}$ $LSKCD34^-Flt3^-$ long-term HSCs, when compared with WT $LSKCD34^-Flt3^-$ cells ($p = 0.7$) (Fig. 4*A*). Next, we examined by FACS each of the $LSKCD34^-Flt3^-$ long-term HSC, $LSKCD34^+Flt3^-$ short-term HSC, and $LSKCD34^+Flt3^+$ MPP compartments of $Kit^{W41/W41}$ and WT BM for expression of active caspase-3, to establish altered propensity toward apoptosis (31). Notably, whereas cytokine-deprived BaF3 cells showed enhanced active caspase-3 expression (Fig. 4*B*), active caspase-3 expression was virtually undetectable and indistinguishable between $Kit^{W41/W41}$ and WT $LSKCD34^-Flt3^-$ long-term HSCs (Fig. 4*C*). However, as apoptotic cells are cleared rapidly in vivo, and apoptosis due to Kit deficiency might selectively affect long-term HSCs, which represent only a fraction of $LSKCD34^-Flt3^-$ cells, these findings could not exclude a role of Kit in suppressing HSC apoptosis. Thus, as overexpression of BCL-2 has previously been shown to rescue the in vivo hematopoietic defects of other cytokine deficiencies (32–34), we next intercrossed $Kit^{W41/W41}$ mice with mice overexpressing BCL-2 under control of the *H2K* promoter, ensuring high levels of BCL-2 in HSCs (27). Although we in agreement with previous studies (27), found *H2K-BCL-2* mice to have a slight expansion of phenotypically defined HSCs (Fig. 4*D*), we found that Kit deficiency resulted in a similar reduction in $LSKCD34^-Flt3^-$ long-term HSCs on a *H2K-BCL-2* background (2.3-fold reduction) as on a WT background (3.1-fold reduction) in steady state adult BM ($p = 0.4$) (Fig. 4*D*). BCL-2 also failed to significantly rescue the reduction observed in $LSKCD34^+Flt3^-$ and $LSKCD34^+Flt3^+$ cells in $Kit^{W41/W41}$ mice ($p = 0.3$ and 0.6, respectively) (Fig. 4, *E* and *F*). However, the long-term competitive multilineage repopulating ability of $Kit^{W41/W41}$ BM cells was significantly better sustained on an *H2K-BCL-2* background than on a WT background ($p < 0.001$) (Fig. 4, *G–J*), supporting a limited role of Kit in suppressing apoptosis of HSCs posttransplantation. In agreement with this observation, also donor LSK numbers in $Kit^{W41/W41}$ recipients were somewhat better maintained on a BCL-2 background (Fig. 4*K*).

As the steady state HSC deficiency in $Kit^{W41/W41}$ mice could not be rescued by BCL-2, other cellular mechanisms than antiapoptotic pathways might be more important in Kit-mediated maintenance of self-renewing long-term HSCs. As previous studies have suggested that uniquely protracted $G_0$ and $G_1$ phases of the cell

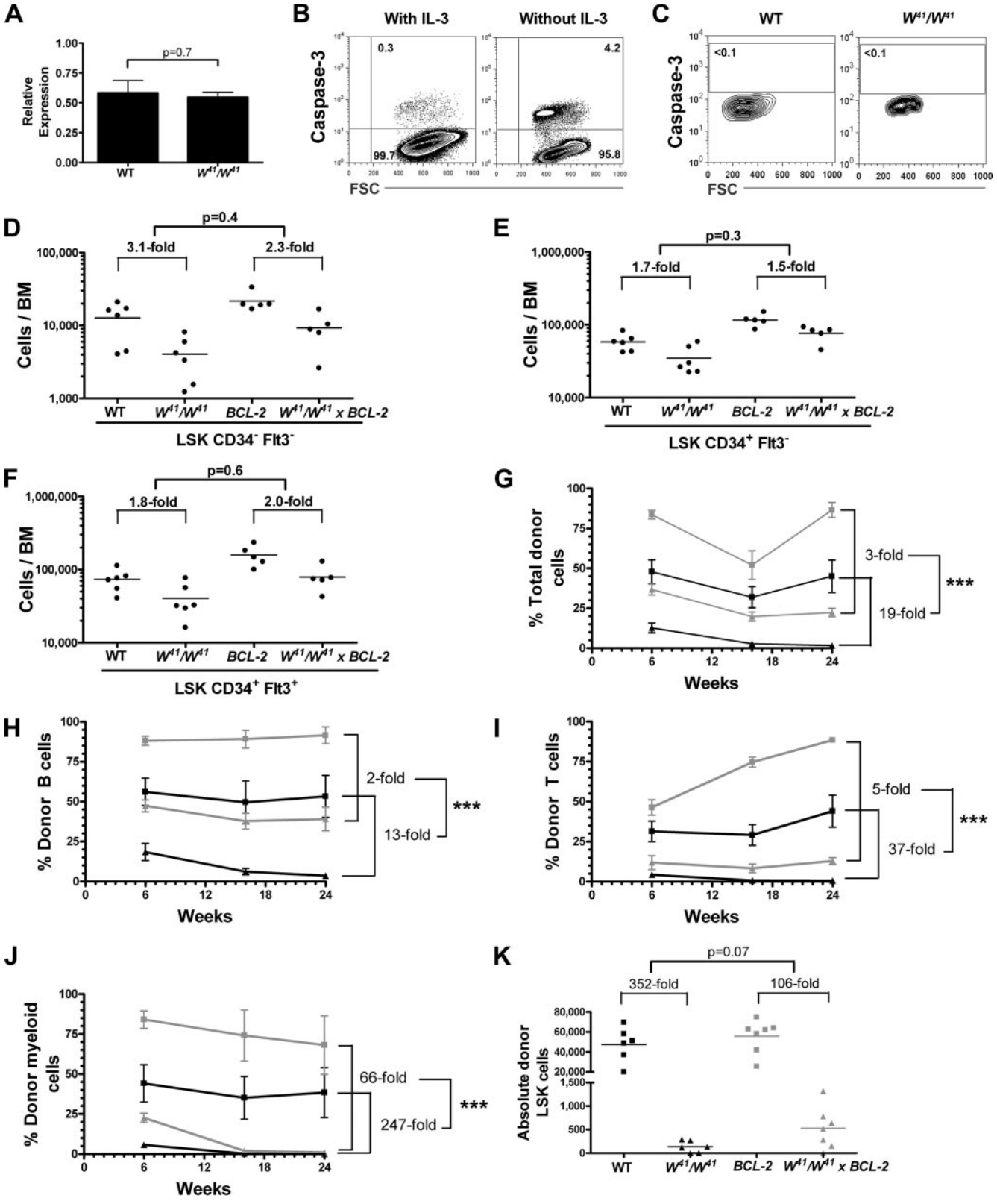


**FIGURE 4.** Involvement of Kit in antiapoptotic regulation of HSCs. *A*, Caspase-3 mRNA expression was determined by quantitative PCR. Results show caspase-3 expression levels relative to HPRT. Mean and SD values from two experiments are shown. *B*, BaF3 cells were starved in culture for 24 h without IL-3 and subsequently fixed/permeabilized and stained with an Ab against active caspase-3. Nonstarved cells served as negative control. *C*, Active caspase-3 expression was measured by intracellular FACS staining within LSKCD34$^-$Flt3$^-$ long-term HSC (LT-HSC) compartment. Representative FACS plots for WT and *Kit*$^{W41/W41}$ LSKCD34$^-$Flt3$^-$ cells ($n = 7$ mice per group). Difference in gate setting between *B* and *C* reflect different FACS instrument settings during analysis. *D–F*, Absolute number per two femurs and two tibias of LSKCD34$^-$Flt3$^-$ long-term HSCs (*D*), LSKCD34$^+$Flt3$^-$ short-term HSCs (*E*), and LSKCD34$^+$Flt3$^+$ MPP (*F*) cells in WT, *Kit*$^{W41/W41}$, *H2K-BCL-2*, and *Kit*$^{W41/W41}$ × *H2K-BCL-2* mice ($n = 5–6$ mice per group). *G–J*, One million unfractionated BM cells from either WT, *Kit*$^{W41/W41}$, *H2K-BCL-2*, or *Kit*$^{W41/W41}$ × *H2K-BCL-2* (CD45.2) mice were transplanted together with $1 \times 10^6$ WT (CD45.1) competitor cells into lethally irradiated WT (CD45.1) recipients and peripheral blood was analyzed at 6, 16, and 24 wk for total (*G*), B cell (*H*),

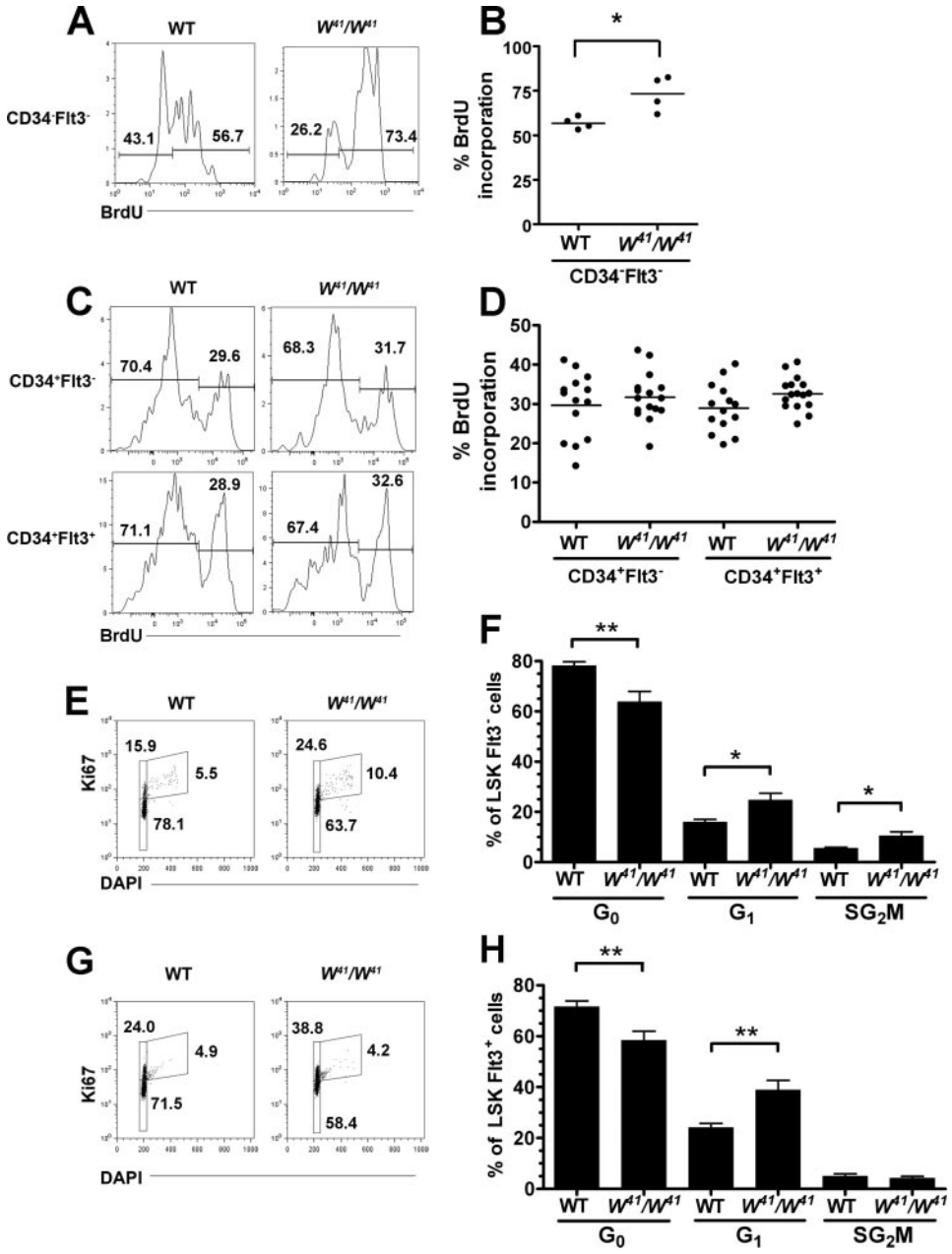


**FIGURE 5.** Increased turnover in $Kit^{W41/W41}$ long-term HSC compartment as measured by BrdU incorporation and Ki67 expression. *A* and *B*, To investigate cycling of long-term HSCs (LSKCD34$^-$Flt3$^-$), BrdU was administrated for 2 wk in the drinking water (1 mg/ml) to 10- 14-wk-old WT and $Kit^{W41/W41}$ mice and BrdU incorporation within the LSKCD34$^-$Flt3$^-$ was analyzed by FACS. *C* and *D*, BrdU was i.p. injected (1 mg/200 $\mu$l), and LSKCD34$^+$Flt3$^-$ and LSKCD34$^+$Flt3$^+$ compartments were analyzed by FACS 1 day after injections. Representative FACS profiles (*A* and *C*) and mean and SD values (*B* and *D*) for 4–15 mice of each genotype are shown from three representative experiments. *E–H*, Cell cycle kinetics in Kit-deficient HSCs. Cell cycle distribution was analyzed by Ki67 and DAPI staining of LSKFlt3$^-$ and LSKFlt3$^+$ cells of 10- to 12-wk-old WT and $Kit^{W41/W41}$ mice. Representative FACS profile of LSKFlt3$^-$ cells (*E*) and the percentage of cells in $G_0$, $G_1$, and $SG_2M$ (*F*) are shown. *G* and *H*, Representative FACS profile of LSKFlt3$^+$ cells (*G*) and the percentage of cells in $G_0$, $G_1$, and $SG_2M$ (*H*) are shown as in *E* and *F*. Mean and SEM of $n = 8$ mice of each genotype from two experiments. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

cycle is a defining property of self-renewing HSCs (10–12, 35), and as several intrinsic regulators of HSC numbers recently have been shown to act in part by maintaining HSC quiescence (36, 37), we next investigated the cell cycle status and cycling kinetics of $Kit^{W41/W41}$ HSCs in 10- to 14-wk-old mice. Strikingly, and in agreement with their selective reduction at this age, LSKCD34$^-$Flt3$^-$ long-term HSCs displayed accelerated BrdU incorporation kinetics ($p < 0.05$) (Fig. 5, *A* and *B*), whereas BrdU incorporation of LSKCD34$^+$Flt3$^-$ short-term HSCs as well as LSKCD34$^+$Flt3$^+$ MPP cells in $Kit^{W41/W41}$ mice were only slightly (nonsignificantly) enhanced (Fig. 5, *C* and *D*). In agreement with this observation, $Kit^{W41/W41}$ mice also had fewer LSKFlt3$^-$ HSCs in $G_0$ ($p < 0.01$), as determined by DAPI and Ki67 staining, and corresponding significant increases of cells in $G_1$ ($p < 0.05$) and $SG_2M$ ($p < 0.05$) (Fig. 5, *E* and *F*). The LSKFlt3$^+$ MPP population showed a similar pattern in support of enhanced cycling (Fig. 5, *G* and *H*). These findings suggest that Kit and its ligand might play a previously unrecognized role in sustaining HSCs in steady state hematopoiesis by ensuring protraction of their cell cycle transit.

## Discussion

In this study, to better establish and understand the potential role of cytokine receptors in regulation of HSCs, we studied in detail the HSC compartment in $Kit^{W41/W41}$ mice, harboring a point mutation in the kinase domain resulting in a partial loss of function of the Kit tyrosine kinase receptor (25).

T cell (*I*), and myeloid (*J*) donor reconstitution. Data for WT (■), $Kit^{W41/W41}$ (▲), *H2K-BCL-2* (▨), and $Kit^{W41/W41}$ × *H2K-BCL-2* (gray triangles) mice are shown as mean and SD ($n = 14$ mice per group). *K*, Absolute donor LSK cells 25 wk after transplantation of WT, $Kit^{W41/W41}$, *H2K-BCL-2* and $Kit^{W41/W41}$ × *H2K-BCL-2* BM cells ($n = 6$–7 mice). Results were expressed in *D–K* as mean and SD. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

In agreement with previous studies (9, 22), we found that the nonsignificant and mild (29%) reduction in absolute numbers of LSKFlt3$^-$ FL cells was exclusively related to the reduced cellularity of *Kit*$^{W41/W41}$ FL, confirming that Kit has little or no role in promoting the extensive expansion of HSCs that takes place during fetal development (2, 9). In contrast, in 10- to 12-wk-old mice we found a 1.7-fold reduction in the long-term HSC compartment, which was further reduced (to 2.3-fold) by 28 wk of age, indicating that, although intact Kit function is not essential for generation and fetal expansion of HSCs, it is important for maintaining the long-term HSC compartment in steady state adult BM. Notably, as WT mice have ~15,000 long-term HSCs in the BM (38), our findings of a 2- to 3-fold reduction in the long-term HSC compartment (as determined by phenotypic analysis and serial transplantation of limiting numbers of BM cells) implies that a partial loss of Kit function in *Kit*$^{W41/W41}$ mice results in loss of >10,000 long-term HSCs in steady state hematopoiesis, within the first 6 mo of life.

In a posttransplantation setting in which HSCs are required to undergo extensive self-renewing divisions, we confirmed a dramatic reduction in the maintenance of *Kit*$^{W41/W41}$ HSCs when competing with WT HSCs, and more importantly we demonstrated for the first time that *Kit*$^{W41/W41}$ HSCs are deficient in their maintenance also in a noncompetitive setting. Because the initial HSC (LSK) reconstitution was unaffected by the Kit deficiency, these findings argue against Kit primarily being important for HSC homing and initial engraftment of the HSC compartment, and rather suggest that Kit might be required for the extensive self-renewing divisions that HSCs must undergo following transplantation into myeloablated recipients.

The mechanisms by which cytokines, such as thrombopoietin and Kit ligand, might regulate HSC maintenance have not yet been established. Although, Kit ligand through in vitro studies has been demonstrated to be a potent viability factor for HSCs (14), the physiological importance of this factor has not been established. Through the use of mice overexpressing the antiapoptotic regulator BCL-2, capable of potently rescuing the phenotype of other cytokine receptor-deficient mice (32–34), we were able to demonstrate in the posttransplantation setting, but not in steady state hematopoiesis, a small but significant rescue effect of BCL-2 on HSC reconstitution in mice transplanted with *Kit*$^{W41/W41}$ BM cells. Importantly, this established that Kit might be promoting HSC maintenance, at least partially in the posttransplantation setting, through suppression of apoptosis. Although the lack of evidence for BCL-2 rescuing the HSC phenotype in steady state *Kit*$^{W41/W41}$ mice and the caspase-3 analysis failed to support a role of Kit in suppressing HSC apoptosis, it is important to emphasize that this lack of evidence does not preclude an antiapoptotic role of Kit in steady state HSC regulation. Other antiapoptotic regulators, not investigated in this study, might prove more important in promoting potential antiapoptotic effects than BCL-2, although overexpression of BCL-2 has proved sufficient to rescue other hematopoietic defects resulting from cytokine deficiencies (32–34).

As the HSC loss due to the *Kit*$^{W41/W41}$ deficiency, in steady state adult BM, could not be rescued by overexpression of BCL-2, it appeared evident that Kit might regulate HSC maintenance through alternative modes of action. In that regard, our finding of enhanced cycling kinetics of *Kit*$^{W41/W41}$ LSKCD34$^-$Flt3$^-$ cells (as demonstrated through cell cycle analysis as well as BrdU incorporation), is of considerable interest, not only because a protracted $G_0/G_1$ progression is uniquely coupled to HSC self-renewal (12, 13), but also because recently identified essential intrinsic regulators of HSC maintenance also appear to act largely by suppressing HSC cell cycle progression (36, 37). It is possible that Kit-Kit ligand interaction might be critical for the anchoring of HSCs in the stem cell niche, and thus the enhanced cycling might be secondary event to the release of the HSCs from the niche in the *Kit*$^{W41/W41}$ mice. In that regard, our studies implicate an important role of cytokines in maintaining quiescent HSCs, perhaps through inhibition of their cell cycle progression. This finding comes as a surprise, as Kit ligand primarily has been described as a synergistic factor that enhances in vitro proliferation of stem and progenitor cells in combination with other cytokines (4). Thus, based on our findings, the strategies by which Kit ligand has been used in attempts to promote ex vivo HSC self-renewal and expansion should perhaps be reconsidered.

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## Disclosures

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