

# Requirements for survivin in terminal differentiation of erythroid cells and maintenance of hematopoietic stem and progenitor cells

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**Survivin, which is the smallest member of the inhibitor of apoptosis protein (IAP) family, is a chromosomal passenger protein that mediates the spindle assembly checkpoint and cytokinesis, and also functions as an inhibitor of apoptosis. Frequently overexpressed in human cancers and not expressed in most adult tissues, survivin has been proposed as an attractive target for anticancer therapies and, in some cases, has even been touted as a cancer-specific gene. Survivin is, however, expressed in proliferating adult cells, including human hematopoietic stem cells, T-lymphocytes, and erythroid cells throughout their maturation. Therefore, it is unclear how survivin-targeted anticancer therapies would impact steady-state blood development. To address this question, we used a conditional gene-targeting strategy and abolished survivin expression from the hematopoietic compartment of mice. We show that inducible deletion of *survivin* leads to ablation of the bone marrow, with widespread loss of hematopoietic progenitors and rapid mortality. Surprisingly, heterozygous deletion of *survivin* causes defects in erythropoiesis in a subset of the animals, with a dramatic reduction in enucleated erythrocytes and the presence of immature megaloblastic erythroblasts. Our studies demonstrate that survivin is essential for steady-state hematopoiesis and survival of the adult, and further, that a high level of survivin expression is critical for proper erythroid differentiation.**

## CORRESPONDENCE

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Abbreviations used: CBC, complete blood count; cdk, cyclin-dependent kinase; CPC, chromosomal passenger complex; E, embryonic day; HE, hematoxylin and eosin; IAP, inhibitor of apoptosis protein; INCENP, inner centromere protein; pI-pC, polyinosinic-polycytidylic acid; RNAi, RNA interference.

At 16.5 kD, survivin is the smallest member of the inhibitor of apoptosis protein (IAP) family. Survivin contains a single BIR domain and a C-terminal  $\alpha$ -helical coiled coil, but no RING finger or other identifiable domain typically found in IAPs (1). Currently, there are three lines of experimental evidence that support a role for survivin in suppression of apoptosis (2). First, overexpression of survivin has been shown to antagonize cell death initiated via the extrinsic or intrinsic apoptotic pathway. Second, transgenic expression of survivin under the *keratin 14* (*K14*) promoter inhibited apoptosis in vivo (3). Conversely, liver cells that were isolated from survivin heterozygous mice exhibited an increase in apoptosis upon treatment with a lower concentration of Fas ligand (4). Third, molecular antagonists of survivin, including antisense, ribozymes, siRNA, and dominant-

negative mutants, resulted in caspase-dependent cell death and enhancement of apoptotic stimuli and anticancer activities in vivo (2). Although the role of survivin in conferring resistance to apoptosis is well established, the context in which it does so and the mechanisms by which it accomplishes this remains debatable. It is likely that survivin mediates its antiapoptotic effect through association with other pro- or antiapoptotic molecules (5–8).

Contrary to its role in apoptosis inhibition, survivin's role in mitosis is unequivocal. Survivin inactivation has been shown to cause defects in chromosome alignment, spindle assembly checkpoint, and cytokinesis, with eventual cell death via mitotic catastrophe (9–12). In *Caenorhabditis elegans*, embryos and oocytes lacking the survivin homologue Bir-1 displayed chromosomal and spindle defects (13). In addition,

homozygous *survivin* gene deletion in mice resulted in embryonic lethality at embryonic day (E) 4.5, with cells exhibiting polyploidy and disrupted microtubules (14).

As expected for a mitotic regulator, survivin is generally expressed in a cell cycle–dependent manner, peaking at G<sub>2</sub>–M and rapidly degraded in G<sub>1</sub> (15). Survivin localizes to the centromeres during prometaphase/metaphase, translocates to the spindle midzone during anaphase/telophase, and eventually localizes to the midbody during cytokinesis (16–18). Consistent with its dynamic localization, numerous studies have shown that survivin is a component of the chromosomal passenger complex (CPC) that functions in the maintenance of an active spindle assembly checkpoint and in cytokinesis. In addition to survivin, the CPC is comprised of inner centromere protein (INCENP) (19), Aurora-B kinase (20), and Borealin/Dasra B (21, 22). RNA interference (RNAi) knockdown of survivin or its partner proteins in U2OS and HeLa cells disrupts proper targeting of the CPC to centromeres and the central spindle, suggesting that these proteins are mutually dependent on each other for proper localization (21, 23–26). It has been proposed that survivin's specific role within the CPC is to target Aurora B kinase to different components of the mitotic apparatus, where it can phosphorylate its substrates.

A growing number of studies indicate survivin is expressed in normal adult cells, particularly human CD34<sup>+</sup> hematopoietic stem and progenitor cells (27–29), T lymphocytes (30, 31), polymorphonuclear neutrophils (32), and vascular endothelial cells (33, 34), where it may regulate their proliferation or survival. Most recently, our laboratory has reported that survivin is differentially required for the development of two closely related blood cells, erythroid cells and megakaryocytes (35). During terminal differentiation, erythroid cells continue to express survivin mRNA and protein throughout their maturation, even after cell cycle exit, whereas megakaryocytes express an approximately fourfold lower level of survivin mRNA and no detectable protein (35, 36). Functionally, a reduction in survivin expression was found to interfere with erythroid cell formation, whereas its overexpression antagonized growth, maturation, and polyploidization of megakaryocytes (35). Furthermore, survivin-deficient hematopoietic progenitors failed to give rise to either erythroid or megakaryocytic colonies in vitro (35). Based on these studies, survivin appears to have critical roles in normal hematopoietic cells, and the physiological impact of survivin-targeted anticancer strategies on the hematopoietic compartment is unclear.

To elucidate precisely the role of survivin in in vivo hematopoiesis, we induced homozygous and heterozygous deletion of the *survivin* gene in the hematopoietic compartment in mice, using the *Mx1-Cre* transgene. We show that complete loss of survivin in homozygous-floxed *Mx1-Cre* animals resulted in rapid lethality. These mutant animals exhibited markedly hypocellular BM and spleen, a plethora of peripheral blood defects, and striking reductions in multiple hematopoietic progenitors, including hematopoietic stem cells. Surprisingly, acute heterozygous deletion of *survivin* led to defects in erythropoiesis in a subset of animals. Analysis of the

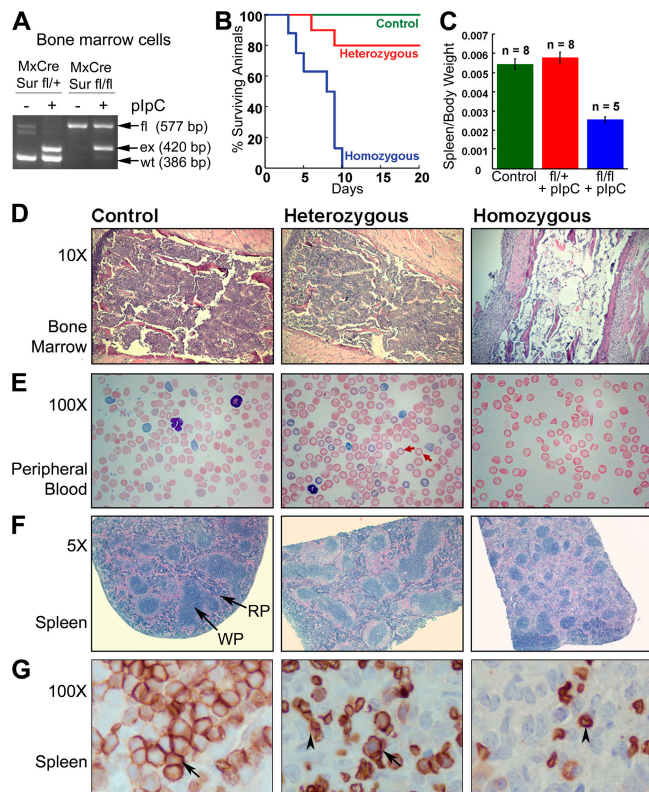
erythroid lineage from spleens of these affected mice displayed a dramatic reduction in enucleated erythrocytes, and the presence of immature erythroblasts that were megaloblastic in nature. Collectively, we show that survivin is crucial for steady-state hematopoiesis in the adult, and that erythroid cells are sensitive to survivin levels.

## RESULTS

### Survivin is essential for adult hematopoiesis

Because *survivin* deficiency results in periimplantation lethality (14), we used a conditional knockout strategy to study the requirement for survivin in in vivo hematopoiesis. Conditional gene-targeted mice, with all four exons of the *survivin* gene flanked by loxP sites (31), were bred to mice expressing Cre recombinase under the control of the endogenous *Mx1* locus, which can be transiently activated in response to type-I IFN (37). Subsequently, mice harboring the *Mx1-Cre* transgene and either heterozygous or homozygous floxed alleles of *survivin* were treated with polyinosinic-polycytidylic acid (pI-pC), which is a synthetic double-stranded RNA, to induce deletion of *survivin* in the hematopoietic compartment. pI-pC treatment of *Mx1-Cre/surv<sup>fl/+</sup>* and *Mx1-Cre/surv<sup>fl/fl</sup>* adult mice resulted in robust excision of the *survivin* floxed allele in BM cells as determined by PCR analysis (Fig. 1 A). To our surprise, administration of pI-pC to *Mx1-Cre/surv<sup>fl/fl</sup>* mice caused rapid death (Fig. 1 B). Indeed, all of the animals became moribund within 10 d of pI-pC treatment. In comparison, the control littermates showed no ill effect after pI-pC injection, whereas the heterozygous-deleted mice showed 20% mortality. Analysis of peripheral blood, BM, and spleen (a prominent site of hematopoiesis in young mice) of survivin-deficient animals revealed a wide spectrum of hematopoietic defects. As clearly evident in Fig. 1 D, there was almost a complete ablation of the BM of *Mx1-Cre/surv<sup>fl/fl</sup>* mice. Consistent with this gross BM depletion, peripheral blood smears from survivin-deleted animals exhibited marked pancytopenia, including reticulocytopenia (Fig. 1 E). In addition, a plethora of erythrocyte abnormalities, such as red cell fragmentation, anisopoikilocytosis, and hypochromia, were visible. Furthermore, *Mx1-Cre/surv<sup>fl/fl</sup>* mice had smaller and extremely hypocellular spleens (Fig. 1, C and F) caused by a reduced white and red pulp. The red pulp, which is a site of normal extramedullary hematopoiesis in young mice, was paucicellular. Most dramatic was the complete absence of nucleated erythroid progenitors that stained positive for the erythroid-specific marker Ter119 in the spleens of *Mx1-Cre/surv<sup>fl/fl</sup>* mice, as shown by immunohistochemical analysis for Ter119 (Fig. 1 G). Control mice that were treated with pI-pC did not display any of these phenotypes (Fig. 1, B–G). Interestingly, however, we observed an intermediate phenotype in survivin heterozygous-deleted mice (as discussed in Haploinsufficiency of *survivin* leads to defects in erythropoiesis).

To better understand why the animals die within 2 wk of survivin depletion, we repeated the pI-pC injections on another group of mice (8 wk of age) and analyzed their complete



**Figure 1. *Survivin* is essential for adult hematopoiesis.** (A) Deletion of the floxed region was monitored by multiplex PCR using three primers to amplify the floxed, excised, and wild-type alleles. PCR of total BM cells from two representative examples 10 d after pl-pC administration. ex, excised; fl, floxed; wt, wild-type. (B) Survival curve for control (*Sur<sup>fl/+</sup>*;  $n = 19$ ), heterozygous (*MxCre.Sur<sup>fl/+</sup>*;  $n = 10$ ), and homozygous (*MxCre.Sur<sup>fl/fl</sup>*;  $n = 8$ ) pups after pl-pC treatment. (C) Spleen weight with respect to body weight  $\pm$  the SEM was recorded for control, heterozygous, and homozygous-deleted mice. (D) HE stained sections of sternum BM from control, heterozygous, and homozygous-deleted mice. (E) Peripheral blood smears from control, heterozygous, and homozygous-deleted mice were stained with May-Grünwald Giemsa. Red arrows, hypochromic erythrocytes. (F) HE spleen sections from mice in D. WP, white pulp; RP, red pulp. (G) Anti-Ter119 immunohistochemical analysis of spleen sections from mice in D. Black arrows, nucleated Ter119<sup>+</sup> erythroid cells; arrowheads, enucleated Ter119<sup>+</sup> erythrocytes.

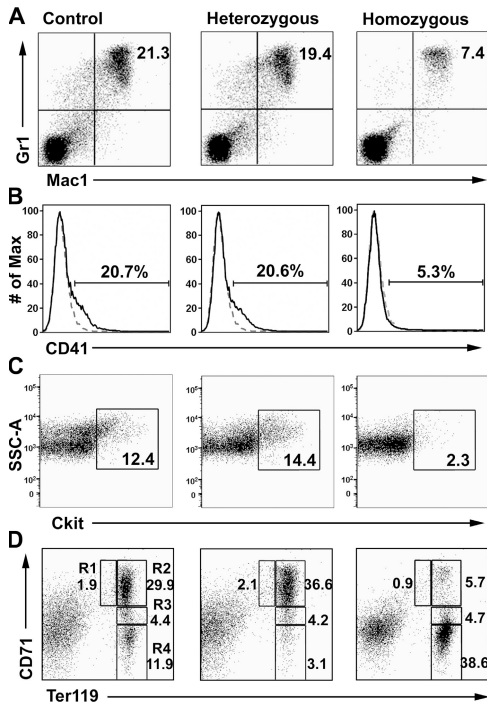
blood counts (CBCs) on days 7 and 11 after treatment. CBC analysis at day 7 revealed that *survivin*-depleted mice had lower hemoglobin and white cell and platelet counts compared with the control animals (unpublished data). By day 11, the *survivin*-depleted mice developed definitive anemia. Thrombocytopenia and leukopenia were reflected in the automated CBC, as well as on the peripheral blood smears shown in Fig. 1 E; homozygous-deleted animals displayed pancytopenia, with multiple defects in circulating erythrocytes. Therefore, we believe that the development of anemia in the periphery is mostly caused by underproduction, which may or may not be exacerbated by some peripheral destruction. Based on the presence of fragmented red cells in the periphery, we hypothesize that a thrombotic microangiopathic process might

contribute to the peripheral destruction of red cells in some of the mice. Furthermore, to investigate if the rapid morbidity and mortality of the *survivin*-depleted mice was caused by injury to nonhematopoietic tissues, gross and microscopic examination was performed on the major organs from control and *survivin*-depleted mice. There was no evidence of organ damage in the kidneys, lungs, livers, and hearts of the *survivin*-depleted mice (unpublished data). Under the circumstances, we speculate that the morbidity and mortality observed in the *survivin*-depleted animals is secondary to BM failure. It is possible that anemia, infections caused by neutropenia, or hemorrhage caused by thrombocytopenia contribute to early morbidity and mortality in these animals.

### Hematopoietic progenitors are susceptible to *survivin* loss

Because the BM histology indicated that *survivin* deletion affected multiple hematopoietic lineages, we used flow cytometry to examine specific cell populations in either the BM or spleen of control, *Mx1-Cre/sur<sup>fl/+</sup>*, and *Mx1-Cre/sur<sup>fl/fl</sup>* mice after pl-pC injection. In contrast to littermate controls, Gr-1<sup>+</sup>Mac-1<sup>+</sup> myeloid cells and CD41<sup>+</sup> megakaryocytes were both reduced in the BM of homozygous-deleted mice as early as 36 h after pl-pC induction (Fig. 2, A and B). C-kit<sup>+</sup> progenitors were also dramatically diminished in the spleen of homozygous-deleted mice by 10 d of pl-pC administration (Fig. 2 C). Consistent with these results, total BM cells, isolated from homozygous-deleted mice 1 d after pl-pC injection, failed to give rise to any myeloid, erythroid, or megakaryocytic colonies in colony-forming assays (unpublished data). The rapid time frame in which hematopoietic progenitors disappear after *survivin* excision suggests *survivin* is critical for the maintenance of hematopoietic cells.

We next focused on analyzing the effect of *survivin* deletion in the erythroid lineage. During their maturation, erythroid cells progressively become smaller and exhibit chromatin condensation, hemoglobinization, cell cycle exit, and enucleation. As erythroblasts undergo terminal differentiation, they also down-regulate expression of the transferrin receptor CD71 and up-regulate the erythroid-specific marker Ter119. Erythroid cells can be segregated into four subsets by their CD71 and Ter119 expression level (R1 proerythroblasts, Ter119<sup>low</sup>CD71<sup>high</sup>; R2 basophilic erythroblasts, Ter119<sup>high</sup>CD71<sup>high</sup>; R3 polychromatophilic erythroblasts, Ter119<sup>high</sup>CD71<sup>med</sup>; and R4 orthochromatophilic erythroblasts and reticulocytes, Ter119<sup>high</sup>CD71<sup>low</sup>) (38). To examine the consequences of *survivin* deletion specifically on erythropoiesis, we analyzed the distribution of erythroid subsets in the spleen of 1-mo-old *Mx1-Cre/sur<sup>fl/fl</sup>* mice 10 d after pl-pC administration. Compared with their littermate controls, homozygous-deleted mice exhibited a marked reduction in the percentages of R1 and R2 subsets and an increase in the percentage of the R4 subset (Fig. 2 D). To investigate the erythroid perturbation more carefully, we isolated R2 and R4 erythroid cells from pl-pC-treated control and *Mx1-Cre/sur<sup>fl/fl</sup>* spleens by FACS for morphological evaluation. Because *survivin* deletion results in rapid BM ablation and early death in *Mx1-Cre/sur<sup>fl/fl</sup>*

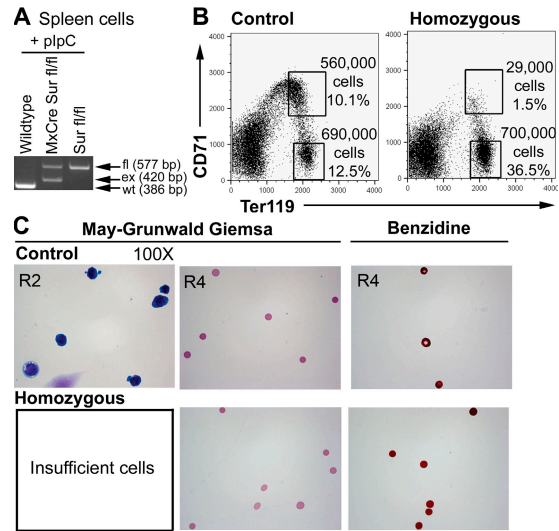


**Figure 2. *Survivin* deletion leads to a reduction in hematopoietic progenitors.** (A) Gr1<sup>+</sup>Mac1<sup>+</sup> BM cells harvested from mice 36 h after treatment with one dose of pI-pC (*n* = 2). (B) CD41<sup>+</sup> BM cells from mice, as described in A. (C) Flow cytometric analysis of c-kit<sup>+</sup>-stained spleen cells from 1-mo-old *Surv<sup>fl/+</sup>*, *MxCre Surv<sup>fl/+</sup>*, and *MxCre Surv<sup>fl/fl</sup>* pups 10 d after 2 doses of pI-pC. Percentages are shown. (D) CD71<sup>+</sup>Ter119<sup>+</sup> spleen cells from mice, as described in C. Results shown are representative of four independent experiments.

mice, the experiment was performed at an earlier time point, 4 d after pI-pC injection. *Survivin* excision in total spleen cells was confirmed by PCR analysis (Fig. 3 A). At this early time point, the spleens from *survivin* homozygous-deleted mice were comparable in size to those of control littermates and harbored similar absolute numbers of R4 cells, but strikingly fewer R2 cells (Fig. 3 B). Morphological evaluation of the R4 population by both May-Grünwald Giemsa and benzidine staining revealed that the majority of cells in *survivin*-deleted animals were mature, hemoglobinized, and enucleated erythrocytes that were indistinguishable from those of controls (Fig. 3 C). These data support an essential role for *survivin* in proliferating proerythroblasts and basophilic erythroblasts. In contrast, R4 cells, which had already exited the cell cycle and extruded their nuclei, were not affected by *survivin* loss, and the total number of cells within the R4 population was not significantly altered, resulting in an apparent relative increase in the percentage of cells in the R4 gate.

**Survivin-depleted erythroblasts exhibit altered cell cycle, increased cell death, and polyploidy**

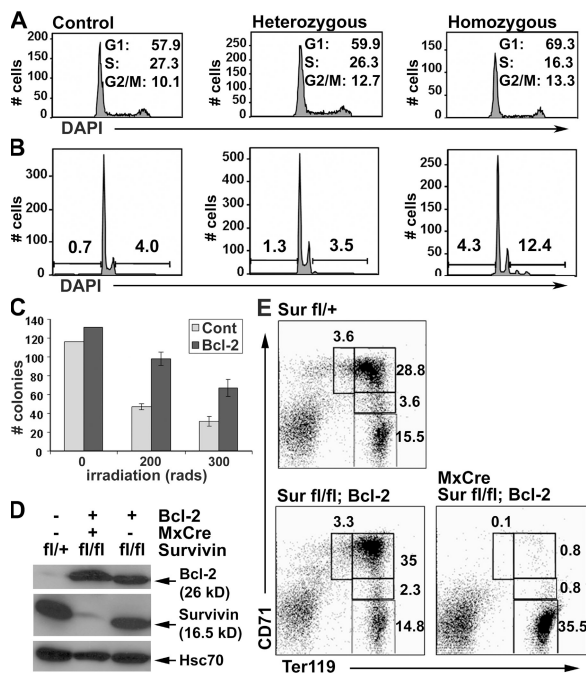
To determine whether aberrant proliferation or increased cell death was responsible for the erythroid defect, we next analyzed the cell cycle profile and DNA content of *survivin*-



**Figure 3. *Survivin* deletion diminishes proliferating CD71<sup>+</sup>Ter119<sup>+</sup> erythroid cells.** (A) *Survivin* deletion in total splenic cells from control (*Surv<sup>fl/fl</sup>*) and homozygous-deleted (*MxCre Surv<sup>fl/fl</sup>*) mice 4 d after pI-pC treatment was confirmed by PCR. (B) FACS profiles for sorted CD71<sup>+</sup>Ter119<sup>+</sup> R2 and R4 erythroid populations are shown with both percentages and absolute cell numbers. (C) Cytospin slides of FACS-sorted R2 and R4 erythroid cells were stained with either May-Grünwald Giemsa or benzidine.

depleted erythroid precursors. BM cells from control and *Mx1-Cre/surv<sup>fl/fl</sup>* mice were harvested 48 h after pI-pC treatment and stained with DAPI and antibodies against CD71 and Ter119. Analysis of *survivin*-deficient erythroblasts showed increased G<sub>1</sub> and decreased S phase cells, indicating that these cells were either cell cycle-arrested or had exited the cell cycle faster (Fig. 4 A). In addition, *survivin*-deficient erythroblasts exhibited an increase in cells with ≥4 N DNA content, as well as a moderate increase in cells with sub-G<sub>1</sub> DNA (Fig. 4 B). The increase in the number of cells with sub-G<sub>1</sub> DNA suggests that some of the cells have undergone cell death, either as a consequence of aberrant cell cycle progression or initiation of apoptosis.

We next investigated whether the death seen in these cells was a consequence of activation of the intrinsic mitochondrial apoptotic pathway. If *survivin* depletion resulted in initiation of this pathway, then ectopic *Bcl-2* expression in these cells might rescue the phenotype caused by *survivin* loss. To address this question, we used *vav-Bcl-2* transgenic mice, in which constitutive expression of the human *Bcl-2* cDNA is restricted to nucleated hematopoietic cells by the *vav* promoter (39). To assess the ability of *vav-Bcl-2* to promote the survival of erythroid progenitors, lineage-negative BM cells from *vav-Bcl-2* transgenic mice or their wild-type littermates were subjected to varying doses of gamma irradiation (0, 200, or 300 rads) and plated in erythroid colony-forming assays with IL3, SCF, and EPO. As demonstrated in Fig. 4 C, *vav-Bcl-2*, indeed, promoted the survival of erythroid progenitors in response to gamma irradiation. In fact, after treatment with 200 or 300 rads of gamma irradiation,



**Figure 4. *Survivin* homozygous-deleted erythroid cells exhibit altered cell cycle, increased cell death, and polyploidy.** (A) Control (*Sur<sup>fl/+</sup>*), heterozygous (*MxCre Sur<sup>fl/+</sup>*), and homozygous (*MxCre Sur<sup>fl/fl</sup>*) mice were treated with one dose of pI-pC. BM cells were isolated 48 h later and stained for CD71, Ter119, and DAPI for flow cytometric cell cycle analysis. Cell cycle phases on gated proliferating CD71<sup>+</sup>Ter119<sup>+</sup> cells were calculated using Watson Pragmatic model. The data are representative of three sample sets from two independent experiments. (B) SubG1 DNA and ploidy analysis for A. (C) Erythroid colony-forming assays of untreated or gamma-irradiated (200 or 300 rads; *n* = 3) lineage-negative BM cells from *vav-Bcl-2* transgenic mice or their wild-type littermates. Mean numbers of pure and mixed erythroid colonies  $\pm$  the SEM are shown. (D) Western blot, probed for survivin and Bcl-2, of spleen extracts prepared from *Sur<sup>fl/+</sup>*, *Sur<sup>fl/fl</sup>*; Bcl-2, and *MxCre Sur<sup>fl/fl</sup>*; Bcl-2 pups 10 d after 2 doses of pI-pC treatment. Heat shock protein 70 (Hsc70) was included as a loading control. (E) Flow cytometric analysis of CD71/Ter119 stained spleen cells from mice, as described in C. Results are representative of five out of seven *MxCre Sur<sup>fl/fl</sup>*; Bcl-2 pups from four independent experiments.

the *vav-Bcl-2* transgene led to a twofold increase in erythroid colony formation in vitro, compared with wild-type controls. Furthermore, *vav-Bcl-2* also prolonged the survival of erythroid progenitors after 3 d of EPO deprivation (unpublished data). To determine whether overexpression of Bcl-2 can rescue the defects caused by survivin deficiency, we crossed *vav-Bcl-2* transgenic mice to *Mx1-Cre/sur<sup>fl/fl</sup>* mice to generate *Mx1-Cre/sur<sup>fl/fl</sup>*; Bcl-2 animals. Bcl-2 and survivin expression were detected by Western blot analysis of protein extracts prepared from the spleens of pI-pC-treated control, Bcl-2 transgenic, or *Mx1-Cre/sur<sup>fl/fl</sup>*; Bcl-2 mice (Fig. 4 D). Although ectopic expression of Bcl-2 under the *vav* promoter protected erythroid cells on a wild-type survivin background, it failed to rescue the smaller spleen, loss of erythroid progenitors, and eventual demise of *Mx1-Cre/sur<sup>fl/fl</sup>*; Bcl-2 mice after survivin deletion (Fig. 4 E).

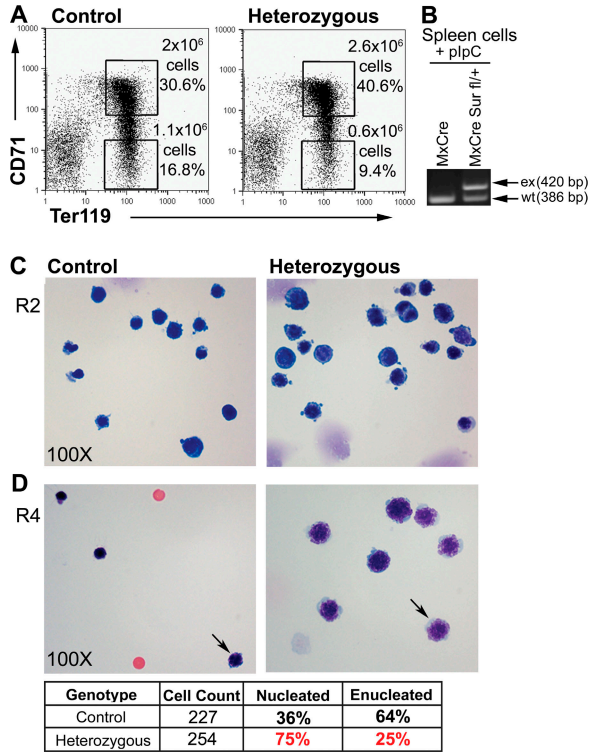
### Haploinsufficiency of *survivin* leads to defects in erythropoiesis

We previously reported that heterozygous loss of survivin caused a nearly 50% reduction in erythroid colony formation, but a much less dramatic change in the number of megakaryocytic colonies (35). These findings suggested that the erythroid lineage might be uniquely sensitive to the dosage of survivin. In this current study, we observed defects in a proportion of the heterozygous-deleted mice that are consistent with a dose-dependence for survivin in erythroid cells. First, 2 out of the 10 *Mx1-Cre/sur<sup>fl/+</sup>* animals, but none of the control animals, died within 10 d of pI-pC treatment (Fig. 1 B). Second, four out of the five BM sections from heterozygous-deleted mice were identified as harboring fewer erythroid precursors in a blinded analysis by a pathologist (S. Gurbuxani; Fig. 1 D and not depicted). Additionally, peripheral blood smears from these mice displayed an increase in hypochromic erythrocytes (Fig. 1 E, red arrows), which were not observed in any pI-pC-treated control animals.

Furthermore, analysis of the CD71/Ter119 erythroid population by flow cytometry revealed that 20% of the heterozygous-deleted animals harbored an altered proportion of erythroid cells, with affected animals displaying an increased proportion of R2 cells and a concomitant decrease in the R4 population (Fig. 2 D and Fig. 5 A). In the data depicted in Fig. 5 A, which are representative of the affected group of animals, nearly half as many R4 cells and a greater number of R2 cells were isolated from the heterozygous-deleted spleen in comparison to the control spleen. To further explore the nature of this defect, R2 and R4 cells from pI-pC-treated control, and *Mx1-Cre/sur<sup>fl/+</sup>* mice were collected by FACS. Excision of the floxed allele was confirmed by PCR analysis (Fig. 5 B). May-Grünwald Giemsa staining of purified cells revealed that although the R2 progenitors appeared similar, the R4 erythroid population was distinctly different in the two groups (Fig. 5, C and D). Heterozygous survivin-deficient R4 cells exhibited a striking increase in the proportion of nucleated erythroblasts, with a concomitant decrease in enucleated erythrocytes. Interestingly, the R4 population of survivin heterozygous-deleted erythroblasts appeared immature and possessed less condensed, larger nuclei, which is indicative of a block in terminal differentiation. Although this phenotype was not fully penetrant, the megaloblastic features observed in a significant proportion of heterozygous-deleted animals suggest that survivin plays a novel role in erythroid terminal differentiation.

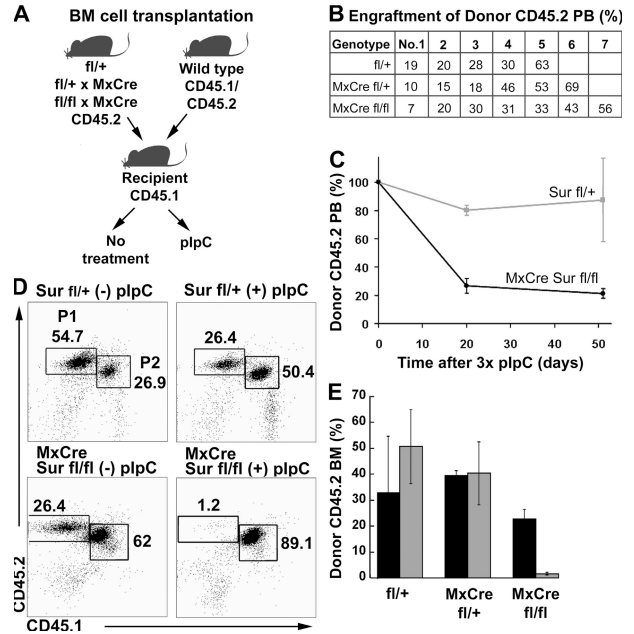
### Defects caused by survivin deletion are cell autonomous

Whereas the preceding data suggest that survivin deficiency results in impaired proliferation and subsequent loss of hematopoietic progenitors, *Mx1-Cre* excision is not absolutely limited to hematopoietic cells (37). Therefore, to determine whether the requirement for survivin is cell autonomous, we transplanted total CD45.2<sup>+</sup>-nucleated BM cells from control, *Mx1-Cre/sur<sup>fl/+</sup>*, or *Mx1-Cre/sur<sup>fl/fl</sup>* mice, along with wild-type CD45.1<sup>+</sup>/CD45.2<sup>+</sup> support cells, into lethally irradiated



**Figure 5. *Survivin* haploinsufficiency in erythroid maturation.** (A) FACS sort profile of CD71/Ter119-stained R2 and R4 erythroid populations from spleens of control (*MxCre*) and heterozygous-deleted (*MxCre Sur<sup>fl/β</sup>*) mice 10 d after pl-pC administration. Percentages and absolute cell numbers are shown. (B) *Survivin* deletion in total splenic cells from *MxCre* and *MxCre Sur<sup>fl/β</sup>* mice was confirmed by PCR. (C) Cytospin slides of FACS-sorted R2 erythroid cells stained with May-Grünwald Giemsa. (D) May-Grünwald Giemsa-stained cytosin slides of FACS-sorted R4 erythroid cells. Arrows, nucleated erythroblasts. (bottom) The table summarizes percentages of erythroblasts and erythrocytes for the sorted R4 population from each mouse.

CD45.1<sup>+</sup> congenic recipients (Fig. 6 A). Engraftment of donor cells was first verified by staining for CD45 surface marker expression in peripheral blood 5 wk after transplantation. The engraftment of CD45.2<sup>+</sup> cells ranged from 7 to 69% (Fig. 6 B). Recipients were then treated with pI-pC, and the contribution of CD45.2<sup>+</sup> cells to the hematopoietic compartment was assessed. *Survivin* excision led to pronounced depletion of CD45.2<sup>+</sup> cells in the peripheral blood, supporting a role for *survivin* in hematopoietic stem and progenitor cells (Fig. 6 C). Administration of pI-pC to *Mx1-Cre/sur<sup>fl/β</sup>* transplanted mice also led to near complete disappearance of the BM CD45.2<sup>+</sup> hematopoietic cells within 2 wk of pI-pC treatment in all five mice analyzed (Fig. 6, D and E). No significant changes were observed in either control or *Mx1-Cre/sur<sup>fl/β</sup>* transplanted mice. These data show that there is a cell autonomous requirement for *survivin* in hematopoietic progenitors. Next, to determine whether HSCs are also susceptible to *survivin* deletion, we elucidated the origin of the HSC populations in control and *Mx1-Cre/sur<sup>fl/β</sup>* transplanted



**Figure 6. Cell-autonomous defects of *survivin* deletion.** (A) Mice with chimeric BM were generated by transplanting conditionally gene targeted CD45.2<sup>+</sup> BM cells with wild-type CD45.1<sup>+</sup>/CD45.2<sup>+</sup> BM support cells into lethally irradiated congenic CD45.1<sup>+</sup> recipients. A subset of mice was treated with pl-pC after BM transplantation. (B) Table depicts percentage of donor CD45.2<sup>+</sup> cells that has engrafted for each mouse before pl-pC administration, as determined by CD45.1/CD45.2 staining of peripheral blood. (C) Mean percentages of CD45.2<sup>+</sup> cells ± the SEM in the peripheral blood of *Sur<sup>fl/+</sup>* (*n* = 3; gray line) and *MxCre Sur<sup>fl/β</sup>* (*n* = 9; black line) mice after pl-pC treatment. Initial engraftment of CD45.2<sup>+</sup> was normalized to 100% for each mouse. (D) Flow cytometric analysis of CD45.1/CD45.2 stained BM cells from representative *Sur<sup>fl/+</sup>* and *MxCre Sur<sup>fl/β</sup>* mice in the absence or presence of pl-pC treatment. Percentages of CD45.2<sup>+</sup> cells (P1) and CD45.1<sup>+</sup>/CD45.2<sup>+</sup> cells (P2) were determined for gated large nucleated cells. (E) Histogram depicts mean contribution of BM CD45.2<sup>+</sup> cells ± the SEM of *Sur<sup>fl/+</sup>* (*n* = 2, untreated; *n* = 3, plus pl-pC), *MxCre Sur<sup>fl/+</sup>* (*n* = 2, untreated; *n* = 3, plus pl-pC), and *MxCre Sur<sup>fl/β</sup>* (*n* = 2, untreated; *n* = 5, plus pl-pC) transplanted mice.

animals 10 wk after pI-pC administration. Flow cytometric analyses revealed that the lin<sup>-</sup>c-kit<sup>+</sup>Sca-1<sup>+</sup> HSC populations in *Mx1-Cre/sur<sup>fl/β</sup>* transplanted mice were exclusively derived from wild-type CD45.1<sup>+</sup>/CD45.2<sup>+</sup> support cells, whereas the HSC populations in control animals expressed either CD45.2<sup>+</sup> alone or CD45.1<sup>+</sup>/CD45.2<sup>+</sup> cell surface markers (Fig. 7).

**DISCUSSION**

We show that *survivin* is an essential gene in the adult and is indispensable for the proliferation and maintenance of both hematopoietic stem and progenitor cells. Similar to inducible deletion of the antiapoptotic Bcl-2 family member myeloid leukemia-1 (MCL-1) in mice, homozygous deletion of *survivin* resulted in rapid death, ablation of the BM, and loss of early BM progenitor populations, including HSCs (40). Consistent with our findings, T lineage-specific *survivin* knockout mice revealed critical requirements for *survivin* in the

development and homeostasis of T cells (30, 31). In response to mitogen-induced proliferation, survivin-deficient T cells exhibited p53-mediated cell cycle arrest, a spindle formation defect, and spontaneous cell death. In addition, p53 inactivation or introduction of Bcl-2 failed to rescue the thymocyte developmental defects caused by survivin deficiency (30). In light of these published works, our study suggests survivin may function as both a chromosomal passenger protein and an inhibitor of apoptosis in the maintenance of hematopoietic stem and progenitor populations.

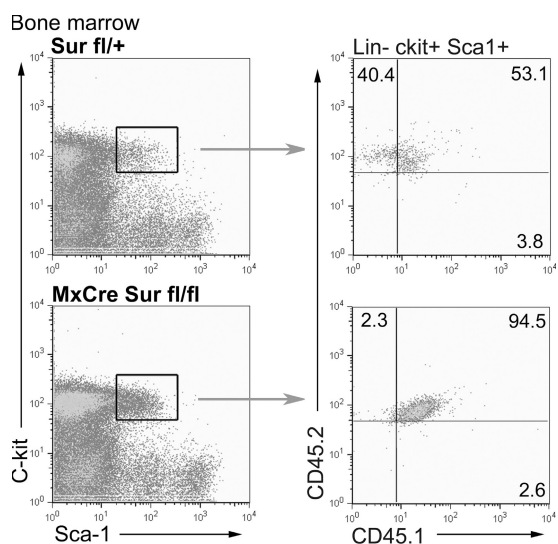
In addition, we demonstrate that haploinsufficiency of *survivin* affects erythroid terminal maturation. Erythroid progenitors undergo a series of three to four rapid “differentiation divisions” to give rise to mature, enucleated, and hemoglobinized erythrocytes (41). During this process, the G1 phase of the cell cycle is reduced from 11 h to 5 h, and the cell size is reduced from a 12- $\mu\text{m}$  proerythroblast to a 4- $\mu\text{m}$  erythrocyte (mouse). This altered cell cycle progression is accompanied by extensive changes in gene expression, including a rapid down-regulation of D-type cyclins and their partner, cyclin-dependent kinase type 4 (cdk4). In contrast, expression levels of S- and G<sub>2</sub>-M-associated cell cycle regulators, such as cyclin A and cdk1/cdc2, are transiently up-regulated. In this study, we show a dose-dependent requirement for survivin in erythropoiesis. We previously reported that survivin levels increase as erythroid cells mature, with both mRNA and protein present in cells throughout even the late orthochromatic stage. In fact, erythroid cells express approximately fourfold higher levels of survivin mRNA and protein than the related megakaryocytic lineage during their terminal differentiation (35). Therefore,

reduced levels of key mitotic regulators, including survivin, may compromise the efficiency of the cell cycle machinery and subsequently, lead to defects in erythroid maturation. Consistent with this model, mice heterozygous for BubR1, which is a spindle assembly checkpoint protein, exhibited decreased erythropoiesis and a higher frequency of anemia (42). Interestingly, stable association of BubR1 to kinetochores and maintenance of the spindle assembly checkpoint is critically dependent on survivin (12, 24). In the presence of reduced survivin levels, Aurora B kinase, INCENP, and Borealin may less efficiently localize to different components of the mitotic apparatus, including the inner centromeres, where the CPC plays an important auxiliary role in spindle checkpoint surveillance. Because Aurora B, INCENP, and survivin form a complex that is required for multiple aspects of mitosis and cytokinesis, a reduction in survivin levels likely disrupts proper targeting of Aurora B kinase to its substrates. It remains to be determined whether survivin has a novel role in erythroid terminal differentiation independent of its mitotic function.

Several preclinical studies have shown that disrupting survivin expression or function in cancer cells decreases their proliferation and enhances apoptosis (43). The absence of significant side effects on hematopoietic cells reported in these studies may be caused by local intratumor injection and minimal systemic dissemination of anti-survivin therapies. More recently, shepherdin, which is a small peptide that destabilizes survivin by blocking its interaction with the molecular chaperone heat shock protein 90 (Hsp90), has been shown to affect human CD34<sup>+</sup> hematopoietic progenitor colony formation in vitro (44). In that study, treatment of CD34<sup>+</sup> cells with shepherdin inhibited precursor erythroid burst-forming unit colony formation and, at slightly higher peptide concentrations, granulocyte-macrophage colony-forming units and granulocyte erythrocyte macrophage megakaryocyte colony-forming units. Our study confirms that the hematopoietic system is extremely sensitive to survivin disruption and emphasizes the need for innovative and selective anti-survivin cancer therapies.

## MATERIALS AND METHODS

**Generation of *Mx1-Cre/Surv<sup>fl/fl</sup>* and *Mx1-Cre/Surv<sup>fl/fl</sup>; Bcl-2* mice.** Conditionally gene-targeted mice, with all four exons of the *survivin* gene flanked by loxP sites, were obtained from A. Winoto (University of California, Berkeley, Berkeley, CA) (31). *Mx1-Cre* transgenic mice harboring *Cre recombinase* under the control of the interferon-responsive *Mx1* promoter in a C57BL/6 background were purchased from The Jackson Laboratory. *Vav-Bcl-2* transgenic mice, in which constitutive expression of a human *Bcl-2* cDNA is driven by the promoter of the *vav* gene, were provided by J. Adams (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia) and K. Macleod (University of Chicago, Chicago, IL) (39). To generate *Mx1-Cre/surv<sup>fl/fl</sup>* mice, *surv<sup>fl/fl</sup>* mice were crossed to *Mx1-Cre* transgenic mice. *Mx1-Cre/surv<sup>fl/fl</sup>* mice were subsequently bred to *surv<sup>fl/fl</sup>* mice to produce littermates with the following genotypes: *surv<sup>fl/fl</sup>*, *surv<sup>fl/fl</sup>*, *Mx1-Cre/surv<sup>fl/fl</sup>*, and *Mx1-Cre/surv<sup>fl/fl</sup>*. To generate *Mx1-Cre/surv<sup>fl/fl</sup>; Bcl-2* mice, *Mx1-Cre/surv<sup>fl/fl</sup>* mice were crossed to *Bcl-2* transgenic mice to obtain *Mx1-Cre/surv<sup>fl/fl</sup>; Bcl-2* mice, which were then bred to *surv<sup>fl/fl</sup>* mice. All animal research was approved by the University of Chicago and Northwestern University Institutional Animal Care and Use Committees.



**Figure 7. Hematopoietic stem cells are susceptible to *survivin* deletion.** The c-kit and Sca-1 profiles of lineage-negative BM cells (negative for CD5, B220, CD11b, Ter119, Gr-1, 7-4) from *Surv<sup>fl/fl</sup>* ( $n = 1$ ) and *MxCre Surv<sup>fl/fl</sup>* ( $n = 2$ ) transplanted mice 10 wk after 3 doses of pl-pC (left plots). Lin<sup>-</sup>ckit<sup>+</sup>Sca-1<sup>+</sup> cells were gated and analyzed for CD45.1 and CD45.2 cell surface markers (right plots). Numbers indicate percentages of cell populations.

**Mouse genotyping.** Mouse genotyping was performed by PCR of tail DNA. For genotyping the *Mx1-Cre* transgene, the *Mx1-Cre* forward (5'-gctgcattaccgctgcatgaacga-3') and *Mx1-Cre* reverse (5'-gtggcagatggcgcg-gcaacaccatt-3') primers were used to amplify a 725-bp PCR product. Three primers (Adv17, Adv25, and Adv28) were used to genotype the wild-type, floxed, and Cre-deleted *survivin* allele, as previously described (31). For genotyping the *vav-Bcl-2* transgene, primers against the SV40 poly(A) DNA sequence were used (39).

**Histological analysis.** Newborn (10–14 d) mice received two intraperitoneal injections (13  $\mu$ g/g body weight) of pI-pC (GE Healthcare) every other day to induce *survivin* deletion. Sternum BM and spleen were isolated 10 d later, fixed overnight in formalin (Sigma-Aldrich), and processed for histological analysis. Serial sections were stained with hematoxylin and eosin (HE), and peripheral blood smears were stained with May-Grünwald Giemsa using standard protocols. Immunohistochemistry was performed on deparaffinized spleen sections with anti-Ter119 antibody (BD Biosciences).

**Flow cytometric analysis and cell sorting.** To obtain BM cells, mouse femurs and tibiae were flushed with PBS, and cells were separated by passage through a 20-gauge syringe. For spleen cells, spleens were cut into small pieces and subsequently disaggregated with an 18-gauge syringe. Cells were resuspended in PBS containing 1 mM EDTA and 1% bovine serum albumin (Sigma-Aldrich) to reduce aggregates and nonspecific staining, respectively. The cells were stained with different combinations of c-kit, Gr-1, Mac-1, CD41, CD71, and Ter119 antibodies, conjugated to APC, PE, or FITC (BD Biosciences). Finally, surface marker expression was analyzed using a FACSCanto flow cytometer (BD Biosciences) and FlowJo software. To isolate discrete erythroid populations, spleen cells were first stained with FITC-conjugated CD71 and PE-conjugated Ter119 antibodies. Then, based on the CD71 and Ter119 expression level, erythroid subpopulations were purified using a MoFlo-HTS cell sorter (Dako-Cytomation). FACS-sorted cells were cytospun onto slides and stained with either May-Grünwald Giemsa or benzidine Wright Giemsa for morphological evaluation.

**Cell cycle analysis.** Adult (6–8 wk) mice were injected intraperitoneally with one dose of pI-pC. BM cells were isolated 48 h later and stained with FITC anti-CD71 and PE anti-Ter119 antibodies. Cells were fixed with 2% paraformaldehyde (Sigma-Aldrich) and 70% ethanol to preserve cell surface antibody-conjugated fluorochrome staining. Subsequently, cells were treated with 2 N HCl/0.5% Triton X-100, followed by 0.1 M sodium tetraborate, and DNA was stained with DAPI (Sigma-Aldrich) at 4°C overnight. The cell cycle profile of CD71<sup>+</sup> Ter119<sup>+</sup> erythroid cells was analyzed using a LSR-II flow cytometer (BD Biosciences) and FlowJo software.

**Erythroid colony-forming assays.** Primary mouse BM cells were collected from *vav-Bcl-2* transgenic mice and their wild-type littermates and enriched for progenitors with the EasySep negative-selection mouse hematopoietic progenitor-enrichment kit (StemCell Technologies). The cells were either left untreated or irradiated with 200 or 300 rads in a Cesium-137 irradiator. To evaluate erythroid colony formation, 25,000 cells were plated in MethoCult 3234 (StemCell Technologies), supplemented with IL3, SCF, and 10 U/ml EPO. Pure burst-forming unit erythroid and mixed colonies were enumerated after 10 d.

**BM transplantation.** C57BL/6 CD45.1<sup>+</sup> congenic recipients (6–8 wk) were purchased from The Jackson Laboratory and placed on Bactrim-containing water 1 wk before lethal irradiation. Mice were irradiated with a total of 1,200 rads in split doses (800 rads, and then 400 rads 4 h later). Total nucleated BM cells (10<sup>6</sup>), marked by the CD45.2<sup>+</sup> allele from untreated control, *Mx1-Cre/surv<sup>fl/fl</sup>*, or *Mx1-Cre/surv<sup>fl/β</sup>* mice, along with CD45.1<sup>+</sup>/CD45.2<sup>+</sup> support cells (0.5 × 10<sup>6</sup>), were injected retroorbitally into lethally irradiated CD45.1<sup>+</sup> recipients that were anesthetized with Nembutal (Abbott Laboratories). CD45.2<sup>+</sup> and CD45.1<sup>+</sup>/CD45.2<sup>+</sup> BM cells were allowed to

engraft for 5 wk after transplantation. After the engrafting, peripheral blood from recipient mice was obtained by tail vein bleeding, subjected to red blood cell lysis, and stained with PE-Cy5.5-conjugated CD45.1 (eBioscience) and FITC-conjugated CD45.2 (BD Biosciences) antibodies to verify donor cell engraftment by flow cytometric analysis. A subset of the recipients was injected with 2 doses of pI-pC, whereas the remainder served as untreated controls. BM cells from pI-pC-treated and control animals were isolated 2 wk after pI-pC administration and analyzed for the presence of CD45.2<sup>+</sup> and CD45.1<sup>+</sup>/CD45.2<sup>+</sup> donor cells. For HSC analysis, BM cells from transplanted mice, 10 wk after 3 doses of pI-pC, were depleted of mature hematopoietic lineages, expressing surface antigens CD5, CD11b, B220, Gr-1, Ter119, and 7–4, with EasySep (StemCell Technologies). Lineage-negative cells were stained for APC anti-c-kit, PE-Cy7 anti-Sca-1 (eBioscience), FITC anti-CD45.2, and PE-Cy5.5 anti-CD45.1 antibodies and analyzed by FACSCanto flow cytometer.

The authors thank Dr. Astar Winoto for the *survivin*-floxed mice, Drs. Jerry Adams and Kay Macleod for the *vav-bcl2* transgenic mice, Dr. Hanno Hock for advice on *Mx1-Cre* targeting, The University of Chicago Cancer Research Center, and the Flow Cytometry Core Facility for technical expertise. Additional thanks to Mitchell Weiss, Iannis Aifantis, Jonathan Licht, and Hanno Hock for critical review of the manuscript.

This research was supported, in part, by the Elsa U. Pardee Foundation and by National Institutes of Health grants CA101774 and DK074693. J.D. Crispino is a Scholar of the Leukemia and Lymphoma Society.

The authors have no conflicting financial interests.

Submitted: 14 November 2006

Accepted: 24 May 2007

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