An anticlastogenic function for the Polycomb Group gene Bmi1

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Bmi1 is a key component of multiprotein Polycomb repression complex 1 (PRC1), and its disruption in mice induces severe aplastic anemia by early adulthood. The contributing mechanisms responsible for this phenotype remain elusive. Here we show that transformed human cell lines as well as primitive hematopoietic cells exhibit a high frequency of spontaneous chromosome breaks upon Bmi1 depletion and are hypersensitive to genotoxic agents. Consistent with these observations, we found that Bmi1 is recruited rapidly to DNA damage foci where it blocks transcriptional elongation. We also show that Bmi1 contributes to homologous recombination DNA repair and is required for checkpoint recovery. Together, our results suggest that Bmi1 is critical for the maintenance of chromosomal integrity in both normal and transformed cells.

The Polycomb group gene (PcG) Bmi1 is known as a key determinant of normal and leukemic hematopoietic stem cell (HSC) function. In its absence HSCs fail to self-renew, leading to bone marrow failure and profound anemia in young mice. Although many functions have been ascribed to Bmi1, the molecular mechanisms underlying its role in HSCs remain uncertain. In mouse and human fibroblasts, Bmi1 genetically interacts with p16ink4a and/or p19arf to prevent senescence (1–4). Bmi1 binds the loci together directly with other PcG proteins leading to changes in histone modifications compatible with gene repression (5, 6). Evidence suggests that the inactivation of ink4a/arf is not the sole mechanism by which Bmi1 regulates HSC activity. In support of this evidence, Bmi1−/− leukemia cell lines lacking expression of p16ink4a and p19arf still require the ectopic expression of Bmi1 to generate leukemia in vivo (7). Moreover, the demonstration that Bmi1 genetically interacts with E4 transcription factor 1 (E4f1), an atypical p53 E3 ubiquitin ligase (8), to regulate the repopulating activity of HSCs in an INK4a/ARF-independent manner further substantiates this hypothesis (9).

Biochemical studies recently showed that the PcG protein ring finger protein 2 (RNF2) is an E3 ubiquitin ligase exhibiting monoubiquitination activity toward histone H2A. Bmi1 has been proposed to protect RNF2 from degradation and to enhance its monoubiquitinating activity (10).

Several studies suggested that PcG proteins, including Bmi1, have a role in the DNA damage response/repair process (11–15). Hong et al. (14) recently reported the immediate recruitment of the Polycomb repressive complex 2 (PRC2)-associated PHD finger protein 1 (PHF1) to double-strand breaks (DSB) following irradiation. PHF1 interacts directly with Ku70/80 and RAD50 proteins, and its knockdown sensitizes cells to irradiation. Similarly, Rouleau et al. (15) showed that poly(ADP ribose)polymerase 3 (PARP-3), which interacts with proteins typically found at DSBs in the nonhomologous end-joining (NHEJ) pathways, is part of the PRC2 complex that includes enhancer of zeste homolog 2 (EZH2) and suppressor of zeste 12 homolog (SUZ12). Together these observations strongly suggest a role for the PRC2 in the NHEJ repair pathway.

Using RNAi, Bergink et al. (16) demonstrated that UV-induced H2A monoubiquitination is reduced in cells engineered to express low levels of the RNF2 PcG protein. Recently, the SUMO E3 ligase PcG gene Pc2, known to interact with Bmi1 and RNF2 within Polycomb repression complex 1 (PRC1), has been shown to be essential for the sumoylation and nuclear localization of centrin 2 required in the nucleotide-excision repair process (17).

Two recent studies have linked Bmi1 to oxidative metabolism. Chatoo et al. (18) reported that Bmi1 promotes intracellular accumulation of reactive oxygen species (ROS) in neurons through repression of p53 pro-oxidant activity. Liu et al. (19) showed that Bmi1 deficiency leads to increased expression of several genes involved in ROS homeostasis and mitochondrial function. They also demonstrated that the activation of ROS-mediated DNA damage response in Bmi1-deficient mice occurs in an ink4a/arf-independent manner. (19) Here, we build on these observations and show that Bmi1 ensures the maintenance of chromosome integrity in both normal and transformed cells by promoting transcriptional repression at the lesions. We also document that the loss of Bmi1 recapitulates several phenotypes associated with genomic instability syndromes (i.e., cell-cycle defects, DNA damage sensitivity, and ROS accumulation) that often result in bone marrow failure, immunodeficiency, congenital abnormalities, and growth retardation.

Results

Correlation Between Survival of Bmi1−/− Mice and Activity of Long-Term Repopulating HSC. Deletion of Bmi1 leads to axial skeleton patterning and hematopoietic defects, severe ataxia, and seizures. Although Bmi1-deficient mice survive after birth, about half of them die before the weaning period (3 wk old) (20). Mice that do survive the first days die within 4–10 wk. By simplifying young ataxic animals’ access to water and nutrients, we were able to extend the median and maximum lifespan of Bmi1-deficient mice significantly, to 150 d (Fig. S1 A), at which time a phenotype of very severe bone marrow failure was observed. Considering the 4- to 5-mo transition period typically associated with HSCs that show short- or intermediate-term versus long-term repopulation (LTR) potential (21), these data are consistent with the hypothesis that Bmi1 deficiency is not compatible with the maintenance of LTR-HSC activity (Fig. S1B).

Cell-Cycle Progression Defect in Bmi1−/− HSCs. By conducting a series of genetic complementation studies (Fig. S1C), we showed that disruption of p53/pRb pathways (E6–E7), prevention of premature senescence (T-box 2, TBX2) (22) or reduction of ROS levels (N-acetyl cysteine, NAC) failed to restore the LTR activity of Bmi1−/− HSCs (Fig. S1 D and E). Although freshly isolated Bmi1+/− fetal liver cells can be fully rescued by Bmi1+/-

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its ΔPEST mutant, it was impossible to rescue Bmi1−/− cells that were kept in culture for 2 d or more. To gain further insights into this observation, we examined the cell-cycle status of primitive Bmi1−/− hematopoietic cells that were kept in culture under growth conditions that normally support fetal liver HSC activity (23). As shown in Fig. S1F, cultures initiated with these cells showed a significant increase of both cycling (fewer cells in G0) and apoptotic (subG1) cells compared with control HSCs. Strikingly, Bmi1+/− HSCs accumulated in G2 (Fig. S1F). This finding indicates that Bmi1-deficient fetal liver HSCs are exquisitely sensitive to ex vivo manipulation and are impaired irreversibly after a few days in culture. Overall, these data suggest that the severe phenotype observed in Bmi1−/− cells is likely to be the result of cumulative effects rather than being attributable only to deregulation of p53 or pRb pathways.

γ-H2AX Foci Formation in the Absence of BMI1. The multiple cell-cycle abnormalities observed in cultured Bmi1−/− cells, together with the growing body of evidence linking PcG genes to DNA damage response, prompted us to investigate further the potential role for BMI1 in this process.

We first performed a series of time-course experiments to characterize the appearance of DNA damage-induced γ-H2AX foci in murine embryonic fibroblasts (MEF) freshly isolated from wild-type or Bmi1−/− mice. As expected, in wild-type MEF, γ-H2AX* foci could be detected as early as 5 min after ionizing radiation (T = 5 min) (Fig. L4). The number of γ-H2AX* foci per cell (Fig. 1C, white bars) as well as the percentage of positive cells (Fig. 1D, black line), increased markedly by 4 h after irradiation. Consistent with a normal DNA-repair process, the number of γ-H2AX foci returned to baseline levels when examined 24 h postirradiation (Fig. 1A–C).

Strikingly, we observed a two- to threefold increase in the number of spontaneous γ-H2AX foci in Bmi1−/− versus wild-type MEF (Fig. 1B Upper and D). The early appearance (T = 5 min) of γ-H2AX foci was not affected by the absence of Bmi1 (Fig. 1B). At 24 h postirradiation, however, we noted a fivefold increase in the proportion of cells with persistent γ-H2AX foci in Bmi1−/− MEF as compared with wild-type (33 ± 5% vs. 6 ± 2%, respectively) (red box in in Fig. 1B Bottom Right and gray line in Fig. 1D).

S-G2/M Checkpoint Recovery Defect in Bmi1 Mutant Cells. To test whether the presence of persistent γ-H2AX foci in Bmi1-deficient MEF is associated with a defect in checkpoint recovery, we performed BrdU pulse-chase experiments to label cells in S phase selectively at the time of camptothecin (CPT) treatment and to track their progression through the cell cycle after CPT removal. Importantly, we did not find obvious differences in cell-cycle distribution and kinetics between untreated wild-type and Bmi1-null cells (Fig. S2, compare panels in rows 1 and 2). As expected, CPT treatment delayed the progression from S to G2/M in wild-type cells (Fig. S2 C1 and C3). By 12 h, most of these CPT-treated cells had moved into G2/M (Fig. S2D3), consistent with a checkpoint release of repaired wild-type cells. In contrast, Bmi1−/− cells failed to recover from CPT treatment, as shown by a prolonged arrest at the S-phase checkpoint (compare progression of Bmi1−/− cells in Fig. S2 C4 and D4 with that of wild-type cells in Fig. S2 C3 and D3).

Spontaneous Chromosome Breaks in Cell Lines with Reduced Bmi1 Levels. The persistence in checkpoint activation and γ-H2AX focus formation along with the aplastic anemia phenotype suggested that BMI1 might be implicated in maintenance of chromosome integrity. To test this hypothesis, we first correlated the frequency of spontaneous chromosome breaks in two well-characterized human cell lines (HCT116 and 293T) in which BMI1 levels are acutely decreased by the use of shRNA vectors. To facilitate cytogenetic analysis, we used the HCT116 cell line, a human near-diploid colon carcinoma cell line with few well-known chromosomal abnormalities.

In both cell lines, Bmi1 knockdown resulted in the formation of radial chromosome forms reminiscent of some chromosomal instability syndromes. We also observed an increase in the rate of spontaneous chromosome breaks in both shBmi1-transfected cell lines (arrows in Fig. S3A). HCT116 cells transfected with a control vector showed a maximum of two breaks per cell, with 54% of cells having no chromosome breaks (black bars in Fig. S3B). In contrast, shBmi1-transfected HCT116 cells were more susceptible to spontaneous chromosome breakage, with a maximum of 16 breaks per cell and with 56% of cells having more than two breaks (white bars). Similarly, although 62% of control 293T cells showed no break, 84% of shBmi1-transfected 293T cells displayed more than two breaks per cell (Fig. S3B Right). shBmi1-transfected 293T cells exhibited a much higher frequency of chromosome breakage than shBmi1-transfected HCT116 cells, with the number of breaks per cell exceeding 20 in 56% of the scored metaphases (Fig. S3B). Together these results link spontaneous chromosome breakage with reduction in BMI1 protein levels.

Loss of Bmi1 Renders Cells Hypersensitive to Clastogenic Agents. To investigate if Bmi1 impairment affects chromosome integrity upon DNA damage, we performed cytogenetic analysis of HCT116 and 293T cells engineered to express low levels of Bmi1 and exposed to different clastogens. As shown in Fig. 2A, Bmi1 knockdown resulted in a marked increase of CPT- and aphidicolin-(APh) induced chromosome breaks in both cell lines (red arrows).
As described in two independent experiments, reduction of BMI1 protein levels increased least 50 Giemsa-stained metaphases per condition. Results shown are from the same as those shown in Fig. 3. Chromosomal aberrations were scored in APH. Control and shBmi1-transfected HCT116 also were exposed to 1293T cells exposed as in and chromatid breaks in control (CT) and shBmi1-transfected HCT116 and exhibit pulverized metaphases. (Arrows indicate radial forms or breaks. Note that only shBmi1-transfected 293T cells that show elevated ROS levels (Fig. S4A). We next observed that sorted Bmi1−/− Lin“Sca1+” cells are more sensitive than the wild-type counterpart to CPT (Fig. S4C). To determine whether Bmi1 deficiency confers increased sensitivity to DNA damage in vivo, we exposed 6-wk-old Bmi1−/− knockout mice to whole-body irradiation. As shown in Fig. 3A, although none of the irradiated wild-type or heterozygote mice showed mortality, all Bmi1−/− mice died within 3 wk after exposure to 500 cGy γ irradiation. We next determined whether in vivo exposure to the ROS scavenger NAC could confer protection to Bmi1−/− deficient mice that show elevated ROS levels (Fig. S4B and ref. 10). This treatment, although reducing the ROS levels in Bmi1−/− hematopoietic cells, failed to prevent mortality of Bmi1−/− mice after irradiation (Fig. 3B, gray line, and Figs. S4B). Moreover, histological analysis revealed that, regardless of NAC pretreatment, bone marrow from irradiated Bmi1−/− mice was severely hypoplastic and replaced by adipocytes (Fig. 3C Lower). Bone marrow specimens isolated from irradiated wild-type littermates were histologically normal (Fig. 3C Upper), indicating a complete recovery of control cells. In agreement with these results, we observed a dramatic reduction in bone marrow progenitor numbers in irradiated Bmi1−/− mice (Fig. 3D, red bars). Irradiation also dramatically increased the proportion of apoptotic Bmi1−/− cells compared with control cells (Fig. 3E).

To monitor the impact of in vivo irradiation on the proliferation of primitive Bmi1−/− bone marrow cells, we performed a CFSE dye-dilution analysis with Lin“ cells (mutant and controls) over a 3-d period (Fig. 3F Upper). Under these conditions, nonirradiated Lin“ wild-type cells underwent multiple rounds of division after a 72-h culture (Fig. 3Fa). Lin“ cells derived from mutant mice initiated with Bmi1−/− bone marrow cells exhibited a much higher proportion of early-generation cells (CFSEhigh) compared with the wild-type counterpart, pointing to a proliferation defect (Fig. 3Fc). After 2-Gy exposure, the wild-type cell population showed significant recovery, because most of Lin“ cells resumed cycling and had progressed through approximately five divisions by 72 h (Fig. 3Fb). In contrast, the majority of Lin“ Bmi1−/− irradiated cells were permanently arrested after one di-

Moreover, radial chromosome forms were elevated significantly in shBmi1-transfected cells compared with control cells, regardless of the treatment. APH treatment of 293T cells engineered to express low levels of Bmi1 resulted in a high incidence of chromosome fragmentation and pulverization (Fig. 2A Lower Right). The frequency and distribution of chromosomal aberrations were evaluated for each population and each condition (Fig. 2B). As expected, CPT treatment significantly increased the yield of chromosome breaks in control HCT116 cells, with 48% of cells showing breaks compared with 16% of cells (with a maximum of one break per cell) in the untreated population (compare blue bars and green bars, Fig. 2B Lower Left). Bmi1 knockdown significantly enhanced the sensitivity of HCT116 cells to CPT treatment, with 100% of cells having breaks at a maximum of 46 breaks per cell (compare green bars and purple bars, Fig. 2B Lower Left). Similarly, HCT116 cells with reduced Bmi1 levels were more susceptible to APH-induced chromosome aberrations than cells with normal Bmi1 levels (compare light blue bars and orange bars, Fig. 2B Center). Bmi1 knockdown in APH-treated 293T cells resulted in a dramatic increase in the number of breaks per cell and in the number of pulverized cells, with 76% of cells displaying more than 20 breaks per cell or pulverization (see orange bars, Fig. 2B Lower Right). Together these data show, in two different human cell lines, that lowering Bmi1 levels results in a high incidence of spontaneous and clastogen-induced chromosome breaks.

**Fig. 2.** Cells with reduced levels of BMI1 are highly susceptible to clastogen-induced chromosome breaks. (A) Control and shBmi1-transfected HCT116 and 293T cells were exposed to APH (0.1 μM and 0.2 μM for HCT116 cells, 0.05 μM and 0.1 μM for 293T cells). (Top) For CPT-sensitivity experiments, control and shBmi1-transfected HCT116 cells were treated with 1 μM CPT for 1 h and allowed to recover for 24 h. (Middle and Bottom) For APH-sensitivity experiments, HCT116 and 293T cells were exposed to APH for 24 h. Representative images of metaphase spreads of each population are shown. Arrows indicate radial forms or breaks. Note that only shBmi1-transfected 293T cells exhibit pulverized metaphases. (B) Distribution of chromosome gaps/breaks and chromatid breaks in control (CT) and shBmi1-transfected HCT116 and 293T cells exposed as in A to no drug (0) or to increasing concentrations of APH. Control and shBmi1-transfected HCT116 also were exposed to 1 μM CPT as described in A. Note that values for untreated cells (blue and red bars) are the same as those shown in Fig. 3. Chromosomal aberrations were scored in at least 50 Giemsa-stained metaphases per condition. Results shown are from two independent experiments. Reduction of BMI1 protein levels increased the frequency of DNA damage-induced chromosomal breaks in both cell lines (P < 0.0001, Mann–Whitney test).
vision (arrow in Fig. 3Fd). Again, NAC pretreatment was largely ineffective in preventing this effect (Fig. 3Fh). These data clearly show that ROS scavenging failed to prevent DNA damage-induced cell-cycle arrest in Bmi1−/− primitive hematopoietic cells.

**Bmi1 Is Required For Efficient Transcriptional Repression at Damaged Foci.** Because elevated ROS levels could not account completely for the DNA damage checkpoint defects observed in knockout cells, we next delineated the precise role of Bmi1 in the established DNA damage-signaling cascade.

As recently shown by others (11, 13), we first confirmed that Bmi1 is recruited rapidly to DNA damage foci after exposure to UV light (Fig. S5), γ irradiation (Fig. S6A–C), and S phase-specific genotoxic agents such as CPT and hydroxyurea (HU) (Fig. S6D). This recruitment is observed in both HeLa and HCT116 cell lines (Figs. S5 and S6D and E) and in primary cells (MEF and primitive hematopoietic cells) (Fig. S6A–C). Moreover, coimmunoprecipitation experiments performed with HCT116 cell extracts showed that endogenous Bmi1 associates with γ-H2AX and mono- and diubiquitin γ-H2AX at the chromatin-bound fraction upon irradiation (Fig. S6F).

Considering the role of PcG proteins in gene silencing, we next investigated whether Bmi1 accumulated to DNA damage foci to repress transcriptional activity at the injured site. RNA polymerase II (pol II)-mediated transcriptional elongation is associated with extensive phosphorylation at the serine 2 (ser2) residue of RNA pol II (Fig. 4A). As recently shown by others (11, 13), we next confirmed that Bmi1 is recruited rapidly to DNA damage foci after exposure to UV light (Fig. S5), γ irradiation (Fig. S6A–C), and S phase-specific genotoxic agents such as CPT and hydroxyurea (HU) (Fig. S6D). This recruitment is observed in both HeLa and HCT116 cell lines (Figs. S5 and S6D and E) and in primary cells (MEF and primitive hematopoietic cells) (Fig. S6A–C). Moreover, coimmunoprecipitation experiments performed with HCT116 cell extracts showed that endogenous Bmi1 associates with γ-H2AX and mono- and diubiquitin γ-H2AX at the chromatin-bound fraction upon irradiation (Fig. S6F).

**Defect in Homologous Recombination in Cells Expressing Low Levels of Bmi1.** We next tested whether Bmi1-deficient cells have a bona fide homologous recombination (HR) defect using the HR reporter assay system established by Jasim and coworkers (25) as depicted in Fig. 5A. In this system, cleavage of the I-SceI site between two incomplete EGFP genes (SceGFP and iGFP)
induces HR, thereby resulting in the restoration of a functional GFP gene. Fig. 5B shows a typical example of GFP expression level in control cells (Upper Right) and shBmi1-transfected cells (Lower Right) following ectopic expression of SceI endonuclease. As summarized in Fig. 5C, the proportion of GFP* cells falls from 11 ± 1.9% in control cells to 3.2 ± 1.3% in cells expressing an shRNA vector to Bmi1, thus supporting its contribution in HR-dependent DNA repair.

Discussion
The ability to maintain genome integrity has emerged as a critical influence on stem cell longevity (26). This capacity relies on the proper execution of a finely tuned molecular network that coordinates DNA repair with cell-cycle progression. In the present study, we uncovered a function for Bmi1 in safeguarding chromosomal integrity of primitive hematopoietic cells and of transformed/immortalized cell lines. Moreover, our results reinforced recent observations documenting a delocalization of BMI1 at DNA lesions upon DNA damage (11–13).

During the course of our study, another group reported the recruitment of multiple members of PcG complexes (PRC1 and PRC2) to DSB. This recruitment occurs independently of ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) kinases (11). Interestingly, BMI1 recruitment to DSB required its really interesting new gene (RING) domain as the fork-head-associated (FHA) and breast cancer gene 1 C-terminal (BRCT) domains of nibrin (NBS1) (13) and occurs in a PARP1/2-dependent manner (11).

Our study also demonstrates that loss of Bmi1 does not prevent or delay the DNA damage-induced formation of γ-H2AX foci, indicating that Bmi1 is dispensable for the initial recognition of DNA breaks.

Considering these observations, one could ask whether BMI1 is recruited to repress transcription of genes located in the damaged area and/or is involved in posttranslational modifications that allow the sequential accrual of many DNA damage/repair proteins.

Strikingly, we observed an aberrant RNA pol II (ser2) phosphorylation, a mark that denotes transcription elongation, at DSBs, strongly suggesting a role for BMI1 in transcriptional repression of the damaged area.

Interestingly, the persistent γ-H2AX foci observed in Bmi1-deficient cells were larger than those detected in wild-type cells. Two main classes of DNA damage-induced foci have been described: the early, small foci that are observed from 10 min to 8 h after induction of DNA damage, and the late, larger foci that appear only after 4 h following DNA damage and persist after the majority of breaks have been repaired. It has been suggested that these late foci mark the location of lesions that are particularly difficult to repair (27, 28). Thus, it is conceivable that loss of Bmi1 impedes the repair of late DNA damage-induced foci. Consistent with a persistence of unrepaired damage and a checkpoint recovery defect, Bmi1 depletion leads to a hyperactivated and sustained ATM/checkpoint kinase 2 (CHK2) pathway (19), raising the possibility that Bmi1-deficient HSCs undergo premature senescence as a result of accumulated DNA damage.

Supporting a role for PRC1 in protein ubiquitination, loss of Bmi1 dramatically diminishes the accumulation of ubiquitin conjugates (including ubiquitin-γ-H2AX) upon DNA damage (13) and hence impinges on the subsequent signal amplification that is indispensable for efficient repair.

p53 and retinoblastoma (Rb) proteins are known to play a critical role in the maintenance of the G2 checkpoint and in the prevention of mitotic catastrophes in response to DNA damage.
occurring between the S and G2 phases (29). In fact, abrogation of the G2 checkpoint in p53-deficient HCT116 cells results in chromosome fragmentation in response to APH-induced DNA damage (30). Therefore, the chromosome pulverization observed in p53-deficient 293T cells (31) may result from the combination of Bmi1 deficiency and impaired G2 checkpoint. Importantly, we also found that Bmi1 deficiency sensitizes MEF and primary hematopoietic cells to clastogenic agents.

Several inherited bone marrow failure syndromes leading to aplastic anemia, such as dyskeratosis congenita or Fanconi anemia, are regarded as genomic instability disorders (32). Here we show that, in addition to the aplastic anemia and HSC exhaustion previously described, Bmi1-deficient cells share several features with genomic instability disorders, including accumulation at the G2/M phase of the cell cycle and chromosome instability. The absence of mitotic cells in splenocytes isolated from Bmi1-deficient mice precludes the analysis of chromosomal instability as classically performed. However, cytogenetic analysis performed on two different cell lines clearly showed that reduction in Bmi1 protein levels results in an increase in spontaneous chromosome aberrations and hypersensitivity to DNA-damaging agents. The presence of radial chromosome structures in cells with reduced Bmi1 levels further emphasizes the similarities with genomic instability disorders.

Overgeneration of ROS has been prominently associated with genomic instability and premature aging phenotypes. Considering the recent role ascribed to Bmi1 in antioxidative defense mechanisms (18), one could ask whether the acute radiation sensitivity of Bmi1-deficient mice might arise from the elevated ROS levels. Furthermore, recent studies revealed an unsuspected role for oxidative metabolism in the regulation of hematopoietic cell fate, because the induction of ROS production in Drosophila led to premature differentiation of all blood cells (33). Taken together, these observations raised the possibility that PCG-mediated function in HSC self-renewal occurs in part through the control of ROS generation. In the present study, we confirmed that Bmi1 deficiency leads to enhanced ROS production in hematopoietic cells. However, although antioxidant agents significantly reduced ROS levels and gave a minor proliferative advantage to Bmi1−/− progenitors, they failed to prevent the hypersensitivity of these cells to DNA damage. Importantly, the alteration of mitochondrial function and the ensuing enhanced ROS production observed in Bmi1-deficient cells occurs independently of the Ink4a/Arf pathway. Moreover, the abrogation of the normally activated DNA-damage response observed in Bmi1-deficient cells, through chk2 deletion, although rescuing some defects of Bmi1-deficient mice, failed to restore the long-term repopulating ability of Bmi1−/− HSC (19).

Together, the results presented here document an anticlastogenic function for Bmi1, further expanding its involvement in multiple cellular processes and possibly explaining its key function in many different types of stem cells.

Materials and Methods

Scoring Technique Used for Analysis of Chromosome Breakage. Analysis was performed on 50 Giemsa-stained metaphases in treated and untreated cultures for the control vector and shBmi1-transfected specimens. The number and type of structural chromosome abnormalities were scored. Chromatid gaps were not included in the final score. Isochromatid gaps, chromatid and isochromatid breaks, deletions, and fragments were scored as a single break. Structural rearrangements including dicentrics, rings, translocations, and radial figures were scored as two breakage events. Pulverized cells were noted. The total chromosome abnormalities and the average number of aberrations per cell were scored for each sample. Dicentrics and translocations were not scored for the aphidicolin test using the 293T transfected cells.

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Supporting Information

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SI Materials and Methods

Antibodies and Plasmids. Monoclonal anti-Polycomb ring finger oncogene (BMI1) (F6) and γ-H2AX antibodies were purchased from Upstate; nibrin (NBS1) and mediator of DNA-damage checkpoint 1 (MDC1) antibodies were from Bethyl Laboratories; and 53BP and meiotic recombination 11 (MRE11) antibodies were from Novus. Anti-RAD51 was from Lifespan Bioscience, and RNA pol II (ser2) was from Abcam. For some costainings rabbit anti-BMI1 antibody from Cell Signaling was used.

DR-GFP and pCBASceI plasmids used for the homologous recombination assay were obtained from Addgene.

Chemicals and Treatments. Gamma irradiation was delivered with a 137Cs irradiator. Camptothecin (CPT) and Aaphidicolin (APH) were purchased from Sigma-Aldrich. For time-course and recovery experiments, cells were washed twice after treatment and processed as described in the figure legends. For APH sensitivity assays, cells were exposed for 24 h to increasing concentrations of drug and processed for cytogenetic analysis.

For in vivo analysis, N-acetyl cysteine (NAC) was added to drinking water (1 mg/mL) 1 wk before irradiation and during the whole experimentation process. For experiments in vitro, cell cultures were treated with 100 μM NAC as indicated in the figure legends.

Cell Culture, shRNA, and Transfection. Murine embryonic fibroblasts were generated according to published methods. Isolation, infection, and transplantation of embryonic day 14.5 (E14.5) fetal liver cells were done as previously reported (1). All cell lines were cultured in DMEM supplemented with 10% FBS. The HCT116 cell line, a human near-diploid colon carcinoma cell line with few chromosomal abnormalities, was studied by standard G-banding techniques before the chromosomal breakage studies. Karyotype was as previously reported: 45,X,Y(8;16)(q13;p13),add(10)(q26),add(18)(p11). The 293T cell line, a near-triploid human embryonic kidney cell line, also was used for the aphidicolin test to confirm results obtained with the HCT116 mismatch repair-deficient cell line.

The shRNA sequence to Bmi1 used in the studies presented in this paper was selected from a group of seven different shBmi1 tested in preliminary experiments. Four of these seven shRNA induced hypersensitivity to CPT as evaluated by chromosome spreads. Notably, the four shRNA vectors to Bmi1 associated with this phenotype provided levels of mRNA knockdown which corresponded to 60% or more of that observed in shLuc (control) cells. The shRNA which provided 30% knockdown did not confer hypersensitivity to CPT. The selected shBmi1 encoding retrovirus selected was a gift from John Dick (Ontario Cancer Institute, Toronto, ON, Canada). With this sequence, levels of knockdown determined by Western blot analysis reached up to 80% of protein reduction. Empty vector was used as control. Experiments showing less than 60% reduction in BMI1 protein levels (e.g., low levels of transfection) have not been taken into consideration.

Transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s recommendations.

Retroviral Generation, Infection, and Transplantation of E14.5 Fetal Liver and Bone Marrow Cells. Details of the different retroviral vectors used in this study are available from the authors on request. Production of vesicular stomatitis virus-pseudotyped (VSV) retroviruses, infection of hematopoietic cells, and transplantation into mice were done as described by Thorsteinsdottir et al. (2).

Preparation of Cell Extracts, Immunoprecipitation, and Western Blotting. Total protein extraction was performed in Laemmli buffer. Core histones were extracted using classical acidic extraction method. Chromatin-bound (CB) and cytoplasmic/nucleoplasmic (CN) fractionation were performed as described by Mladenov and colleagues (3). To determine the purity of CB and CN protein fractions, membranes were probed with histone H3 (Abcam) and α-tubulin (Cell Signaling Technology) antibodies. Immunoprecipitations and immunoblots were done according to published methods. As negative controls for immunoprecipitation experiments, premum rabbit serum or normal mouse IgG were used.

Immunofluorescence and Confocal Microscopy. For immunofluorescence studies, cells grown on glass coverslips (Fisher Scientific) were fixed in 4% paraformaldehyde, permeabilized in 0.25% Triton X-100, and immunostained with the combination of antibodies specified in the figure legends. Where indicated, the cells were preextracted before fixation for 5 min at 4 °C in a buffer containing 25 mM Hepes at pH 7.5, 50 mM NaCl, 1 mM EDTA, 3 mM MgCl2, 300 mM sucrose, and 0.5% Triton X-100. Confocal images were captured using a Zeiss LSM510 laser-scanning microscope.

FACS Analysis. Flow cytometry analyses were performed using a LSRII cytometer (Becton Dickinson). For intracellular antigens, cells were first fixed with 3.7% paraformaldehyde and permeabilized with 0.25% Triton X-100. Cell-cycle status of hematopoietic cells was determined by staining for 45 min at 37 °C with Hoescht 33342 (Molecular Probes) and pyronin Y (Sigma), followed by intracellular staining with phospho (Ser10)-histone H3 antibody (Upstate). BrdU pulse-chase experiments were performed as described by Strom et al. (4).

For analysis of intracellular reactive oxygen species, splenocytes were incubated with 5 μM dichlorofluorescein diacetate (DCFDA) (Invitrogen) and incubated in a shaker at 37 °C for 30 min, followed immediately by flow cytometry analysis. CFSE-labeling experiments were performed as described by Strom et al. (4).

Histological Analysis. Bone samples were fixed, decalcified in EDTA (14%) for 12 d, and embedded in paraffin. Sections then were stained with H&E for morphological observation.

Metaphase Spreads. Cells from the different experimental groups were collected after colcemid pretreatment (0.2 μg/mL for 1 h). Metaphase spreads were prepared after hypotonic treatment (0.075 M KCL solution for 15 min at 37 °C) and three fixations with Carnoy’s fixative (3:1 methanol:acetic acid).

Fig. 51. (A) Extended survival of Bmi1−/− mice in modified feeding conditions. Kaplan–Meier survival curves of Bmi1-knockout mice (n = 23) compared with heterozygotes (n = 103) and wild-type (n = 76) littermates over a period of 7 mo. Median lifespan of Bmi1−/− mice is 150 d when food and water are in the bottom of the cage (Log rank test, P = 0.002). (B) Bmi1 appears critical for the maintenance of long-term repopulating hematopoietic stem cells (LTR-HSC). Despite a normal HSC contingent in Bmi1−/− LTR-HSC in E14.5 fetal liver cells, loss of engraftment correlated with the pattern of reconstitution typically associated with LTR-HSC. STR, short-term repopulating. (C) Overview of experimental strategy for complementation studies. Bmi1−/− fetal liver cells (the equivalent of one fetal liver) were transduced with control, Bmi1, or candidate gene (lists in D and E) retroviral vectors. The equivalent of half of a fetal liver was kept in culture for 3 wk and assayed at different time points for content of colony-forming cells (CFC). The other half was transplanted in irradiated mice (three mice for each candidate gene) for in vivo studies. Q-PCR, quantitative RT-PCR. (D) FACS profile showing donor contribution (GFP+ Ly5.2+) at 12 and 32 wk posttransplantation in the peripheral blood of recipients of Bmi1−/− fetal liver cells engineered to express the candidate genes. Representative results of three independent experiments performed in triplicate are shown. (E) Summary of the results shown in D and comparison with rescue of phenotype of colony-forming cells (second column) and p16+/− and p19+/− expression (fifth column) as evaluated by quantitative RT-PCR analysis of infected cells. +, partial or complete rescue; −, no rescue. (F) Bmi1+/+ and Bmi1−/− fetal liver cells were isolated and cultured for 4 d in conditions that sustain LTR-HSC activity. Cultured cells then were stained with lineage (Gr1, B220, and Ter119), CD48, and CD150 antibodies to analyze the HSC-enriched population. Simultaneous staining with the DNA dye (Hoescht), RNA dye (Pyronin), and mitotic marker (phospho-histone H3) was used to determine the cell-cycle status of the Lin−CD48+ primitive subpopulation. The figure shows representative FACS profiles from three independent experiments.
Fig. S2. Bmi1+/+ and Bmi1−/− mouse embryonic fibroblasts (MEF) were pulse-labeled with 50 μM BrdU for 30 min, washed, and then treated or not treated with 1 μM CPT for 30 min. CPT was removed, and the S-phase population was monitored immediately after treatment (T = 0) and at the indicated times after CPT removal. Bivariate dot plots (BrdU incorporation versus DNA content) show representative data from three independent cultures.
Fig. S3. (A) Representative pictures of metaphase spreads from HCT116 and 293T cells transfected with control (CT) or shBmi1-expressing vector. Arrows indicate chromosomal aberrations including radial forms and chromosome and chromatid breaks. (B) Distribution of chromosome breaks in HCT116 and 293T cells expressing normal (CT) or reduced protein level (shBmi1) of BMI1 (as evaluated by Western blot analysis). At least 50 Giemsa-stained metaphases were scored for each population. Only experiments showing more than 60% reduction in BMI1 protein levels have been analyzed. The nonparametric Mann–Whitney test was performed to calculate the differences between the groups. Reduction of BMI1 protein levels increased the frequency of spontaneous chromosomal breaks in two independent experiments. $P < 0.001$. 
**Fig. S4.** (A) Effect of various DNA-damaging agents on survival of Bmi1+/+ and Bmi1−/− MEF. Cells were treated with increasing amounts of clastogens for 24 h. Cell survival was assessed by annexin V/propidium iodide (PI) staining 48 h after treatment. Each point represents the average of three independent experiments. (B) Distribution of chromosome gaps/breaks and chromatid breaks in young Bmi1+/+ and Bmi1−/− MEF exposed to no drug (DEB 0) or diepoxibutane (DEB 500 ng/mL) for 48 h. Chromosomal aberrations were scored in at least 50 Giemsa-stained metaphases per condition. Results are from two independent experiments. Loss of Bmi1 increased the frequency of diepoxibutane-induced chromosomal breaks (P = 0.0035; Mann–Whitney test). (C) Comet assay was performed on lineage-marker-negative (Lin−) fetal liver cells isolated from Bmi1+/+ and Bmi1−/− embryos infected with an empty vector (CT) or a retrovirus encoding Bmi1 (for Bmi1−/− cells) and cultured for 2 d. Histograms show percentage of cells with undamaged DNA (no tail) and damaged DNA (tail). (D) Lin−Sca+ fetal liver cells isolated from Bmi1+/+ and Bmi1−/− embryos were treated with increasing concentrations of CPT for 24 h. Cell survival was assessed by annexin V/PI staining 48 h after treatment.
HeLa cells were exposed to local UV irradiation (60 J/m²) through 5-μm pore filters (hence the large size of the foci). Cells were fixed within 5 min and coimmunostained with antibodies to BMI1 (green) and γ-H2AX, MRE11, or NBS1 (red). Nuclei were visualized by DAPI staining (blue).

**Fig. S5.** HeLa cells were exposed to local UV irradiation (60 J/m²) through 5-μm pore filters (hence the large size of the foci). Cells were fixed within 5 min and coimmunostained with antibodies to BMI1 (green) and γ-H2AX, MRE11, or NBS1 (red). Nuclei were visualized by DAPI staining (blue).
Fig. S6. (A) Colocalization of endogenous BMI1 (green) and γ-H2AX (red) proteins in primary young MEF (passage 2) untreated or exposed to 1 μM CPT (1 h), 2 mM hydroxyurea (HU) (1 h), or ionizing radiation (10 Gy). Cells were preextracted, immediately after treatment, with Triton X-100 before fixation to visualize only the chromatin-bound proteins. Nuclei are visualized using DAPI (blue). (B) Cellular distribution of BMI1 (green) and γ-H2AX (red) proteins in Lin-Sca1+ fetal liver cells either untreated or irradiated at 1 Gy after 2 d in culture. Cells then were fixed, permeabilized, and analyzed by confocal microscopy. (C) MEF were irradiated (10 Gy), allowed to recover for 30 min, and coimmunostained for BMI1 (green) and NBS1, MDC1, 53BP, or rad51 (red). Nuclei are visualized using DAPI (blue). (D) HCT116 cells were untreated or were treated with CPT (1 μM, 1 h) and processed as in A. (E) Coimmunoprecipitation of Bmi1 with γ-H2AX in the soluble CN and CB fractions of HCT116 cells irradiated at 10 Gy. Input represents 10% of total protein used for immunoprecipitation.