

# Hmgb3 regulates the balance between hematopoietic stem cell self-renewal and differentiation

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**Hmgb3 is an X-linked member of a family of sequence-independent chromatin-binding proteins that is preferentially expressed in hematopoietic stem cells (HSC). *Hmgb3*-deficient mice (*Hmgb3*<sup>-/-</sup>) contain normal numbers of HSCs, capable of self-renewal and hematopoietic repopulation, but fewer common lymphoid (CLP) and common myeloid progenitors (CMP). In this study, we tested the hypothesis that *Hmgb3*<sup>-/-</sup> HSCs are biased toward self-renewal at the expense of progenitor production. Wild-type and *Hmgb3*<sup>-/-</sup> CLPs and CMPs proliferate and differentiate equally *in vitro*, indicating that CLP and CMP function normally in *Hmgb3*<sup>-/-</sup> mice. *Hmgb3*<sup>-/-</sup> HSCs exhibit constitutive activation of the canonical Wnt signaling pathway, which regulates stem cell self-renewal. Increased Wnt signaling in *Hmgb3*<sup>-/-</sup> HSCs corresponds to increased expression of *Dvl1*, a positive regulator of the canonical Wnt pathway. To induce hematopoietic stress and a subsequent response from HSCs, we treated *Hmgb3*<sup>-/-</sup> mice with 5-fluorouracil. *Hmgb3*<sup>-/-</sup> mice exhibit a faster recovery of functional HSCs after administration of 5-fluorouracil compared with wild-type mice, which may be due to the increased Wnt signaling. Furthermore, the recovery of HSC number in *Hmgb3*<sup>-/-</sup> mice occurs more rapidly than CLP and CMP recovery. From these data, we propose a model in which *Hmgb3* is required for the proper balance between HSC self-renewal and differentiation.**

hematopoiesis | Wnt

**H**ematopoietic stem cells (HSC) are a rare population of cells that predominantly reside within adult bone marrow and are responsible for life-long replenishment of all blood cell types (1). HSCs differentiate into common lymphoid (CLP) and common myeloid progenitors (CMP) that are capable of exponential proliferation and differentiation. To maintain their numbers, HSCs undergo self-renewal, which generates progeny that retain the ability for multilineage differentiation. The mechanisms by which the fate of HSCs, either self-renewal or differentiation, is determined are still unclear.

The proteins that comprise the high-mobility group (HMG) superfamily are among the most abundant nonhistone proteins in the eukaryotic nucleus (2). *Hmgb3* is an X-linked member of this superfamily and is classified with *Hmgb1* and -2 into the Hmg-box subfamily, defined by the presence of DNA-binding Hmg-box domains (3). The 80% identity between Hmg-box proteins (4), suggests similar functions at the molecular level. *Hmgb1* and -2 have been shown to bind DNA without sequence specificity and can bend linear DNA (5, 6). They also interact directly with Hox and Oct family transcription factors, which have been reported to be important regulators of stem cell differentiation (7–10).

*Hmgb1*, -2, and -3 exhibit different patterns of gene expression in the adult mouse. *Hmgb1* expression is ubiquitous, *Hmgb2* is primarily expressed in the thymus and testes, and *Hmgb3* expression is localized to the bone marrow (4, 11, 12). The tissue-specific expression patterns may be responsible for the phenotypes observed in mouse knockout models. *Hmgb1* deficiency results in lethal hypoglycemia in newborn mice and, whereas *Hmgb2*<sup>-/-</sup> mice are viable, homozygous males exhibit decreased fertility because of defective spermatogenesis (4, 11).

In adult bone marrow, *Hmgb3* is found in maturing Ter119<sup>+</sup> erythroid cells and in populations enriched for HSCs, CLPs, and CMPs (12). *Hmgb3* is a marker for HSCs, whereas *lin*<sup>-</sup>, *c-kit*<sup>HI</sup>, and *Hmgb3*<sup>NEG</sup> cells were incapable of long-term bone marrow repopulation. *Hmgb3*-deficient mice (*Hmgb3*<sup>-/-</sup>) are viable and hematologically normal except for a mild erythrocythemia (13). *Hmgb3*<sup>-/-</sup> mice contain normal numbers of HSCs, which are capable of self-renewal and long-term repopulation. However, there are reduced numbers of CLPs and CMPs in *Hmgb3*<sup>-/-</sup> mice, which is compensated by increased proliferation of more mature progenitors, resulting in normal numbers of hematopoietic cells.

The canonical Wnt signaling pathway regulates proliferation and differentiation of several cellular systems, including multiple types of stem cells (reviewed in Logan and Nusse, ref. 14). In this pathway, binding of Wnt ligand to its cognate receptor leads to activation of the Dishevelled (*Dvl1*) protein, which ultimately causes stabilization and nuclear translocation of  $\beta$ -catenin and the subsequent induction of target gene expression. Activation of this pathway has been shown to increase HSC and leukemia stem cell self-renewal (15, 16).

In this study, we have focused on the role of *Hmgb3* in regulating cell-fate decisions of HSCs. We hypothesized that the reduction in CLP and CMP numbers in *Hmgb3*<sup>-/-</sup> mice may be because of either more rapid differentiation of CLPs and CMPs or decreased production of CLPs and CMPs. We observed that CLPs and CMPs from wild-type and *Hmgb3*<sup>-/-</sup> mice proliferate and differentiate at similar rates, leading us to hypothesize that the decreased numbers of CLPs and CMPs were because of their impaired production by the HSC. We observed enhanced activation of the canonical Wnt signaling pathway in *Hmgb3*-deficient HSCs. Consistent with this observation, *Hmgb3*-deficient HSCs contain increased levels of *Dvl1* mRNA. We compared the effects of 5-fluorouracil (5-FU), which transiently eliminates *c-kit*<sup>HI</sup> cells, on HSC, CLP, and CMP populations in wild-type and *Hmgb3*<sup>-/-</sup> mice (17). After 5-FU treatment, *c-kit*<sup>HI</sup> HSCs in *Hmgb3*<sup>-/-</sup> mice recovered more rapidly than in wild-type mice. Furthermore, recovery of *c-kit*<sup>HI</sup> hematopoietic progenitors in *Hmgb3*<sup>-/-</sup> mice was biased in favor of HSCs over CLPs and CMPs. Together, our data suggest that *Hmgb3* deficiency biases HSCs toward self-renewal at the expense of differentiation, and that the normal function of *Hmgb3* is to regulate the balance between these cell-fate decisions.

## Results

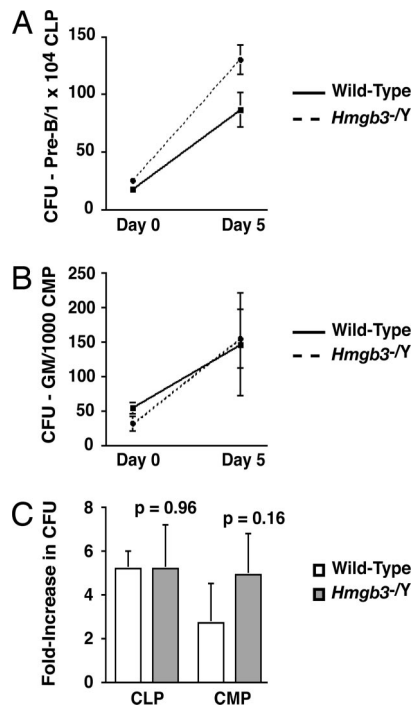
**Analysis of CLP and CMP Function in *Hmgb3*-Deficient Mice.** We hypothesized that the reduced number of phenotypic CLP and CMP in *Hmgb3*<sup>-/-</sup> mice may be caused by rapid differentiation of *Hmgb3*<sup>-/-</sup> CLP and CMP, resulting in fewer cells with the typical CLP or CMP phenotype. To test this hypothesis, we performed *in*

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Abbreviations: 5-FU, 5-fluorouracil; CFU-GM, CFU-granulocyte-macrophage; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; HMG, high-mobility group; HSC, hematopoietic stem cell; SP, side population.

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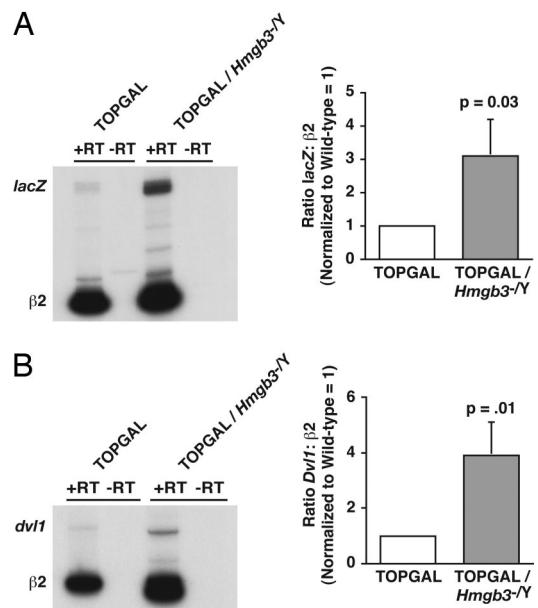


**Fig. 1.** Expansion of CLP and CMP *in vitro*. (A) Mean CFU-Pre-B frequency generated from CLPs ( $lin^{-}$ ,  $c-kit^{LO}$ ,  $Sca-1^{LO}$ , and  $IL-7R\alpha^{+}$ ) before (day 0) and after 5 days in culture (day 5). CFU-Pre-B frequency was determined by scoring CFU-Pre-B per  $1 \times 10^4$  CLP at day 0 and at day 5. Wild-type,  $n = 12$ , 212 colonies counted on day 0, 1,038 colonies counted on day 5; *Hmgb3*<sup>-/-</sup>,  $n = 12$ , 306 colonies counted on day 0, 1,563 colonies counted on day 5. The data represent the pooled results of three independent experiments. Error bars represent standard deviations. (B) Mean CFU-GM frequency generated from CMPs ( $lin^{-}$ ,  $c-kit^{+}$ ,  $Sca-1^{-}$ , and  $IL-7R\alpha^{-}$ ) before (day 0) and after 5 days in culture (day 5). CFU-GM frequency was determined by scoring CFU-GM per 1,000 CMP at day 0 and at day 5. Wild-type,  $n = 12$ , 646 colonies counted on day 0, 1,755 colonies counted on day 5; *Hmgb3*<sup>-/-</sup>,  $n = 12$ , 377 colonies counted on day 0, 1,854 colonies counted on day 5. The data represent the pooled results of three independent experiments. Error bars represent standard deviations. (C) Average expansion in CFU generated by CLP and CMP before and after 5 days in culture ( $n = 3$ ). The expansion in the number of CFU for each individual experiment was calculated by dividing the average CFU frequency at day 5 by the average CFU frequency at day 0. Error bars represent standard error of the mean. *P* values were generated by Student's *t* test.

*in vitro* "delta" assays (18, 19), in which the formation of clonogenic colonies derived from CLPs ( $lin^{-}$ ,  $c-kit^{+}$ ,  $Sca-1^{+}$ , and  $IL-7R\alpha^{+}$ ; ref. 20) and CMPs ( $lin^{-}$ ,  $c-kit^{+}$ ,  $Sca-1^{-}$ ,  $IL-7R\alpha^{-}$ ; ref. 21) isolated from *Hmgb3*<sup>-/-</sup> and littermate control mice were compared before and after 5 days of expansion in serum-free culture.

Cultured *Hmgb3*<sup>-/-</sup> and wild-type CLPs proliferated equivalently in culture ( $1.3 \pm 0.2$ - and  $1.4 \pm 0.6$ -fold, respectively;  $P = 0.7$ ). CFU-pre-B formation by wild-type and *Hmgb3*<sup>-/-</sup> CLP also increased equivalently after 5 days in culture ( $5.2 \pm 0.8$ - and  $5.2 \pm 2.0$ -fold, respectively;  $P = 0.96$ ; Fig. 1A and C). Wild-type and *Hmgb3*<sup>-/-</sup> CMPs proliferated equivalently ( $6.5 \pm 2.2$ - and  $5.7 \pm 1.5$ -fold, respectively;  $P = 0.6$ ). There was no significant difference in CFU-granulocyte-macrophage (CFU-GM) formation after 5 days in culture by *Hmgb3*<sup>-/-</sup> CMP compared with wild-type ( $4.9 \pm 1.9$ - and  $2.7 \pm 1.8$ -fold, respectively;  $P = 0.16$ ; Fig. 1B and C). These data indicate that under identical *in vitro* conditions, wild-type and *Hmgb3*<sup>-/-</sup> CLP and CMP are functionally similar in regard to proliferation and differentiation.

**Activation of Wnt Signaling Through Overexpression of Dvl1 in *Hmgb3*-Deficient HSC.** Based on these data, we hypothesized that *Hmgb3*-deficient HSCs are biased toward self-renewal, resulting in

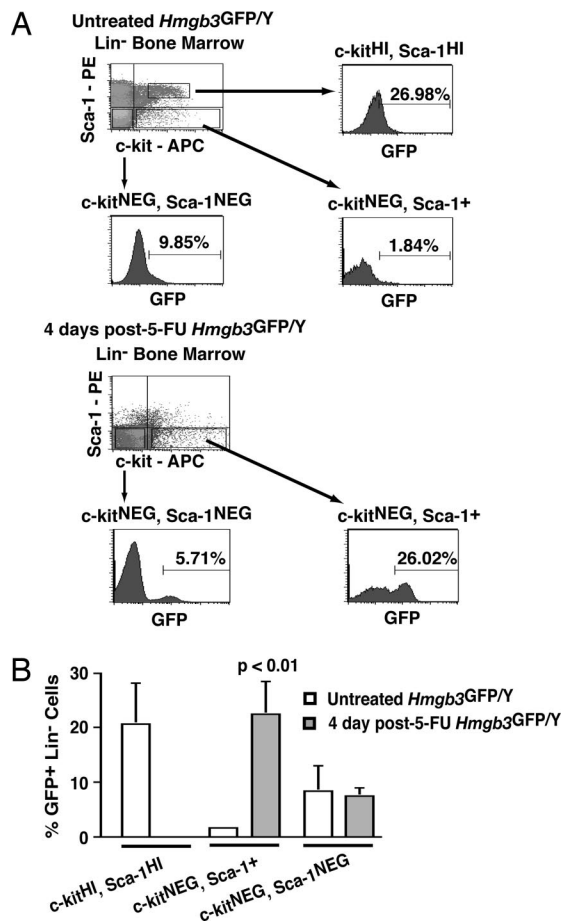


**Fig. 2.** Increased canonical Wnt signaling in *Hmgb3*<sup>-/-</sup> HSCs after 5-FU treatment. (A) (Left) Semiquantitative duplex RT-PCR analysis of  $\beta$ -galactosidase mRNA in TOPGAL (wild-type) and TOPGAL  $\times$  *Hmgb3*<sup>-/-</sup> HSCs ( $lin^{-}$ , SP<sup>+</sup>,  $c-kit^{HI}$ ,  $Sca-1^{HI}$ , and  $IL-7R\alpha^{-}$ ). Test gene mRNA levels were quantified within the linear range of amplification by densitometry and normalized to  $\beta$ 2-microglobulin expression. (Right)  $\beta$ -Galactosidase mRNA levels relative to  $\beta$ 2-microglobulin. The amount of  $\beta$ -galactosidase (*lacZ*) mRNA in TOPGAL ( $n = 3$ ) and TOPGAL  $\times$  *Hmgb3*<sup>-/-</sup> ( $n = 3$ ) HSCs was normalized to  $\beta$ 2-microglobulin mRNA ( $\beta$ 2) by the formula (densitometry value, test; densitometry value,  $\beta$ 2-microglobulin). The normalized TOPGAL  $\beta$ -galactosidase mRNA level was then set to 1. The data represent the pooled results of three independent experiments by using mRNA isolated from sorted HSCs pooled from three mice. *P* values were determined by Student's *t* test. (B) (Left) Semiquantitative duplex RT-PCR analysis of *Dvl1* mRNA in wild-type TOPGAL and TOPGAL  $\times$  *Hmgb3*<sup>-/-</sup> HSCs ( $lin^{-}$ , SP<sup>+</sup>,  $c-kit^{HI}$ ,  $Sca-1^{HI}$ , and  $IL-7R\alpha^{-}$ ). Test gene mRNA levels were quantified within the linear range of amplification by densitometry and normalized to  $\beta$ 2-microglobulin expression. (Right) *Dvl1* mRNA levels relative to  $\beta$ 2-microglobulin. The amount of *Dvl1* mRNA in TOPGAL ( $n = 3$ ) and TOPGAL  $\times$  *Hmgb3*<sup>-/-</sup> ( $n = 3$ ) HSCs was normalized to  $\beta$ 2-microglobulin mRNA as described above.

a deficiency in CLP and CMP. The canonical Wnt signaling pathway has been reported to activate self-renewal pathways in HSCs and leukemia stem cells (15, 16). To test our hypothesis, we examined the status of the canonical Wnt signaling pathway in wild-type and *Hmgb3*-deficient HSCs containing a *lacZ* reporter gene that is specifically expressed when the canonical Wnt pathway is activated (TOPGAL; ref. 22). Using RT-PCR, we observed a  $3.1 \pm 1.1$ -fold ( $P = 0.03$ ) increase in the amount of *lacZ* mRNA in steady-state HSCs [ $lin^{-}$ , side population<sup>+</sup> (SP; ref. 23),  $Sca-1^{+}$ ,  $c-kit^{+}$ , and  $IL-7R\alpha^{-}$ ] isolated from TOPGAL  $\times$  *Hmgb3*<sup>-/-</sup> F<sub>1</sub> mice compared with TOPGAL controls (Fig. 2A), indicating a higher level of Wnt signaling in *Hmgb3*-deficient HSCs.

To determine the mechanism of increased Wnt signaling in *Hmgb3*-deficient HSCs, we analyzed the mRNA levels of several components of the canonical Wnt pathway. Using RT-PCR, we observed a  $3.9 \pm 1.2$ -fold increase in *Dvl1* mRNA levels in *Hmgb3*-deficient HSCs compared with wild-type (Fig. 2B). We were unable to detect any differences in the mRNA levels of *Axin*, *APC*, and *Btrc*, which are negative regulators of Wnt signaling (data not shown).

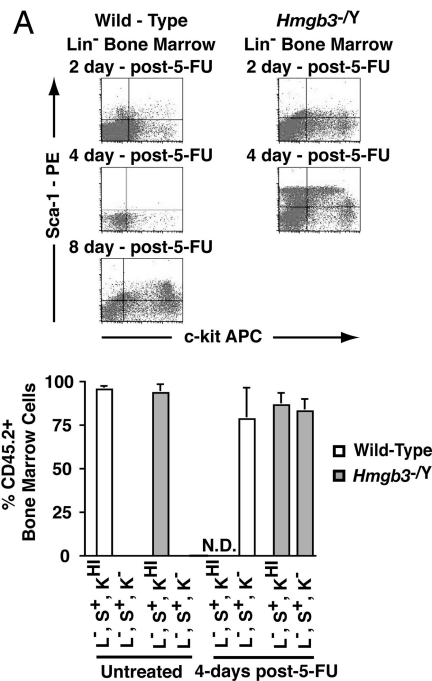
***Hmgb3* Is Expressed Specifically in  $c-kit^{-}$  Cells with Long-Term Repopulating Activity After 5-FU Treatment.** Randall and Weissman (17) have shown that 5-FU transiently alters the HSC phenotype



**Fig. 3.** Analysis of *Hmgb3* expression in primitive hematopoietic cells after 5-FU. (A) Representative FACS analysis of GFP<sup>+</sup> cells within various c-kit and Sca-1 populations in lin<sup>-</sup> bone marrow cells isolated from untreated *Hmgb3*<sup>GFP/Y</sup> mice and from *Hmgb3*<sup>GFP/Y</sup> mice 4 days after 5-FU administration. Regions were drawn based on isotype controls. (B) Average percentage of GFP<sup>+</sup> cells in lin<sup>-</sup> populations (c-kit<sup>HI</sup>, Sca-1<sup>HI</sup>; c-kit<sup>NEG</sup>, Sca-1<sup>+</sup>; c-kit<sup>NEG</sup>, and Sca-1<sup>NEG</sup>) from untreated *Hmgb3*<sup>GFP/Y</sup> mice and from *Hmgb3*<sup>GFP/Y</sup> mice 4 days after 5-FU administration. Bone marrow cells from three mice were pooled for each independent experiment; data represent the average of three experiments. Error bars represent standard deviation. *P* values were generated by Student's *t* test.

from c-kit<sup>HI</sup> to c-kit<sup>LO/NEG</sup> followed by a reappearance of the c-kit<sup>HI</sup> HSC after 8 days. According to our model, the HSCs in *Hmgb3*-deficient mice are actively engaging in self-renewal and should recover faster after 5-FU treatment than wild-type HSCs. We first established whether *Hmgb3* was expressed in c-kit<sup>NEG</sup> HSCs after 5-FU treatment by monitoring *Hmgb3* expression in lin<sup>-</sup> cells isolated from phenotypically normal *Hmgb3*<sup>GFP/Y</sup> knockin mice before and after treatment with 5-FU. *Hmgb3*<sup>GFP/Y</sup> mice contain an internal ribosomal entry site-GFP cassette inserted into the 3' UTR of *Hmgb3*, enabling the tracing of *Hmgb3* expression through GFP (12).

Before 5-FU treatment, 20.6 ± 1.7% of lin<sup>-</sup>, c-kit<sup>HI</sup>, and Sca-1<sup>HI</sup> cells (HSCs) were GFP<sup>+</sup> as compared with 1.6 ± 0.4% of lin<sup>-</sup>, c-kit<sup>-</sup>, and Sca-1<sup>+</sup> cells and 8.3 ± 4.7% of lin<sup>-</sup>, c-kit<sup>-</sup>, and Sca-1<sup>-</sup> cells (Fig. 3 A and B). Four days after 5-FU treatment, lin<sup>-</sup>, c-kit<sup>HI</sup>, and Sca-1<sup>HI</sup> cells were absent. There was a 14.5-fold increase in the number of lin<sup>-</sup>, c-kit<sup>-</sup>, and Sca-1<sup>+</sup> cells that were GFP<sup>+</sup> (*P* < 0.01). There was no change in the percentage of lin<sup>-</sup>, c-kit<sup>-</sup>, and Sca-1<sup>-</sup> cells that were GFP<sup>+</sup> (Fig. 3B). The increased proportion of GFP<sup>+</sup> lin<sup>-</sup>, Sca-1<sup>+</sup>, and c-kit<sup>-</sup> cells after 5-FU treatment is consistent with previous findings



**Fig. 4.** Analysis of competitive repopulation assays. (A) Representative FACS profiles of c-kit<sup>+</sup> and Sca-1<sup>+</sup> populations within lin<sup>-</sup> bone marrow cells isolated from wild-type and *Hmgb3*<sup>-/-</sup> mice 2, 4, and 8 days after treatment with 5-FU. Regions were drawn based on isotype controls. (B) FACS analysis was performed on bone marrow cells to determine the contribution to hematopoietic repopulation made by lin<sup>-</sup>, Sca-1<sup>+</sup>, c-kit<sup>HI</sup>, or c-kit<sup>NEG</sup> cells (CD45.2) isolated from untreated wild-type (c-kit<sup>HI</sup>, *n* = 3; c-kit<sup>NEG</sup>, *n* = 3) and *Hmgb3*<sup>-/-</sup> (c-kit<sup>HI</sup>, *n* = 3; c-kit<sup>NEG</sup>, *n* = 3) mice and wild-type (c-kit<sup>HI</sup>, *n* = 8) and *Hmgb3*<sup>-/-</sup> (c-kit<sup>HI</sup>, *n* = 8; c-kit<sup>NEG</sup>, *n* = 8) mice 4 days after 5-FU treatment. No experiment was performed by using c-kit<sup>HI</sup> cells from wild-type mice 4 days after 5-FU treatment (ND) because of the lack of cells. Error bars represent standard deviation.

that repopulating activity resides within the c-kit<sup>LO/NEG</sup> population after 5-FU treatment, and that *Hmgb3* serves as a marker for long-term repopulating activity (12, 17). Furthermore, these results indicate that loss of *Hmgb3* has the potential to affect the c-kit<sup>NEG</sup> population after 5-FU administration

We then compared the effects of 5-FU on *Hmgb3*-deficient and wild-type HSCs. As expected, 4 days after treatment of wild-type and *Hmgb3*<sup>-/-</sup> mice with 5-FU, the c-kit<sup>HI</sup> population was absent from wild-type lin<sup>-</sup> cells. However, numerous c-kit<sup>HI</sup> and Sca-1<sup>+</sup> cells were present in *Hmgb3*<sup>-/-</sup> lin<sup>-</sup> cells (Fig. 4A). To determine whether this effect was because of 5-FU resistance caused by higher levels of thymidylate synthase, we measured the levels of thymidylate synthase mRNA in wild-type and *Hmgb3*-deficient HSCs and found that the levels were identical (data not shown).

We analyzed repopulating activity of HSCs after 5-FU treatment isolated from untreated wild-type and *Hmgb3*<sup>-/-</sup> mice and from wild-type and *Hmgb3*<sup>-/-</sup> mice 4 days after 5-FU treatment. Lin<sup>-</sup>, Sca-1<sup>+</sup>, IL-7Rα<sup>-</sup>, c-kit<sup>HI</sup>, or c-kit<sup>NEG</sup> candidate HSCs (CD45.2) were transplanted along with congenic bone marrow (CD45.1) cells into irradiated recipients. Twelve weeks after transplant, recipient bone marrow cells were analyzed by flow cytometry for contribution of donor (CD45.2) and competitor (CD45.1) HSCs to repopulation. Untreated wild-type and *Hmgb3*<sup>-/-</sup> c-kit<sup>HI</sup> cells repopulated recipients (95.1 ± 2.2% CD45.2<sup>+</sup> and 93.1 ± 5.0% CD45.2<sup>+</sup>, respectively), but untreated c-kit<sup>NEG</sup> cells did not (Fig. 4B). In agreement with previous results, 4 days after 5-FU treatment, lin<sup>-</sup>, Sca-1<sup>+</sup>, and c-kit<sup>NEG</sup> cells isolated from wild-type mice provided all of the repopulating activity (78.4 ± 18.0% CD45.2<sup>+</sup>; ref. 17). In contrast, 4









the average number of CFU-GM/1000 CMP at day 0. *P* values were generated by Student's *t* test.

**Analysis of Primitive Hematopoietic Populations in *Hmgb3*<sup>-Y</sup> Mice After 5-FU Treatment.** Eight- to ten-week-old *Hmgb3*<sup>-Y</sup> and littermate wild-type mice were injected i.p. with 150 mg/kg 5-FU (the standard dose for all experiments; Sigma, St. Louis, MO). Two, four, six, and eight days after treatment, mice were killed and their bone marrow analyzed for the presence of HSC, CLP, and CMP populations. Cell staining and detection of the HSC (lin<sup>-</sup>, c-kit<sup>HI</sup>, Sca-1<sup>HI</sup>, and IL-7Rα<sup>-</sup>), CLP (lin<sup>-</sup>, c-kit<sup>LO</sup>, Sca-1<sup>LO</sup>, and IL-7Rα<sup>+</sup>), and CMP (lin<sup>-</sup>, c-kit<sup>+</sup>, Sca-1<sup>-</sup>, and IL-7Rα<sup>-</sup>) populations were performed as described (13). Unless specified, flow cytometry analysis for this and all other experiments was performed on a FACSCalibur (Becton Dickinson) by using 488-nm argon and 633-nm helium-neon lasers. *P* values were generated by Student's *t* test.

**Analysis of Primitive Hematopoietic Populations in *Hmgb3*<sup>GFP/Y</sup> Mice After 5-FU Treatment.** Lin<sup>-</sup> cells were isolated from groups of three untreated 8- to 10-week-old *Hmgb3*<sup>GFP/Y</sup> and littermate wild-type mice 4 days after 5-FU administration and were stained with APC-conjugated c-kit (2B8; PharMingen, San Diego, CA) and phycoerythrin-conjugated Sca-1 (E13-161.7; PharMingen), as described (12), before analysis by flow cytometry. *P* values were generated by Student's *t* test.

**Competitive Bone Marrow Transplants.** Bone marrow cells were pooled from groups of three untreated wild-type and *Hmgb3*<sup>-Y</sup> mice and wild-type and *Hmgb3*<sup>-Y</sup> mice 4 days after 5-FU treatment. Lineage depletion and staining for c-kit and Sca-1 were performed as described (12). Cells were sorted into lin<sup>-</sup>, Sca-1<sup>+</sup>, and c-kit<sup>HI</sup> or c-kit<sup>NEG</sup> populations. Sorted cells (1 × 10<sup>4</sup>) were transplanted along with 1 × 10<sup>6</sup> B6.SJL-*Ptprca*<sup>a</sup>/BoAiTac bone marrow cells into lethally irradiated (990 rad, Cs<sup>137</sup> source) 129SvEvTac × B6.SJL-*Ptprca*<sup>a</sup>/BoAiTac F1 recipients. Twelve weeks later, contribution of donor and competitor HSCs toward repopulation was determined by staining recipients' bone marrow with phycoerythrin-conjugated anti-CD45.1 (A20, PharMingen) and PerCP-Cy5.5-conjugated anti-CD45.2 (104, PharMingen) before analysis by flow cytometry. *P* values were generated by Student's *t* test.

**Analysis of HSC Cell Cycle Status.** Bone marrow cells from groups of five 8- to 10-week-old *Hmgb3*<sup>-Y</sup> and littermate wild-type mice were pooled together 2 and 4 days after 5-FU treatment. Lineage depletion was performed as described, with the exception that purified anti-IL-7Rα was included in the depletion, and purified

anti-Mac-1 was not (17, 24). Staining for the SP phenotype by using Hoescht dye was performed as described (23). After Hoescht staining, cells were stained with phycoerythrin-conjugated anti-Sca-1. Cells were sorted on a FACS Vantage SE (Becton Dickinson) by using UV (100 mW) and 488-nm argon lasers. Sorted lin<sup>-</sup>, SP<sup>+</sup>, Sca-1<sup>+</sup>, and IL-7Rα<sup>-</sup> cells were immediately stained with propidium iodide by using the NuCycl PI staining kit (Exalpha Biologicals, Watertown, MA) according to the manufacturer's instructions before analysis by flow cytometry. Cell cycle data analysis was performed by using ModFit software (Verity Software, Topsham, ME). *P* values were generated by Student's *t* test.

**Analysis of HSC Proliferation.** Groups of three *Hmgb3*<sup>-Y</sup> or littermate wild-type mice were injected with a single dose of 1 mg of BrdU (PharMingen) i.p. 2 and 4 days after treatment with 5-FU. Eighteen hours later, bone marrow was harvested and lin<sup>-</sup>, SP<sup>+</sup>, and IL-7Rα<sup>-</sup> sorted as described above. Sorted cells were stained with APC-conjugated anti-Sca-1 (D7, eBioscience) before the cells were fixed and stained with a FITC-conjugated anti-BrdU antibody by using the FITC BrdU Flow Kit (PharMingen) according to the manufacturer's instructions before analysis by flow cytometry. *P* values were generated by Student's *t* test.

**RT-PCR.** Bone marrow cells were harvested from 8- to 10-week old TOPGAL × *Hmgb3*<sup>-Y</sup> or littermate TOPGAL wild-type mice. Lineage depletion (which included purified anti-IL-7Rα), Hoescht dye staining for the SP phenotype, and antibody staining with anti-c-kit and Sca-1 were performed as described above. RNA was isolated from sorted HSCs (Lin<sup>-</sup>, SP<sup>+</sup>, c-kit<sup>+</sup>, Sca-1<sup>+</sup>, and IL-7Rα<sup>-</sup>) by using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR was performed as described by using a duplex-PCR with one primer pair amplifying the test gene and the second primer pair amplifying β2-microglobulin as an internal control. Limiting dilutions of HSC mRNA were used to ensure amplifications remained within the linear range. Primer pairs used for gene amplification were: 5'-GGCTGTAGAGCGAGTGTTC-3' (sense) and 5'-GTAGAGGTTGACAGTGTAGAT-3' (anti-sense) for *lacZ* (27), 5'-AAGCACAAATGCGCGTCGTC-3' (sense) and 5'-CAGCGTCGTCATTGCTATG-3' (anti-sense) for *Dvl1*, and 5'-TGGGCAACATGATGGTTGTG-3' (sense) and 5'-GCTGTCTTGAGACTCATGGA-3' (anti-sense) for β2-microglobulin (28). Reactions were performed for 32 cycles at 94° for 30 sec, 64° for 30 sec, and 72° for 30 sec. *LacZ* and *Dvl1* mRNA levels were measured and normalized to β2-microglobulin by performing densitometry on relative band intensities. RT reactions performed without enzyme served as negative controls for genomic DNA amplification.

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