Increased c-Myc activity and DNA damage in hematopoietic progenitors precede myeloproliferative disease in Spa-1-deficiency

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Mice deficient for Spa-1 encoding Rap GTPase-activating protein develop myeloproliferative disorder (MPD) of late onset with frequent blast crises. The mechanisms for MPD development as well as the reasons for long latency, however, remain elusive. We demonstrate here that preleukemic, disease-free Spa-1^{-/-} mice show reduced steady-state hematopoiesis and attenuated resistance to whole body γ -ray irradiation, which are attributable to the sustained p53 response in hematopoietic progenitor cells (HPCs). Preleukemic Spa-1^{-/-} HPCs show c-Myc overexpression with increased p19Arf as well as enhanced yH2AX expression with activation of Atm/Chk pathway. We also show that deregulated Rap signaling in the absence of Spa-1 enhances post-transcriptional c-Myc stability and induces DNA damage in a p38MAPK-dependent manner, leading to p53 activation. Genetic studies indicate that the introduction of p53+/- and p53-/- mutations in Spa-1-/mice results in the acceleration of typical MPD and rapid development of blastic leukemia, respectively. These results suggest that increased c-Myc expression and DNA damage in HPCs precede MPD development in Spa-1^{-/-} mice, and the resulting p53 response functions as a barrier for the onset of MPD and blast crises progression. (Cancer Sci 2011; 102: 784–791)

C hronic myelogenous leukemia (CML) is a disorder of hematopoietic stem cells (HSCs). It is initiated as chronic myeloproliferative disease (MPD) but eventually progresses to acute leukemia called blast crisis (BC).⁽¹⁾ Initiation of human CML is considered to involve comprehensive interaction of Bcr-Abl with host factors intrinsic to HSCs, whereas BC is associated with additional genomic changes of hematopoietic progenitor cells (HPCs).⁽²⁾ Although Bcr-Abl *per se* is suggested to be responsible for the genomic instability and disease progression, underlying mechanisms remain elusive.⁽³⁾ MPD resembling human CML has been reported in several mouse models, including Nf1^{-/-} mice and transgenic mice expressing oncogenic *K-ras*.⁽⁴⁻⁶⁾ MPD in these mice, however, was not transferable to normal mice and rarely progressed to BC.^(5,7)

We previously reported that deregulated activation of Rap, a Ras-family GTPase, in HPCs caused a spectrum of hematologic malignancy.^(8–11) Typically, mice deficient for *Spa-1* encoding a principal Rap-specific GTPase-activating protein showed deregulated Rap activation in HPCs and developed chronic MPD with progression to BC.⁽⁸⁾ We also reported that Rap was activated in human CML and CML-BC cells,⁽¹²⁾ and that mouse Spa-1^{-/-} HPCs expressing *Bcr-Abl* showed aggravated MPD phenotype with accelerated BC progression.⁽¹³⁾ On the other hand, recent results indicate that endogenous Rap signaling plays crucial roles in normal lymphohematopoiesis. Thus, conditional abrogation of Rap activation in T- and B-lineage cells caused developmental arrest of thymic pre-T cells and defective bone marrow (BM) pre-B cell development, respectively.^(14,15)

A most recent report indicated that the deficiency of Rap guanine nucleotide exchange factor 2 resulted in the complete defect of embryonic hematopoiesis.⁽¹⁶⁾

One of the features of MPD in Spa-1^{-/-} mice was a quite long latency for the disease onset, suggesting the involvement of secondary genetic hits.⁽⁸⁾ In the present study, we investigated the features of Spa-1^{-/-} HPCs prior to overt hematologic disorder. We demonstrate that preleukemic Spa-1^{-/-} HPCs show marked increase of c-Myc and DNA damage causing sustained p53 activation, which significantly delays the disease onset.

Materials and Methods

Mice. Spa-1^{-/-} mice were backcrossed to C57BL/6 (B6) mice for more than 15 generations. B6 and $p53^{-/-}$ mice with a B6 background were purchased from SLC (Shizuoka, Japan) and Taconic Farm (Hudson, NY, USA), respectively. All mice were maintained under pathogen-free conditions at the Institute of Laboratory Animals, Kyoto University Graduate School of Medicine. All animal procedures were performed according to the guidelines for animal experiments of Kyoto University.

Hematological assays. Peripheral blood leukocytes were monitored with the use of an automated cell counter (Nihon Kohden, Tokyo, Japan). BM cells were cultured in methylcellulose medium in the presence of stem cell factor (SCF; 50 ng/mL; PeproTech Inc., Rocky Hill, NJ, USA) and interleukin-3 (IL-3, 10 ng/mL) for 7 days. For spectral karyotyping (Sky) analysis, cells were treated with corcemid (Karyo Max; Life Technologies, Carlsbad, CA, USA).

Flow cytometry and cell separation. BM cells were five-color stained with propidium iodide (PI), a cocktail of Lin-marker antibodies (BD Bioscience, Franklin Lakes, NJ, USA) with additional anti-IL-7R, anti-Sca-1, anti-c-Kit, anti-CD16/32 and anti-CD34 antibodies. Common myeloid progenitors, megakaryoerythroid progenitors, and granulocyte-macrophage progenitors were defined as CD16/32^{low} CD34⁺, CD16/32^{low} CD34⁻, and CD16/32^{high} CD34⁺ populations, respectively, in a Lin⁻ c-Kit⁺ Sca-1⁻ IL7-R⁻ gate. For BrdU/7-AAD staining, the cells were first three-color stained with anti-c-Kit, anti-Sca-1 and anti-Lin marker antibodies, fixed and permealized, treated with DNase I, and then incubated with anti-BrdU antibody and 7-AAD. Lin⁻ HPCs were isolated using FACSAria II (BD Biosciences) or AutoMax (Miltenyl Biotec, Bergisch Gladbach, Germany).

Immunoblotting and immunostaining. Cells were lysed with RIPA buffer (150 mM NaCl, 25 mM Tris-HCl [pH 7.6], 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, protease and phosphatase inhibitors) and immunoblotted. Antibodies used were as follows; anti-Spa-1, anti-p53, anti-phosphorylated (S15) p53, anti-phosphorylated (S1981) Atm, anti-c-Myc,

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anti-Foxo3a, (Cell Signaling Technology, Beverly, MA, USA); anti-p21Cip1, anti-p16Ink4a, anti-Pten, anti-Actin, anti-Atm, anti-Chk2, anti-C3G, anti-Rap1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-Trp53inp1, anti-phosphorylated (T68) Chk2, anti-phosphorylated (S62, T58) c-Myc (Abcam, Cambridge, UK); anti-p19Arf (Calbiochem, Billercia, MA, USA): anti-Mdm2 (R&D Systems, Mineapolis MN, USA): anti-Trp53BP1(Bethyl Laboratories, Montogomery, TX, USA); antiyH2AX (S129) (Upstate); anti-Brm (BD Transduction Laboratories, Franklin Lakes, NJ, USA). Rap-GTP was detected by pull-down assay. For immunostaining, cells were fixed with methanol, permealized, blocked, and incubated with antiyH2AX antibody (Upstate Co., Billercia, MA, USA) and DAPI. The images were visualized using a ZEISS Axiovert200M microscope equipped with an Axiocam MRm camera (Carl Zeiss MicroImaging, Gottingen, Germany).

Quantitative PCR. Total RNA was extracted from the sorted lin⁻ BM cells with a NucleoSpin RNA kit (Macherey-Nagel, Dueren, Germany) followed by cDNA synthesis with Super-Script III (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed with a LightCycler SYBR Green I marker kit on a LightCycler instrument (Roche, Basel, Switzerland). The primer pairs were as follows—*Trp53*: sense, 5'-GCCCATGCTACAG-AGGAGTC-3'; anti-sense, 5'-AGACTGGCCCTTCTTGGTCT-3'; *c-Myc*: sense, 5'-CGAAACTCTGGTGCATAAACTG-3'; anti-sense, 5'-GAACCGTTCTCAG-3'; *Arf*: sense, 5'-CGACGGGCATAGCTTCAG-3'; anti-sense, 5'-GCTCTGC-TCTTGGGATTGG-3'.

Retrovirus production and infection. PLAT-E cells were transfected with a *pMCs-ires-EGFP* retroviral vector containing

farnesylated *C3G* (*C3G-F*) or *Rap1A17* mutant cDNA and pSI-REN-Retro Q vector (Clontech Lab, Mountain View, CA, USA) containing Spa-1 shRNA, and the culture supernatants were harvested for the recombinant retrovirus. Spa-1 shRNA oligomers were (sense) GATCCGGCAAGTACTTCTATGGCAATTCAA-GAGA<u>TTGCCATAGAAGTACTTGCCTTTTTG</u> and (antisense) AATTCAAAAAG<u>GCAAGTACTTCTATGGCAA</u>TCT-CTTGAATTGCCATAGAAGTACTTGCCG_

Bone marrow chimeras. BM cells $(2 \times 10^7 \text{ cells/head})$ were injected intravenously into mice lethally irradiated with 13 Gy γ -ray (6.5 Gy twice with a 4-h interval).

Statistical analysis. Statistical analysis was performed using the Log-rank (Mantel–Cox) test for Kaplan–Meier analysis and two-tailed Student's *t*-test for other experiments.

Results

Reduced steady-state hematopoiesis in preleukemic Spa-1^{-/-} mice. We first investigated the hematopoietic activity of young, disease-free Spa-1^{-/-} mice. Rather unexpectedly, 8-week-old Spa-1^{-/-} mice were leukopenic compared with age-matched littermate control (LMC) mice (13, 340 ± 356/µL in LMC, n = 43 versus 8, 651 ± 262/µL in Spa-1^{-/-} mice, n = 28; P < 0.001). Total BM nucleated cell numbers were also diminished significantly (Fig. 1a). Flow cytometric analysis indicated that the numbers of Lin⁻ c-Kit⁺ Sca-1⁺ (LKS) as well as committed progenitor (Lin⁻ c-Kit⁺ Sca-1⁻, LKS⁻) cells for all myeloid cell lineages were diminished (Fig. 1a). To examine steady-state cycling status of HPCs *in vivo*, we injected BrdU 4 h before sacrifice. Both LKS and LKS⁻ populations of



Fig. 1. Preleukemic Spa-1^{-/-} mice show reduced steady-state hematopoiesis. (a) Bone marrow (BM) cells from disease-free Spa-1^{-/-} and littermate control (LMC) mice were multicolor analyzed, and total nucleated cell numbers as well as those of Lin⁻ c-kit+ Sca-1+ (LKS), Lin⁻ c-kit+ Sca-1⁻ (LKS⁻), megakaryo-erythroid progenitors (MEP), common myeloid progenitors (CMP) and granulocyte-macrophage progenitors (GMP) were determined. (b) Spa-1^{-/-} and LMC mice were injected with BrdU, and 4 h later the BM cells were multicolor analyzed with Lin cocktail, anti-c-Kit, anti-Sca-1, anti-BrdU and 7-AAD. Representative staining profiles in LKS and LKS⁻gates as well as the means and SE of cell cycle stages are shown (n = 6 for each group). A portion of LKS⁻ population was in the second round of cell cycle (triangles).

Spa-1^{-/-} mice revealed significantly higher proportions of the cells at G0/G1 phase than those of LMC mice with few apoptotic sub-G1 cells (Fig. 1b).

Preleukemic Spa-1^{-/-} mice show attenuated resistance to total body γ -ray irradiation. The findings prompted us to investigate the hematopoietic activity of Spa-1^{-/-} mice in response to extrinsic stress. Young Spa-1^{-/-} mice showed remarkably increased mortality after otherwise sublethal total body irradiation (TBI) with 7 or 8 Gy γ -ray (Fig. 2a). Although LCM BM cells began to regenerate on day 7 with nearly complete recovery by day 13 after 7 Gy TBI, no evidence for the regeneration was detected in Spa-1^{-/-} BM until moribund (Fig. 2b). No pathological evidence was found in other tissues including brain and intestine in both Spa-1^{-/-} and LMC mice, suggesting that the enhanced mortality was attributable to BM failure. In agreement with the findings, SCF/IL-3-induced colony-forming unit (CFU) numbers in LMC BM recovered close to the normal level by 14 days, whereas those in Spa-1^{-/-} BM were progressively reduced until moribund (Fig. 2c). Spa-1^{-/-} HPCs also showed significantly enhanced susceptibility to γ -ray irradiation *in vitro* than LCM HPCs with respect to the SCF/IL-3-induced CFU activity (Fig. 2d).

Enhanced radio-susceptibility of Spa-1^{-/-} mice is attributable to sustained p53 activation in HPCs. To confirm that the attenuated radio-resistance of Spa-1^{-/-} mice was HPC-autonomous, we generated BM chimeric mice. As anticipated, Spa-1^{-/-} mice reconstituted with Spa-1^{-/-} BM all died after 7 Gy TBI, whereas LCM mice reconstituted with LCM BM all survived. On the other hand, LCM mice reconstituted with Spa-1^{-/-} BM all died following 7 Gy TBI similar to Spa-1^{-/-} mice, while five out of nine Spa-1^{-/-} mice reconstituted with LCM BM survived (Fig. 3a). The results indicate that the enhanced radio-susceptibility of Spa-1^{-/-} mice is primarily HPC-autonomous, although additional contribution of the hematopoietic microenvironment



Fig. 2. Spa-1^{-/-} mice show attenuated resistance to total body γ -ray irradiation. (a,b) Preleukemic Spa-1^{-/-} and littermate control (LMC) mice received total body irradiation (TBI) (7Gy or 8Gy), and their survival was monitored for 3 months. BM histology of femoral bones was examined at various days after 7Gy TBI. H&E × 100. (c) Spa-1^{-/-} and LMC mice received TBI (7Gy), and varying weeks later stem cell factor/interleukin-3 (SCF/IL-3)-induced colony-forming unit (CFU) numbers in the BM cells were determined. The means and SE of three mice are shown. (d) BM cells from Spa-1^{-/-} and LMC mice were irradiated *in vitro* with varying doses of γ -ray and the numbers of SCF/IL-3-induced CFUs were determined. The means and SE of triplicate culture are shown. BMC, bone marrow cells.



Fig. 3. Attenuated radio-resistance of Spa-1^{-/-} mice is hematopoietic progenitor cells (HPC)autonomous and attributable to p53 response. (a) BM chimeric mice of indicated combinations received 7Gy total body irradiation (TBI), and the survival was monitored for 3 months. (b) Lin⁻ BM cells from unirradiated Spa-1^{-/-} and littermate control (LMC) mice were immunoblotted with indicated antibodies. (c) Indicated numbers of LMC, p53^{-/-}, Spa-1^{-/-}, Spa-1^{-/-} p53^{+/-} and Spa-1^{-/-}p53^{-/-} mice received TBI (7Gy), and the survival was monitored for a month.

is also suggested. Considering the crucial role of p53 in radiation-induced myelosuppression, we investigated the expression of p53 in HPCs. Freshly isolated Lin⁻ BM cells from unirradiated Spa-1^{-/-} mice expressed remarkably increased p53 with reduced Mdm2 compared with those of LCM and accordingly increased expression of potential p53-target proteins such as p21, Pten, and Trp53inp1 (Fig. 3b). Expression of p16INK4a was largely comparable (Fig. 3b). To directly examine the contribution of p53, we generated Spa-1^{-/-} mice with p53^{+/-} or

 $p53^{-/-}$ backgrounds. Whereas Spa- $1^{-/-}p53^{+/-}$ mice died comparably with Spa- $1^{-/-}$ mice after 7 Gy TBI, all Spa- $1^{-/-}$ p53^{-/-} mice survived (Fig. 3c). These results suggested that the attenuated radio-resistance of preleukemic Spa- $1^{-/-}$ mice was attributable to the sustained p53 activation in HPCs.

Increased DNA damage and c-Myc expression underlie p53 activation in Spa-1^{-/-} HPCs. Freshly isolated Lin⁻ BM cells (HPCs) from preleukemic Spa-1^{-/-} mice showed significant expression of γ H2AX along with the increase of Brm that



Fig. 4. Preleukemic Spa-1^{-/-} hematopoietic progenitor cells (HPCs) show increased c-Myc activation and DNA damage response. (a–c) Lin⁻ BM cells isolated from littermate control (LMC) and Spa-1^{-/-} mice were immunoblotted with indicated antibodies. (d) Transcripts of indicated genes were assessed by quantitative RT-PCR in Lin⁻ BM cells from LMC and Spa-1^{-/-} mice. The values were normalized to those of *cyclophilin*, and relative transcripts in Spa-1^{-/-} to LMC HPCs are shown. NS, not significant.

promotes γ H2AX induction⁽¹⁷⁾ and Trp53BP1 that plays a crucial role in activating DNA damage response (Fig. 4a,b).⁽¹⁸⁾ Moreover, Spa-1^{-/-} HPCs exhibited enhanced expression and phosphorylation of Atm, phosphorylation of its substrate Chk2, and prominent N-terminal phosphorylation of p53 at S15 (Fig. 4b), suggesting the sustained activation of DNA damage response. In addition, Spa-1^{-/-}HPCs also showed remarkably increased c-Myc and reduced Fbw7 with concomitantly enhanced expression of Foxo3a and p19ARF (Fig. 4c). The transcripts of *c-myc*, however, were unchanged, although *Arf* transcripts were increased (Fig. 4d). The transcripts of *p53* were not changed either, whereas *Pten* transcripts were increased (Fig. 4d). These results suggest that deregulated c-Myc expression and increased DNA damage underlie the sustained increase and activation of p53 in preleukemic Spa-1^{-/-} HPCs.

Basal Rap signaling regulates c-Myc expression and DNA damage in a p38MAPK dependent manner. We next addressed whether or not deregulated Rap signaling in Spa-1-deficiency was involved in the sustained p53 activation. Toward this end, we generated NIH3T3 cells with varying levels of endogenous Rap-GTP; namely, NIH3T3 expressing dominant negative Rap1A17, empty vector (Vect), constitutive active Rap-GEF C3G (C3G-F), and Spa-1 shRNA (shSpa-1) in addition to C3G-F, with increasing basal Rap-GTP levels in this order (Fig. 5a). It was confirmed that the expression levels of p53 were increased in proportion to the increasing levels of basal Rap-GTP, while the transcripts were unchanged (Fig. 5a). The effect was paralleled with the increase of c-Myc expression, with minimal change in *c*-myc transcripts (Fig. 5a). It was noted that the increase of c-Mvc was associated with the enhanced S62 and diminished T58 phosphorylation, particularly in the cells showing the highest Rap activation (Fig. 5a). Moreover, the cells with the highest Rap-GTP also exhibited increased expression of vH2AX and Trp53BP1 with characteristic chromatin condensation (Fig. 5b), indicative of increased DNA damage. These cells also showed significantly increased Nox2 expression (Fig. 5b).

Spa-1^{-/-} HPCs showed enhanced p38MAPK, but not ERK, activation, and, in agreement, NIH3T3 with deregulated Rap activation also showed enhanced p38MAPK activation (Fig. S1). We then investigated the effect of a p38MAPK inhibitor (SB202190) on c-Myc expression and DNA damage in NIH3T3 cells with deregulated Rap activation. Abundant c-Myc expression in NIH3T3/C3G-F:shSpa-1 cells was suppressed by the treatment with SB202190 in a dose-dependent manner, and the effect was associated with the abrogation of S62 and the restoration of T58 phosphorylation of c-Myc as well as increase Fbw7 expression (Fig. 5c). Expression of Nox2, yH2AX, and Trp53BP1 was also suppressed by SB202190 treatment in parallel, and consequently p53 expression was diminished significantly (Fig. 5c). The stability of c-Myc protein in the presence of cycloheximide was significantly increased in NIH3T3/C3G-F:shSpa-1 cells compared with NIH3T3/Vect cells (Fig. 5d), suggesting the increased c-Myc expression was attributable to the enhanced protein stability at least in part. The results suggest that deregulated Rap signaling in Spa-1-deficiency induces the increase of c-Myc expression and DNA damage via p38MAPK pathway, leading to sustained p53 activation.

Sustained p53 response functions as a barrier for MPD and BC in Spa-1^{-/-} mice. MPD in Spa-1^{-/-} mice was successively transferable into Rag2^{-/-} mice and progressed to blastic leukemia during *in vivo* passage (Fig. S2), confirming the intrinsic propensity for BC progression. We then finally validated the role of sustained p53 response in preleukemic Spa-1^{-/-} HPCs. In the current cohort, 16 out of 17 Spa-1^{-/-} mice developed MPD only after 50 weeks (the mean survival time: 84 weeks), while

 $p53^{-/-}$ mice died of either sarcoma (62%) or T-ALL (24%) by 35 weeks of age with no case of MPD (the mean survival time: 26 weeks, n = 21) (Fig. 6a). Spa-1^{-/-}p53^{-/-} mice all died significantly earlier than $p53^{-/-}$ mice (the mean survival time: 17 weeks, n = 25, P < 0.0001) with increased proportion of T-ALL (48%) (Fig. 6a). Notably, 28% of Spa-1^{-/-}p53^{-/-} mice developed non T-lineage blastic leukemia, apparently bypassing the MPD phase, with gross chromosomal translocations (Fig. 6b), whereas $\text{Spa-1}^{+/+}\text{p53}^{-/-}$ mice never developed non T-lineage leukemia (Fig. 6a). Furthermore, although $\text{p53}^{+/-}$ mice developed no hematologic disorder, eight out of 31 Spa $p53^{+/-}$ mice died within a year, and five of them were 1 confirmed by autopsy to show typical MPD with severe anemia, extramedullary hematopoiesis, and marked increase of mature myeloid cells (Fig. 6c). The results suggest that sustained p53 response in HPCs plays a pivotal role as a barrier for the development of MPD and BC progression in Spa-1-/-mice, significantly delaying the disease onset.

Discussion

Rap signaling plays an important role in normal lymphohematopoiesis; deregulated Rap activation in HPCs may cause MPD and leukemia, whereas defective Rap activation results in impaired lymphohematopoiesis.^(10,11) In current study, we investigated the underlying mechanisms of late-onset MPD in Spamice, which showed deregulated Rap activation in the HPCs.⁽⁸⁾ Prior to overt MPD development, Spa-1^{-/-} mice showed rather reduced steady-state hematopoiesis and attenuated radio-resistance, which were attributable to the sustained p53 activation in HPCs. The reduced hematopoiesis was attributable primarily to the diminished cell cycle progression of HPCs with little evidence of cell death. In either case, the results strongly suggest that the HPCs of preleukemic Spa- $1^{-/-}$ mice are under the intrinsic "stress" that provokes p53 response. We demonstrated that Spa-1^{-/-} HPCs showed increased expression of yH2AX and activation of Trp53BP1/Atm/Chk2 pathway, suggesting that the increased DNA damage may in part contribute to the p53 activation. In addition, Spa-1^{-/-} HPCs showed c-Myc overexpression along with a prominent increase of p19Arf, which antagonizes Mdm2 activity and suppresses the degradation of p53 protein.^(19–21) It was noticed that Mdm2 expression *per se* tended to be reduced in Spa-1^{-/-} HPCs, and it remained to be investigated whether or not the effect was also attributable to the increased p19Arf. It is shown that deregulated c-Myc expression may induce DNA damage directly via aberrant DNA replication and/or indirectly by inducing reactive oxygen species generation.^(22,23) Current results indicate that $\text{Spa-1}^{-/-}$ HPCs show increased expression of Nox2 capable of generating reactive oxygen species. In either case, present results suggest that deregulated c-Myc expression and increased DNA damage underlie the sustained p53 response in preleukemic Spa- 1^{-7-} HPCs.

With the use of NIH3T3 cells with varying levels of basal Rap activation, we confirmed that deregulated Rap activation caused marked activation of p53 in association with the increase of c-Myc and γ H2AX expression. The increase of c-Myc was not related to the increase of *c-myc* transcripts, suggesting the post-transcriptional effect. Stability of c-Myc protein is crucially regulated by phosphorylation at two N-terminal residues, S62 and T58; thus, S62 phosphorylation confers stability, whereas its dephosphorylation followed by T58 phosphorylation targets c-Myc to the ubiquitin-dependent degradation.^(21,24,25) Overexpressed c-Myc in the cells with deregulated Rap activation showed strong S62 with minimal T58 phosphorylation. MAPKs such as ERK and JNK2 are reported to function as a S62 kinase of c-Myc.⁽²¹⁾ The current study, however, indicated that a p38MAPK inhibitor



Fig. 5. Deregulated Rap signaling induces enhanced c-Myc activation and DNA damage response. (a) NIH3T3 cells expressing *Rap1A17*, empty vector, *C3G-F*, or *C3G-F* + *shSpa-1* were immunoblotted with indicated antibodies. Because Rap-GTP levels varied markedly, pull-down assay was done separately in two sets with different conditions. Transcripts of *c-myc* and *p53* were assessed with quantitative RT-PCR. (b) NIH3T3/Vect and NIH3T3/C3G-F:shSpa-1 cells were immunoblotted with indicated antibodies and immunostained with anti- γ H2AX (red) and DAPI (blue). (c) NIH3T3/C3G-F:shSpa-1 cells were cultured in the presence of varying concentrations of SB202190 for 24 h and immunoblotted with indicated antibodies. (d) NIH3T3/Vect and NIH3T3/C3G-F:shSpa-1 cells were cultured in the presence of cycloheximide (CHX; 100 µg/mL). At various time points later, the cells were recovered and immunoblotted with indicated antibodies (upper). Relative signal intensities of c-Myc normalized to those of corresponding Gapdh are plotted (lower). NS, not significant.

significantly suppressed the expression of c-Myc in these cells, and the effect was associated with the reduced S62 and increased T58 phosphorylation and increased Fbw7. In agreement, c-Myc protein in the cells with deregulated Rap activation showed increased half life at steady state. It is thus suggested that Rap signaling regulates c-Myc protein stability via p38MAPK pathway, although exact mechanisms for the modification of c-Myc phosphorylation status by p38MAPK remain to be verified. The results are consistent with the increased p38MAPK activation in Spa-1^{-/-} HPCs.⁽²⁶⁾ Myc proteins play an essential role in normal hematopoiesis,^(27,28) and a recent report indicates that c-Myc abundance during normal HSC differentiation and proliferation is almost exclusively regulated by post-translational regulation.⁽²⁹⁾ Also, we reported



Fig. 6. The sustained p53 response functions as a barrier for myeloproliferative disorder (MPD) and blast crisis in Spa-1^{-/-} mice. (a) Kaplan–Meier analysis of the survival of indicated numbers of Spa-1^{-/-}, Spa-1^{-/-}p53^{+/-}, Spa-1^{-/-}p53^{-/-}, p53^{-/-} and p53^{+/-} mice (left). Although not shown, 17 out of 18 Spa-1^{-/-} mice died in the second year of life. Causes of death by the autopsy of moribund mice are summarized (right). (b,c) Peripheral blood cells from the representative diseased Spa-1^{-/-}p53^{-/-} and Spa-1^{-/-}p53^{+/-} mice were cytospun and stained with Giemsa solution. Arrows indicate blast cells. The mean values of white blood cells (WBC), red blood cells (RBC) and spleen weights in disease-free Spa-1^{-/-}mice were 8.7 (×10⁻³/µL), 1010 (×10⁻⁴/µL) and 150 (mg), respectively. Sky analysis of the typical blastic leukemia cells in Spa-1^{-/-}p53^{-/-} mice is shown.

that conditional transgenic mice expressing dominant negative Rap1A17 showed markedly diminished c-Myc expression in B progenitor cells and defective B-cell development.⁽¹⁵⁾ Thus, it may be an intriguing possibility that the crucial role of Rap signaling in normal lymphohematopoiesis is mediated in part by the regulation of c-Myc expression levels in HPCs.

Constitutive c-Myc overexpression is detected in a wide spectrum of human cancers and leukemia by genomic changes such as mutations, gene duplication and chromosomal translocations, and its role in leukemia genesis has been explored by a number of animal models.⁽²⁰⁾ Current results, however, suggest that deregulated Rap activation induces c-Myc overexpression in HPCs primarily via post-translational mechanisms, leading to the sustained p53 response. We have demonstrated that the p53 activation in Spa-1^{-/-} HPCs functions as a significant barrier for the development of MPD and BC. Although p53 deficiency alone rarely resulted in hematological malignancy except for thymic T-cell leukemia, a significant proportion of Spa-1^{-/-} p53^{+/-} mice developed characteristic MPD much earlier than Spa-1^{-/-} mice, and one-thirds of Spa-1^{-/-} p53^{-/-} mice rapidly developed blastic leukemia of non-T-cell lineages, apparently bypassing the MPD phase. The blastic leukemia cells in Spa-1^{-/-} p53^{-/-} mice expressed abundant c-Myc with gross chromosomal translocations, although the direct role of c-Myc in MPD and BC remains to be formerly proved. Human CML cells expressing Bcr-Abl show increased DNA damage and genomic

instability, and the majority of BC cells show the anomalies in the genes related to DNA damage response, such as p53, ARF, and Rb.^(1,30) On the other hand, we previously reported that human CML and BC cells showed constitutive Rap activation,⁽¹²⁾ and also Spa-1-deficiency strongly accelerated the BC progression of Bcr-Abl-induced CML in a mouse model.⁽¹³⁾ Thus, current results suggest that Rap signaling may link Bcr-Abl to the sustained DNA damage in HSC/HPCs, and may provide a strategic clue to control human CML and its inevitable progression to BC.

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Disclosure Statement

The authors have no financial conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Activation of p38MAPK, but not ERK, in Spa-1^{-/-} hematopoietic progenitor cells (HPCs).

Fig. S2. Myeloproliferative disorder (MPD) in Spa-1^{-/-} mice is transferable to Rag2^{-/-} mice and progresses to blast crisis (BC) during the passage *in vivo*.

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