

MYELOID NEOPLASIA

Normal ABL1 is a tumor suppressor and therapeutic target in human and mouse leukemias expressing oncogenic ABL1 kinases

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Key Points

- Normal ABL1 is a tumor suppressor in BCR-ABL1–induced leukemia.
- Allosteric stimulation of the normal ABL1 kinase activity enhanced the antileukemia effect of ABL1 tyrosine kinase inhibitors.

Leukemias expressing constitutively activated mutants of ABL1 tyrosine kinase (BCR-ABL1, TEL-ABL1, NUP214-ABL1) usually contain at least 1 normal ABL1 allele. Because oncogenic and normal ABL1 kinases may exert opposite effects on cell behavior, we examined the role of normal ABL1 in leukemias induced by oncogenic ABL1 kinases. BCR-ABL1-*Abi1*^{-/-} cells generated highly aggressive chronic myeloid leukemia (CML)-blast phase–like disease in mice compared with less malignant CML-chronic phase–like disease from BCR-ABL1-*Abi1*^{+/+} cells. Additionally, loss of ABL1 stimulated proliferation and expansion of BCR-ABL1 murine leukemia stem cells, arrested myeloid differentiation, inhibited genotoxic stress-induced apoptosis, and facilitated accumulation of chromosomal aberrations. Conversely, allosteric stimulation of ABL1 kinase activity enhanced the antileukemia effect of ABL1 tyrosine kinase inhibitors (imatinib and ponatinib) in human and murine leukemias expressing BCR-ABL1, TEL-ABL1, and NUP214-ABL1. Therefore, we

postulate that normal ABL1 kinase behaves like a tumor suppressor and therapeutic target in leukemias expressing oncogenic forms of the kinase. (*Blood*. 2016;127(17):2131-2143)

Introduction

The ABL1 protein is a ubiquitously expressed nonreceptor tyrosine kinase markedly influenced by subcellular localization and post-translational modifications.¹⁻³ Cytoplasmic expression of ABL1 leads to increased cell proliferation and survival. In response to genotoxic stress, ABL1 is translocated into the nucleus and/or mitochondria where its activity contributes to modulation of DNA repair, induction of apoptosis/necrosis, and inhibition of cell growth.

Normal ABL1 kinase activity is essential for B- and T-cell development, but expendable in hematopoietic stem cells (HSCs) and the myeloid compartment.^{4,6} Constitutively activated oncogenic mutants of the ABL1 tyrosine kinase play a central role in the pathogenesis of acute and chronic leukemias. Activation usually occurs as a consequence of chromosomal translocations (*BCR-ABL1*, *TEL-ABL1*, and others) or episomal amplification (*NUP214-ABL1*).¹

The *BCR-ABL1* fusion oncogene, the product of t(9;22)(q34;q11) is found in all patients with chronic myeloid leukemia (CML), in ~25% of pre-B acute lymphocytic leukemia (ALL) and occasionally in de novo acute myeloid leukemia (AML).⁷ BCR-ABL1 kinase is

leukemogenic only when expressed in an HSC with self-renewal capacity, thereby transforming it to a leukemia stem cell (LSC).⁸ In CML–chronic phase (CML-CP), LSCs are capable of generating large numbers of leukemia early progenitor cells (LPCs): leukemia common myeloid (LCMPs) and leukemia granulocyte/macrophage (LGMPs), which cannot self-renew and eventually differentiate to mature cells. Thus, CML-CP is a stem cell–derived but progenitor-driven disease.⁸ Transition of a relatively benign CML-CP to the aggressive and fatal blast phase (CML–blast phase [CML-BP]) is associated with expansion of LSCs, enhanced proliferation, arrested differentiation, drug resistance, and accumulation of additional genetic and epigenetic aberrations.^{9,10}

NUP214-ABL1 fusion is generated by circularization of the 500-kb genomic region from *ABL1* to *NUP214* and subsequent extrachromosomal (episomal) amplification.¹¹ The *NUP214-ABL1* gene is found in ~4% of all cases of adult ALL. Other *ABL1* fusion genes have been described but are uncommon. For example, the *ETV6(TEL)-ABL1* fusion gene is the product of a t(9;12)(q34;p13) and is found

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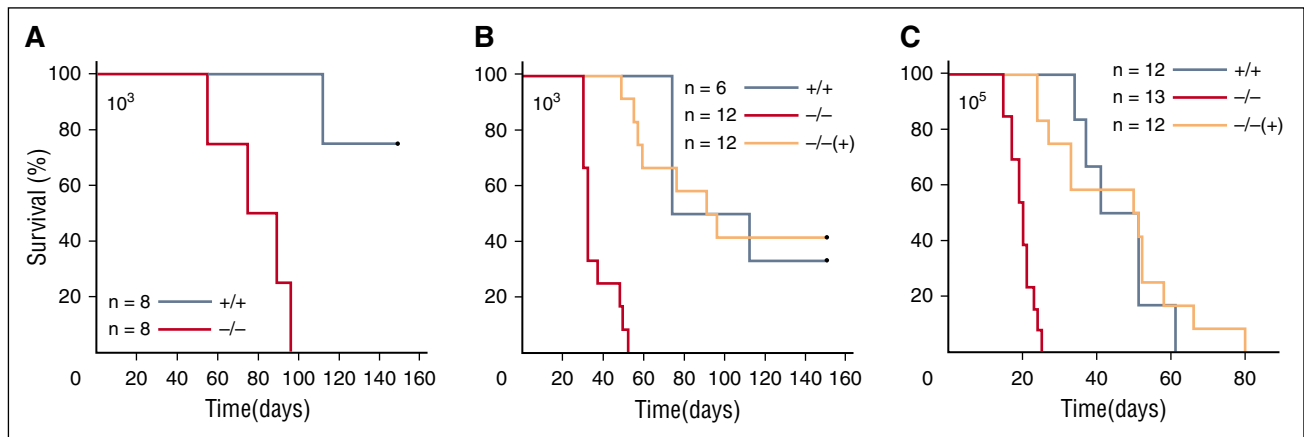


Figure 1. Normal ABL1 kinase plays a tumor suppressor role in BCR-ABL1-induced leukemogenesis. BCR-ABL1 *Abli*^{-/-} [-/-], BCR-ABL1 *Abli*^{+/+} [+/+], and BCR-ABL1 *Abli*^{-/-} leukemia cells reconstituted with YFP-ABL1 [-/-(+)] were inoculated IV into NOD/SCID mice. Kaplan-Meier survival curve of mice injected with (A) 10³ freshly transfected cells [(-/-) and (+/+): n = 8] and (B) 10³ [(+/+): n = 6, (-/-) and (-/-+): n = 12] or (C) 10⁵ [(-/-): n = 13, (+/+) and (-/-+): n = 12] 5-week-old tissue-cultured cells; n = number of mice per group.

occasionally in patients with acute leukemias or myeloproliferative disorders. *EML1*, *ZMIZ1*, and *RCSD1* were identified as *ABL1* partners in ALLs.¹

Leukemias expressing oncogenic forms of the ABL1 kinase usually contain the nonmutated allele encoding normal ABL1 kinase which may play an important role in pathogenesis of disease and/or in response to treatment, given its prominent role in regulation of cell motility, adhesion, autophagy, response to DNA damage, apoptosis, and proliferation.¹⁻³ This possibility is supported by previous observations that loss of normal ABL1 expression resulting from interstitial deletion in the normal chromosome 9 [del(9q34)] and/or transcriptional silencing of the alternative *ABL1* promoter within *BCR-ABL1* translocation occurs during progression of CML-CP to CML-BP.^{12,13} Of note, in the absence of ABL1, BCR-ABL1 cells displayed reduced sensitivity to tyrosine kinase inhibitors (TKIs) such as imatinib.¹⁴ Therefore, we hypothesized that normal ABL1 is a tumor suppressor in CML-CP and therapeutic target in leukemias induced by oncogenic forms of ABL1 kinase.

Materials and methods

BCR-ABL1-positive *Abli*^{-/-} and *Abli*^{+/+} cells

BCR-ABL1-positive *Abli*^{-/-} and *Abli*^{+/+} bone marrow cells (BMCs) expressing YFP-ABL1 fusion protein or yellow fluorescent protein (YFP) only were obtained and maintained as described in supplemental Methods (see supplemental Data available at the *Blood* Web site).

Leukemogenesis in vivo

Green fluorescent protein (GFP)-positive or GFP/YFP-positive cells were injected into the tail vein of sublethally irradiated NOD/SCID mice. Animals were killed when first signs of disease were apparent and leukemia development was confirmed at necropsy. These studies were approved by the Temple University institutional animal care and use committee.

Immunostaining

LSCs and LPCs were identified as described before¹⁵ and detailed in supplemental Methods.

Colony formation assay

Freshly transfected Lin⁻c-Kit⁺Sca-1⁺ BCR-ABL1 *Abli*^{+/+} and BCR-ABL1 *Abli*^{-/-} cells were cultured for 5 weeks in vitro and simultaneously plated in

MethoCult H4230 (StemCell Technologies, Vancouver, BC, Canada) in absence of growth factors. Colonies were scored after 5 to 7 days, and replated in fresh Methocult and scored again after 5 to 7 days. Three rounds of serial replating (representing 5 weeks in culture) were performed. Five-week-old tissue-cultured BCR-ABL1 *Abli*^{+/+} and BCR-ABL1 *Abli*^{-/-} cells were also plated in Methocult. Colonies were scored after 5 to 7 days.

Competitive growth assay

A mixture of GFP-positive BCR-ABL1 *Abli*^{-/-} and GFP/YFP-positive BCR-ABL1 *Abli*^{-/-} cells restored with YFP-ABL1 was maintained in Iscove modified Dulbecco medium (IMDM) supplemented with fetal bovine serum (FBS), stem cell factor (SCF), and interleukin-3 (IL-3) and also simultaneously injected into the tail vein of NOD/SCID mice. After 5 weeks, the in vitro and in vivo cell mixtures were analyzed by flow cytometry to determine the percentage of GFP and GFP/YFP double-positive cells.

Histologic and cytologic analysis

Tissue sections of spleen from leukemia-sick mice were fixed in 10% formalin, paraffin embedded, cut into 0.4-mm sections, transferred to glass slides, and stained with hematoxylin and eosin. Cytospin preparations of cells cultured for 7 days in the presence of pretested threshold concentrations of IL-3 + SCF or granulocyte colony-stimulating factor (G-CSF; 10 ng/mL) were stained with Wright-Giemsa.

Apoptosis induced by genotoxic treatment

Indicated concentrations of cisplatin (Platinol-AQ; Bristol-Myers Squibb Co, Princeton, NJ), mitomycin C (Sigma-Aldrich, St. Louis, MO), etoposide (Bedford Laboratories, Bedford, OH), methylnitronitrosoguanidine (MNNG; Sigma-Aldrich), and hydrogen peroxide (Sigma-Aldrich) were added to cells growing in MethoCult H4230 (10³/mL) supplemented with IL-3. Colonies were scored after 7 days. Results are represented as the percentage of colony-forming cells after drug treatment in comparison with the untreated control group.

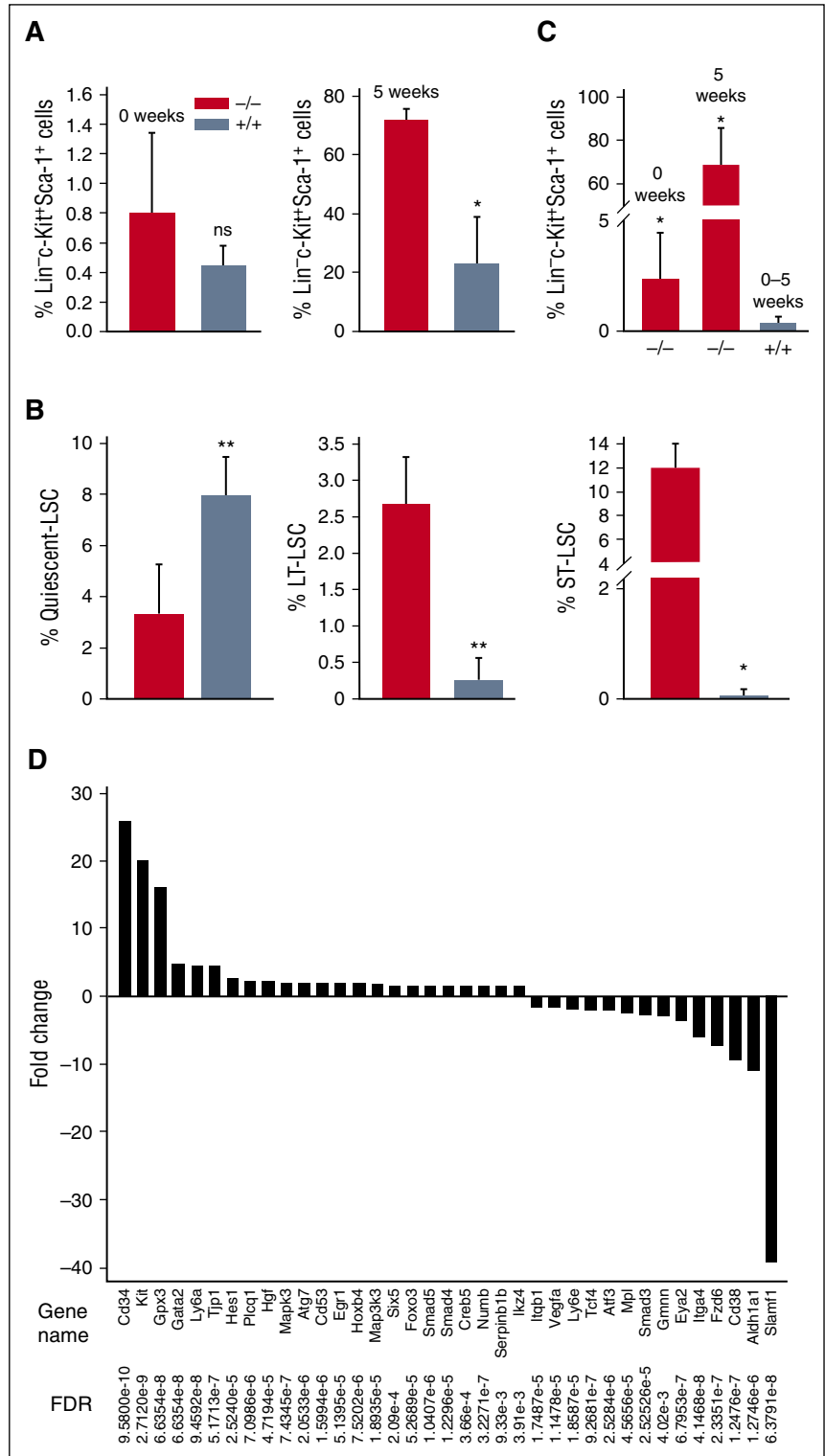
ROS, oxidative DNA damage, and genomic instability

Reactive oxygen species (ROS), 8-oxoguanine (8-oxoG), γ -H2AX nuclear foci, and chromosomal aberrations were detected as described before¹⁶ and detailed in supplemental Methods.

DPH treatment

CML-CP cells from freshly diagnosed patients, B-ALL xenograft cells, Baf3-TEL-ABL1 cell lines, and murine BMCs expressing NUP214-ABL1

Figure 2. ABL1 inhibits expansion of BCR-ABL1-positive LSCs. (A) Mean percentage \pm SD of GFP⁺Lin⁻c-Kit⁺Sca-1⁺ LSCs in freshly established (0 weeks) and 5-week-old (5 weeks) BCR-ABL1 *Ab1*^{-/-} and BCR-ABL1 *Ab1*^{+/+} cells. (B) Mean percentage \pm SD of quiescent LSCs (CPD^{max} Lin⁻c-Kit⁺Sca-1⁺), LT-LSCs (Lin⁻c-Kit⁺Sca-1⁺CD34⁺Flt3⁻), and ST-LSCs (Lin⁻c-Kit⁺Sca-1⁺CD34⁺Flt3⁻) in BCR-ABL1 *Ab1*^{-/-} and BCR-ABL1 *Ab1*^{+/+} leukemia cell populations. (C) Bars represent mean percentage \pm SD of GFP⁺Lin⁻c-Kit⁺Sca-1⁺ LSCs in BMCs isolated from moribund SCID mice transplanted with freshly established (0 weeks) and 5-week-old cultured (5 weeks) BCR-ABL1 *Ab1*^{-/-} and BCR-ABL1 *Ab1*^{+/+} cells (see Figure 1). ***P* < .05, **P* < .001 as determined by the unpaired Student *t* test; ns = not significant. (D) Statistically significant (false discovery rate [FDR] < 0.05) fold changes (>1.5) of expression of indicated genes regulating “stemness” in BCR-ABL1 *Ab1*^{-/-} vs BCR-ABL1 *Ab1*^{+/+} leukemia cells maintained with SCF + IL-3.



oncogene were treated with 5-[3-(4-fluorophenyl)-1-phenylpyrazol-4-yl]imidazolidine-2,4-dione (DPH; Sigma-Aldrich), imatinib and ponatinib (both from Selleck Chemicals) and evaluated as described in supplemental Methods. These studies were approved by the Temple University institutional review board.

Western analyses

Total, cytoplasmic, and nuclear cell lysates were obtained as described before¹⁷ and analyzed as detailed in supplemental Methods.

Genome-wide expression array

The Affymetrix Mouse gene 1.0ST array containing 28 815 probe sets (Affymetrix) was used to measure messenger RNA (mRNA) expression levels. Affymetrix arrays were processed and analyzed as described in supplemental Methods.

Statistical analysis

Results are presented as mean \pm standard deviation (SD) and were analyzed by the 2-tailed paired Student *t* test and the Mann-Whitney rank-sum test

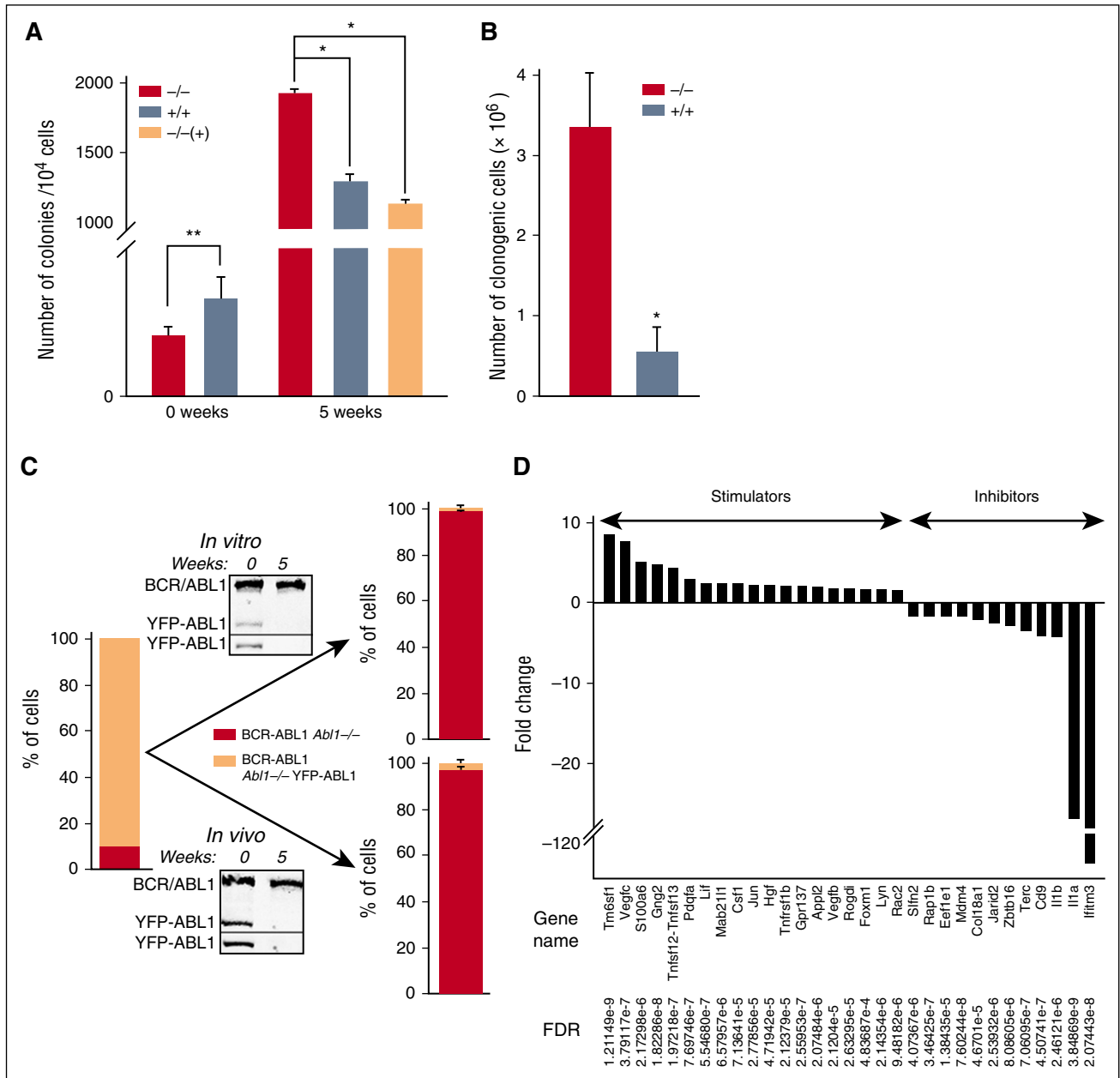


Figure 3. ABL1 inhibits proliferation of BCR-ABL1 leukemia cells. (A) Mean number of colonies ± SD from BCR-ABL1 *Abi1*^{-/-}, BCR-ABL1 *Abi1*^{+/+}, and BCR-ABL1/YFP-ABL1 *Abi1*^{-/-} [-/-(+)] leukemia cells; **P* < .001, ***P* < .05. (B) Mean number of total clonogenic cells ± SD generated by 10⁶ BCR-ABL1 *Abi1*^{-/-} and BCR-ABL1 *Abi1*^{+/+} cells during 5 weeks in vitro culture; **P* < .001. (C) The composition of initial cell mixture (left bar) and those after 5 weeks of in vitro and in vivo expansion (right bars). Western blot analyses using anti-ABL1 and anti-GFP/YFP antibodies (top and bottom boxes in each panel, respectively) show expression of BCR-ABL1 as well as YFP-ABL1 protein in the initial cell mixture (0) and after 5 weeks (5). (D) Statistically significant (FDR < 0.05) fold changes (>1.5) of expression of indicated genes regulating cell proliferation in BCR-ABL1 *Abi1*^{-/-} vs BCR-ABL1 *Abi1*^{+/+} leukemia cells maintained with SCF + IL-3.

when appropriate. Median survival time (MST) of the mice was calculated by Kaplan-Meier log-rank survival analysis. *P* < .05 was considered statistically significant.

Results

Normal ABL1 kinase plays a tumor suppressor role in BCR-ABL1-induced leukemia

To determine the role of ABL1 in CML, BCR-ABL1 kinase was expressed in *Abi1*^{-/-} and *Abi1*^{+/+} murine BMCs (BCR-ABL1 *Abi1*^{-/-}

and BCR-ABL1 *Abi1*^{+/+} cells, respectively); in addition, ABL1 kinase expression was restored in BCR-ABL1 *Abi1*^{-/-} cells by ectopic expression of YFP-ABL1 kinase as described before.¹⁴ Although 100% of NOD/SCID mice injected IV with BCR-ABL1 *Abi1*^{-/-} cells succumbed to leukemia, only 25% of mice injected with BCR-ABL1 *Abi1*^{+/+} cells developed lethal disease (MST = 78.8 ± 5.9 days and 140.5 ± 8.2 days, respectively; *P* < .001) (Figure 1A). When BCR-ABL1-transduced cells were cultured in limited growth factor conditions for 5 weeks to achieve growth factor independence (5-week-old cells), all (100%) mice inoculated with 10³ BCR-ABL1 *Abi1*^{-/-} cells and only 67% of those injected with BCR-ABL1 *Abi1*^{+/+} cells developed deadly disease (Figure 1B; MST = 36.2 ± 2.4 days

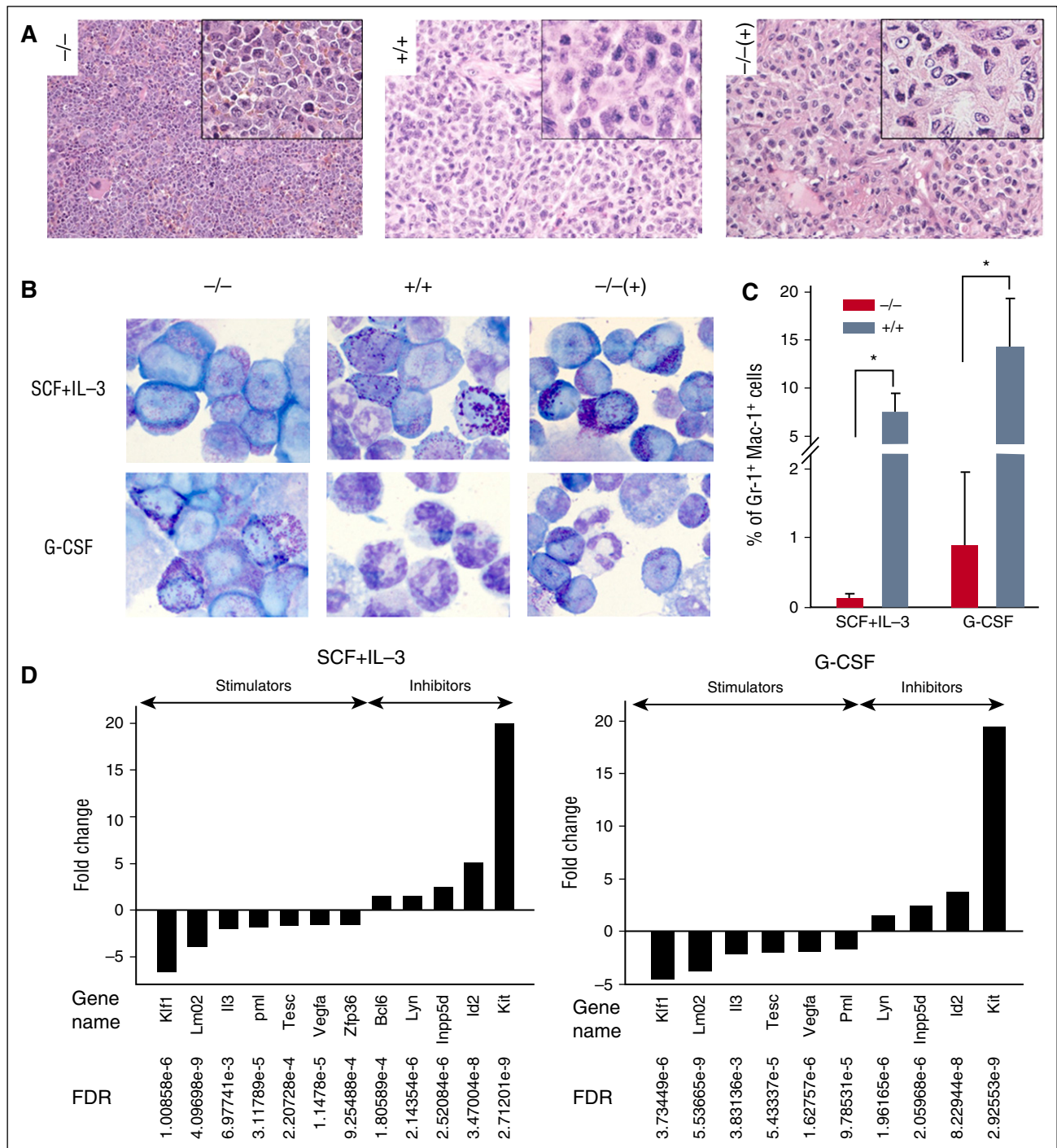


Figure 4. ABL1 promotes myeloid differentiation of BCR-ABL1 leukemia cells. (A) Representative hematoxylin-eosin–stained spleen sections from moribund SCID mice injected with BCR-ABL1 *Abi1*^{-/-} [-/-], BCR-ABL1 *Abi1*^{+/+} [+/+] and BCR-ABL1/YFP-ABL1 *Abi1*^{-/-} [-/-(+)] cells; magnification, ×20, inset, ×40. (B) Wright-Giemsa staining of BCR-ABL1 *Abi1*^{+/+} [+/+], BCR-ABL1 *Abi1*^{-/-} [-/-] and BCR-ABL1/YFP-ABL1 *Abi1*^{-/-} [-/-(+)] leukemia cells maintained in the presence of SCF + IL-3 (top panels) or G-CSF (bottom panels). Representative images are shown; magnification, ×100. (C) Mean percentages of Gr-1⁺/Mac-1⁺ cells ± SD in BCR-ABL1 *Abi1*^{-/-} and BCR-ABL1 *Abi1*^{+/+} leukemia cells maintained in the presence of SCF + IL-3 or G-CSF; **P* < .002. (D) Statistically significant (FDR < 0.05) fold changes (>1.5) of expression of genes regulating myeloid differentiation in BCR-ABL1 *Abi1*^{-/-} vs BCR-ABL1 *Abi1*^{+/+} leukemia cells maintained with SCF + IL-3 or G-CSF.

and 105.7 ± 15.3 days, respectively; *P* < .001). Even though injection of 10⁵ of 5-week-old BCR-ABL1 *Abi1*^{-/-} and BCR-ABL1 *Abi1*^{+/+} cells resulted in a deadly leukemia in all mice, the disease was accelerated in the absence of ABL1 (Figure 1C; MST = 19.7 ± 0.9 and 57.4 ± 5.5, respectively; *P* < .001). Injection of 10³ or 10⁵ of BCR-ABL1 *Abi1*^{-/-} cells reconstituted with YFP-ABL1 [BCR-ABL1 *Abi1*^{-/-}(+)] induced deadly leukemias in 58% and 100% mice,

respectively, with latency (MST = 102.8 ± 12.7 and 45.8 ± 5.1, respectively) similar to that in animals injected with corresponding numbers of BCR-ABL1 *Abi1*^{+/+} counterparts (Figure 1B-C).

ABL1 inhibits expansion of BCR-ABL1–expressing LSCs

Because uncontrolled outgrowth of LSCs is associated with CML-BP progression and/or therapeutic resistance,¹⁸ we sought to

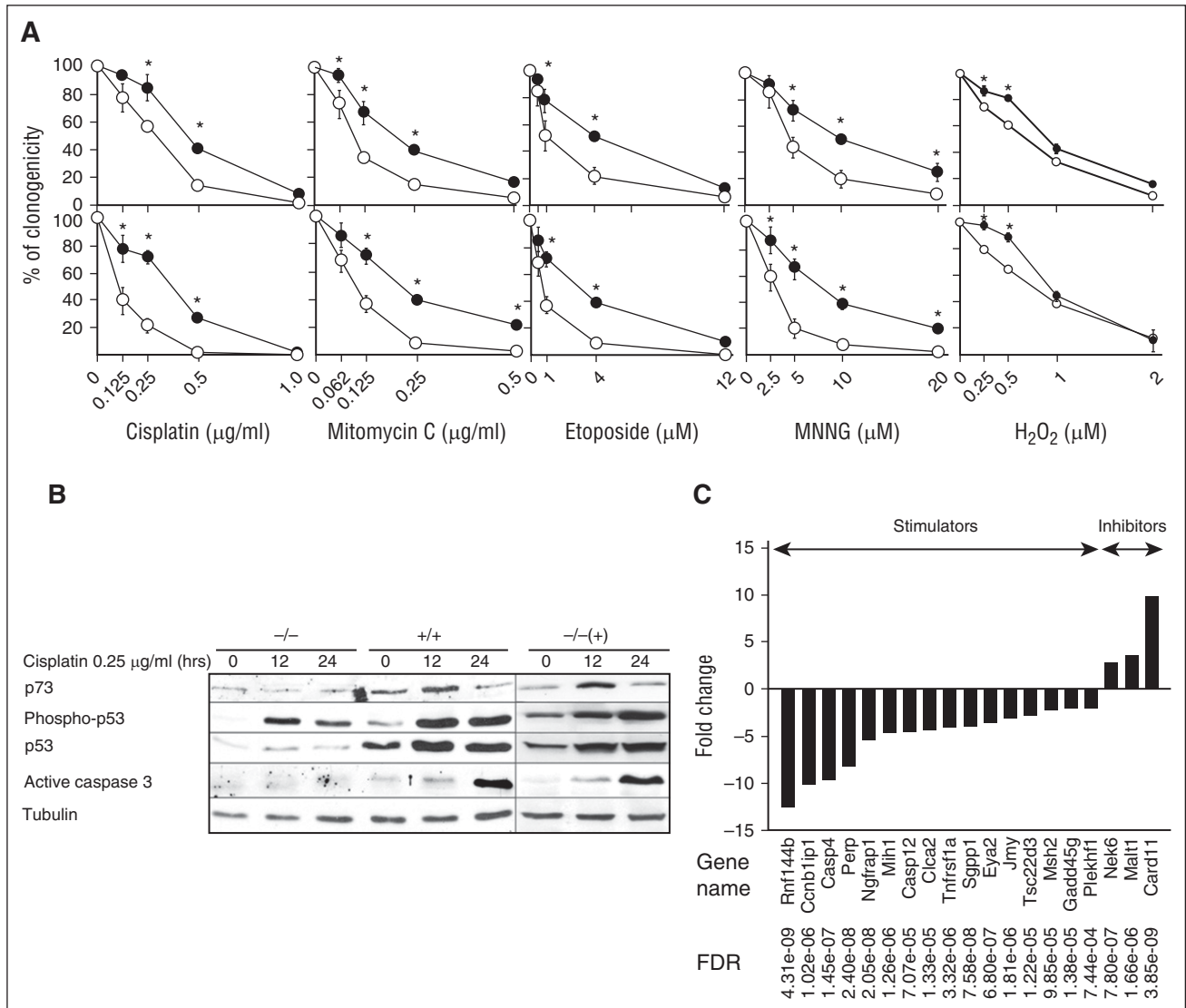


Figure 5. ABL1 promotes apoptosis in BCR-ABL1 leukemia cells in response to genotoxic agents. (A) Results represent mean percentage of clonogenic activity \pm SD of BCR-ABL1 *Abi1*^{-/-} (●) and BCR-ABL1 *Abi1*^{+/+} (○) leukemia cells after treatment with indicated DNA damage-inducing agents in the presence (top row) or absence (bottom row) of SCF + IL-3; **P* < .05. (B) Western blot analysis of p73, p53, phospho-serine 15 of p53 (phospho-p53) and activated caspase 3 in BCR-ABL1 *Abi1*^{-/-} [-/-], BCR-ABL1 *Abi1*^{+/+} [+/+], and BCR-ABL1/YFP-ABL1 *Abi1*^{-/-} [-/- (+)] leukemia cells treated with cisplatin (0.25 μg/ml); tubulin served as loading control. (C) Statistically significant (FDR < 0.05) fold changes (>2.0) of expression of indicated genes in BCR-ABL1 *Abi1*^{-/-} vs BCR-ABL1 *Abi1*^{+/+} leukemia cells maintained with SCF + IL-3.

determine whether ABL1 affects the accumulation of stem cells in the BCR-ABL1-transformed BMC population. We found no significant difference in the percentage of Lin⁻c-Kit⁺Sca-1⁺ HSCs in freshly harvested *Abi1*^{-/-} (1.8 \pm 0.5) and *Abi1*^{+/+} (1.9 \pm 0.6) bone marrow. In concordance, the percentage of Lin⁻c-Kit⁺Sca-1⁺ in freshly transduced GFP⁺ BCR-ABL1 *Abi1*^{-/-} and GFP⁺ BCR-ABL1 *Abi1*^{+/+} cells did not differ (Figure 2A 0 weeks). However, after 5 weeks of in vitro culture, the percentage of LSCs derived from GFP⁺ BCR-ABL1 *Abi1*^{-/-} cells was over threefold higher than those derived from GFP⁺ BCR-ABL1 *Abi1*^{+/+} cells (Figure 2A 5 weeks). Next, we analyzed GFP⁺ BCR-ABL1-positive Lin⁻c-Kit⁺Sca-1⁺ LSCs cultured for 5 weeks to quantify quiescent (Lin⁻c-Kit⁺Sca-1⁺eFluor670^{high}), long-term (LT) (Lin⁻c-Kit⁺Sca-1⁺CD34⁻) and short-term (ST) (Lin⁻c-Kit⁺Sca-1⁺CD34⁺) LSC subpopulations.¹⁵ An approximate threefold reduction of the percentage of quiescent LSCs was accompanied by ninefold and >100-fold increase in LT-LSCs and

ST-LSCs, respectively, derived from GFP⁺ BCR-ABL1 *Abi1*^{-/-} cells in comparison with GFP⁺ BCR-ABL1 *Abi1*^{+/+} cells (Figure 2B).

GFP⁺ BMCs obtained from mice with advanced leukemia originally injected with either freshly transduced or 5-week-old GFP⁺ BCR-ABL1 *Abi1*^{+/+} cells contained 0.4% \pm 0.3% GFP⁺Lin⁻c-Kit⁺Sca-1⁺ LSCs, whereas those inoculated with GFP⁺ BCR-ABL1 *Abi1*^{-/-} counterparts accumulated 2.4% \pm 2.0% and 68.7% \pm 16.9% GFP⁺Lin⁻c-Kit⁺Sca-1⁺ LSCs demonstrating sixfold and 60-fold expansion, respectively (Figure 2C).

Transcriptome analysis by microarrays comparing BCR-ABL1 *Abi1*^{-/-} vs BCR-ABL1 *Abi1*^{+/+} cells revealed differential expression of 33 genes potentially involved in regulating stem cell-like characteristics (Figure 2D). In general, genes positively regulating "stemness" including *Cd34*, *Kit*, *Ly6a*, *Hoxb*, *FoxO3*, *Smad5*, and *Smad4* were upregulated and genes inhibiting "stemness" such as *Ly6e*, *Fzd6*, *Eya2*, and *Slamf1* were downregulated in BCR-ABL1 *Abi1*^{-/-} vs BCR-ABL1 *Abi1*^{+/+} cells. Moreover, Ingenuity Pathway Analysis

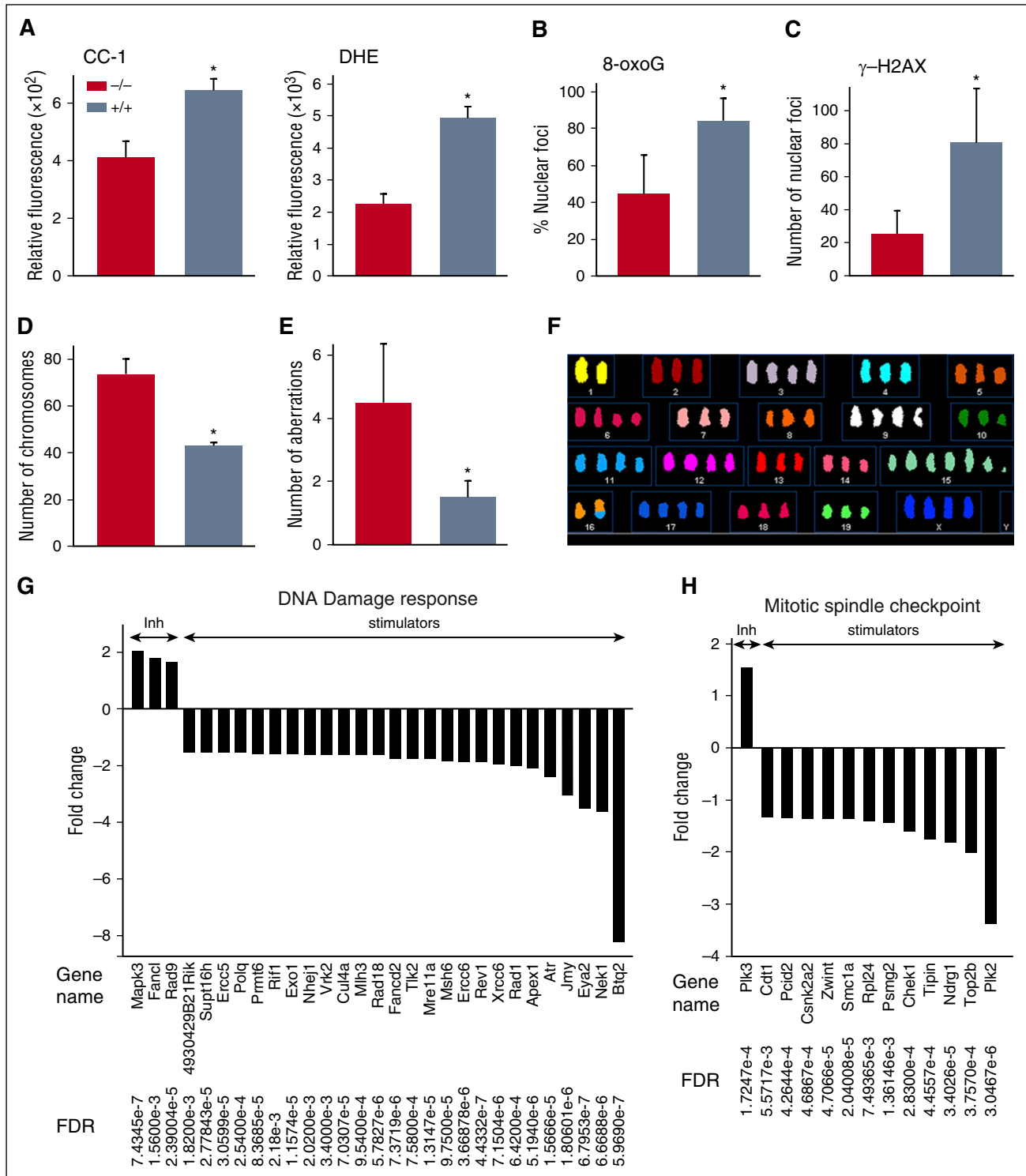
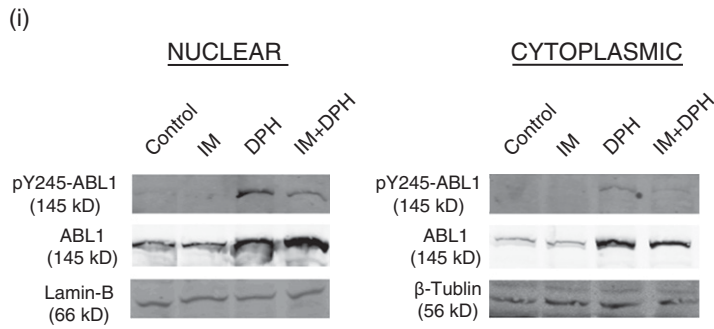


Figure 6. ABL1 prevents accumulation of chromosomal aberrations in BCR-ABL1 leukemia cells. (A) Mean ± SD of relative fluorescence of ROS; CC-1 (left panel) and DHE (right panel) in BCR-ABL1 *Abi1*^{-/-} and BCR-ABL1 *Abi1*^{+/+} leukemia cells; **P* < .001. (B-C) Mean percentage ± SD of (B) 8-oxoG foci and (C) γ-H2AX foci in BCR-ABL1 *Abi1*^{-/-} and BCR-ABL1 *Abi1*^{+/+} leukemia cells assessed by immunofluorescence in 4,6 diamidino-2-phenylindole (DAPI)-counterstained nuclei; **P* < .001. (D-F) SKY analysis of BCR-ABL1 *Abi1*^{-/-} and BCR-ABL1 *Abi1*^{+/+} leukemia cells based on the (D) number of chromosomes and (E) number of aberrations; results represent mean ± SD; **P* < .001. (F) Representative image showing chromosome analysis of metaphase spread of BCR-ABL1 *Abi1*^{-/-} leukemia cell using SKY. (G-H) Statistically significant (FDR < 0.05) fold changes (>1.5) of expression of indicated genes regulating (G) DNA damage response and (H) mitotic spindle assembly checkpoint in BCR-ABL1 *Abi1*^{-/-} vs BCR-ABL1 *Abi1*^{+/+} leukemia cells maintained with SCF + IL-3.

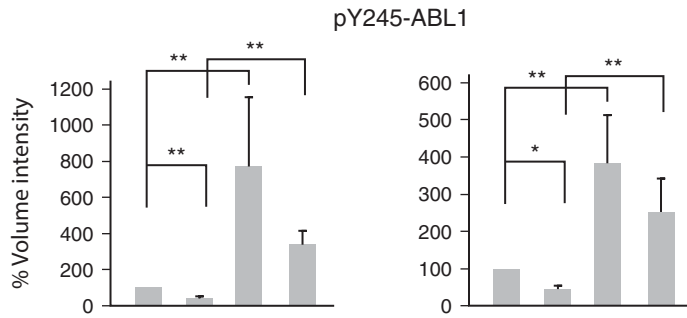
(IPA) suggests that upregulation of *Smad4/5* stimulatory activity and downregulation of *Smad3* inhibitory activity downstream of the transforming growth factor β (TGFβ) superfamily signaling

(supplemental Figure 1) combined with overexpression of the receptor tyrosine kinase *Kit* may contribute to abundant expansion of BCR-ABL1 *Abi1*^{-/-} Lin⁻c-Kit⁺Sca-1⁺ LSCs.¹⁹

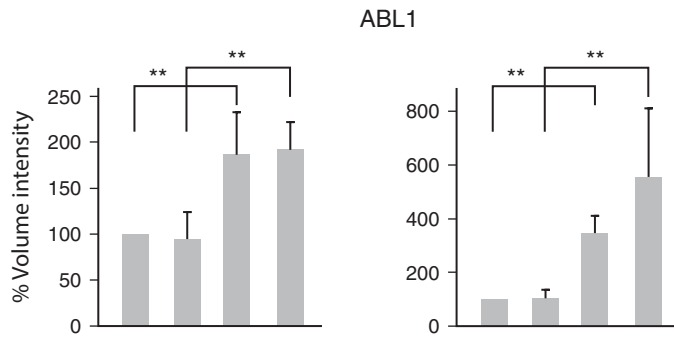
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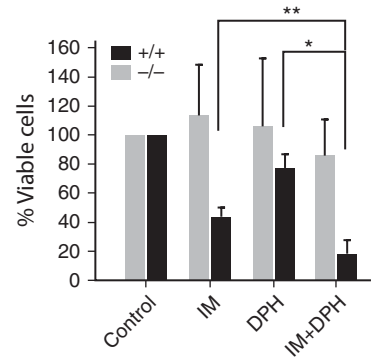
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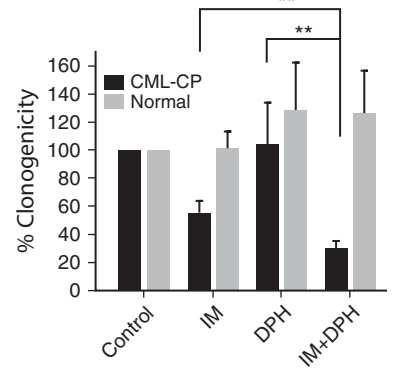
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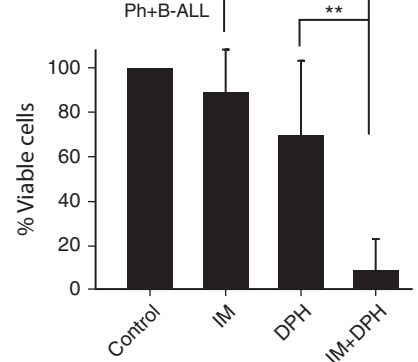
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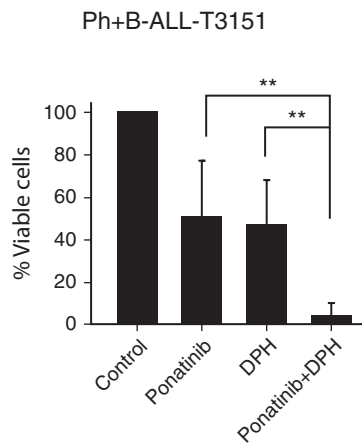
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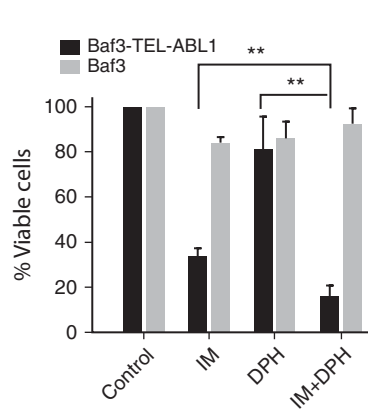
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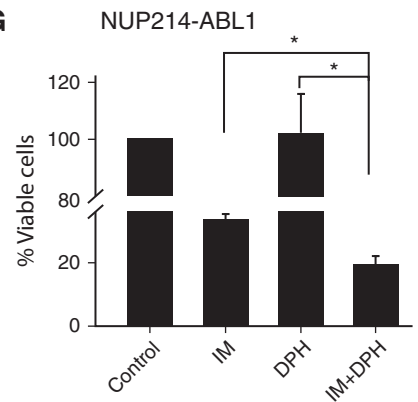
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ABL1 inhibits proliferation of BCR-ABL1-expressing leukemia cells

Although BCR-ABL1 stimulates proliferation, ABL1 is known to both promote as well as inhibit cell proliferation depending on the cellular context.^{1,20} Therefore, we examined whether the tumor-suppressive function of ABL1 is mediated through regulation of cell proliferation. Freshly transduced Lin⁻c-Kit⁺Sca-1⁺ BCR-ABL1 *Abl1*^{+/+} cells displayed 1.5 times higher clonogenic potential than BCR-ABL1 *Abl1*^{-/-} counterparts (Figure 3A 0 weeks), in concordance with other reports indicating that cytoplasmic ABL1 promoted cell cycle progression in CML-CP cells.²¹ Conversely, 5-week-old cultured BCR-ABL1 *Abl1*^{-/-} cells formed ~1.5 times more colonies when compared with BCR-ABL1 *Abl1*^{+/+} and BCR-ABL1/YFP-ABL1 *Abl1*^{-/-} counterparts (Figure 3A 5 weeks). Overall, during 5 weeks of continuous culture, BCR-ABL1 *Abl1*^{-/-} cells generated ~6 times more clonogenic cells than BCR-ABL1 *Abl1*^{+/+} cells (Figure 3B).

To test directly whether loss of ABL1 provides a growth advantage, competition assays were performed in which 10⁵ freshly transduced cells consisting of 10% GFP-positive BCR-ABL1 *Abl1*^{-/-} cells and 90% GFP/YFP-double positive BCR-ABL1/YFP-ABL1 *Abl1*^{+/+} cells were maintained for 5 weeks either in liquid culture or in NOD/SCID mice. Flow cytometry detected ~95% of GFP-positive and 5% of GFP/YFP-positive cells in the mixtures obtained in vitro and in vivo, indicating that the absence of ABL1 provided ~200-fold growth advantage for BCR-ABL1-transformed cells (Figure 3C bars). Overgrowth of BCR-ABL1 *Abl1*^{-/-} cells was confirmed by western blot analysis demonstrating undetectable YFP-ABL1 expression in cell mixtures harvested after 5 weeks (Figure 3C western blot insets).

Transcriptome analysis by microarrays was consistent with a growth advantage of BCR-ABL1 *Abl1*^{-/-} cells; 19 proliferative genes (eg, *Vegfc*, *Vegfb*, *Pdgfa*, *Lif*, *Hgf*, *Lyn*, *Rac2*) were upregulated and 12 antiproliferative genes (eg, *Ifitm3*, *Illa*, *Il1b*) were downregulated in these cells as compared with BCR-ABL1 *Abl1*^{+/+} cells (Figure 3D). In addition, IPA suggests that enhanced signaling from phospholipase Cγ1 (PLCγ1) and upregulation of cytoplasmic tyrosine kinases such as *Lyn*, *Fyn*, and *Syk* may contribute to accelerated proliferation rate of BCR-ABL1 *Abl1*^{-/-} cells overexpressing receptor tyrosine kinase *Kit* (supplemental Figure 2).²²⁻²⁵ Moreover, upregulation of *Pdgfa*, *Vegfb*, *Vegfc*, and *Csfl* expression in BCR-ABL1 *Abl1*^{-/-} cells implicates autocrine stimulation of cell growth.

ABL1 promotes myeloid differentiation of BCR-ABL1-positive leukemia cells

Because moderate expression levels of BCR-ABL1 in the presence of ABL1 promote differentiation in CML-CP and differentiation arrest in CML-BP is associated with enhanced BCR-ABL1 expression and downregulation of ABL1, we sought to determine the role of normal ABL1 in the maturation of the leukemic cells.^{26,27} Although all moribund mice injected with BCR-ABL1 cells developed splenomegaly (supplemental Figure 3), hematoxylin/eosin staining

of spleen sections obtained from mice injected with BCR-ABL1 *Abl1*^{-/-} cells displayed myeloblastic morphology (Figure 4A -/-). Conversely, spleens harvested from moribund animals injected with BCR-ABL1 *Abl1*^{+/+} and BCR-ABL1/YFP-ABL1 *Abl1*^{-/-} cells revealed moderate to complete myeloid differentiation of leukemic cells ranging from promyelocytes to myelocytes, metamyelocytes, and mature neutrophils (Figure 4A +/+ and -/-(+), respectively).

In vitro, in the presence of IL-3 + SCF, cultures of either BCR-ABL1 *Abl1*^{-/-} or BCR-ABL1 *Abl1*^{+/+} cells were composed almost exclusively of myeloid precursors having deep blue cytoplasm, but the BCR-ABL1 *Abl1*^{+/+} population contained more mature myeloid cells displaying abundance of cytoplasmic granules including seemingly fully differentiated neutrophils with hypersegmented multilobed nuclei (Figure 4B SCF + IL-3). In the presence of G-CSF, cultures of BCR-ABL1 *Abl1*^{+/+} cells were predominantly composed of mature neutrophils with very few myeloid precursors whereas cultures of BCR-ABL1 *Abl1*^{-/-} cells contained mostly myeloid precursors and few neutrophils (Figure 4B G-CSF). Reconstitution of ABL1 expression in BCR-ABL1/YFP-ABL1 *Abl1*^{-/-} leukemia cells facilitated accumulation of myeloid precursors and/or mature neutrophils in the presence of SCF + IL-3 and G-CSF, respectively (Figure 4B, -/-(+)). Flow cytometry detected <1% of Gr-1⁺Mac-1⁺ cells in BCR-ABL1 *Abl1*^{-/-} population whereas >7% and >14% of BCR-ABL1 *Abl1*^{+/+} cells were Gr-1⁺Mac-1⁺ in the presence of SCF + IL-3 and G-CSF, respectively (Figure 4C).

Transcriptome microarray analysis supported a myeloid differentiation arrest profile of BCR-ABL1 *Abl1*^{-/-} cells when compared with BCR-ABL1 *Abl1*^{+/+} cells; 7 genes promoting differentiation were inhibited (*Klf1*, *Lmo2*, *Il3*, *Pml*, *Tesc*, *Vegfa*, and *Zfp36*) whereas 5 genes associated with differentiation arrest were preferentially expressed (*Bcl6*, *Lyn*, *Id2*, *Inpp5d*, and *Kit*) (Figure 4D).

ABL1 promotes DNA damage-induced apoptosis in BCR-ABL1-expressing leukemic cells

BCR-ABL1 kinase promotes cell survival after genotoxic stress whereas ABL1 facilitates apoptosis by exerting opposite effects on caspase 9-mediated activation of caspase 3.^{28,29} To elucidate the impact of ABL1 loss in BCR-ABL1 cells, BCR-ABL1 *Abl1*^{-/-} and BCR-ABL1 *Abl1*^{+/+} cells were exposed to genotoxic agents such as cisplatin, mitomycin C, etoposide, MNNG, or hydrogen peroxide. Results of the clonogenic assay demonstrated that ABL1 enhanced the toxicity of DNA-damaging agents (Figure 5A). ABL1 has been reported to modulate survival/apoptosis by regulation of p53 and p73.³⁰⁻³³ Western blot followed by densitometry analysis show more abundant expression of p53 (11- to 16-fold), phospho-serine 15 p53 (1.5-fold to twofold), and p73 (fivefold), resulting in caspase 3 activation in cisplatin-treated BCR-ABL1 *Abl1*^{+/+} when compared with BCR-ABL1 *Abl1*^{-/-} leukemic cells (Figure 5B). BCR-ABL1 *Abl1*^{-/-} cells reconstituted with YFP-ABL1 [BCR-ABL1 *Abl1*^{-/-}(+)] display a similar pattern of expression of these proteins to BCR-ABL1 *Abl1*^{+/+} cells.

Figure 7. Activation of native ABL1 enhances the efficacy of TKIs against leukemias expressing oncogenic ABL1 kinase mutants. (A) Phoenix cells overexpressing ABL1 were treated with dimethylsulfoxide (DMSO) (control), DPH, imatinib (IM), and a combination of imatinib followed by DPH (IM+DPH). (i) Representative western blot analysis of phospho-tyrosine 245-ABL1 (pY245-ABL1), ABL1, lamin-B and β-tubulin in nuclear (left panel) and cytoplasmic (right panel) cell lysates. Quantification of normalized pY245-ABL1 (ii) and ABL1 (iii) levels to lamin-B and β-tubulin. Bars represent mean percentage volume intensity ± SD. (B-G) Percentage of viable cells or colonies ± SD from cells treated for 72 hours with diluent (Control), imatinib (IM), ponatinib, DPH, and combinations: (B) BCR-ABL1 *Abl1*^{-/-} and BCR-ABL1 *Abl1*^{+/+} cells, (C) Lin⁻CD34⁺ cells from 6 CML-CP patients and 3 healthy donors, (D) xenograft cells from 3 freshly diagnosed BCR-ABL1 B-ALL patients, (E) xenograft cells from 3 relapsed B-ALL patients carrying BCR-ABL1(T315I) mutation, (F) Baf3 and Baf3-TEL-ABL1 cells, (G) NUP214-ABL1-positive murine cells; **P* < .001, ***P* < .05 as determined by the unpaired Student *t* test.

The proapoptotic gene expression signature of BCR-ABL1 *AblI*^{+/+} cells was also detected by transcriptional microarrays. Several proapoptotic genes including *Rnf144b*, *Ccnblip1*, *Casp4*, *Casp12*, *Eya2*, and *Gadd45g* were downregulated and 3 antiapoptotic genes *Nek6*, *Malt1*, and *Card11* were upregulated in BCR-ABL1–positive *AblI*^{-/-} cells compared with BCR-ABL1 *AblI*^{+/+} cells (Figure 5C). In addition, IPA suggests that proapoptotic signaling involving JNK1-dependent phosphorylation of p53 and BCL2 is downregulated in BCR-ABL1–positive *AblI*^{-/-} cells (supplemental Figure 4).^{34,35}

ABL1 prevents accumulation of chromosomal aberrations in BCR-ABL1–expressing leukemic cells

BCR-ABL1–positive LSCs and LPCs contain high levels of ROS-induced oxidative DNA damage such as 8-oxoG and DNA double strand breaks (DSBs), which contribute to accumulation of chromosomal aberrations.^{16,36} By contrast, ABL1 prevents genomic instability.³⁷ To evaluate the role of ABL1 in modulating ROS-induced oxidative DNA damage in BCR-ABL1 leukemia cells, endogenous ROS were measured with RedoxSensor Red CC-1 dye which detects superoxide anion ($\cdot\text{O}_2^-$) and hydrogen peroxide (H_2O_2) and with dihydroethidium (DHE) which detects $\cdot\text{O}_2^-$. BCR-ABL1 *AblI*^{-/-} cells contained 1.5 and 2.5 times less ROS detected by CC-1 and DHE, respectively, when compared with BCR-ABL1 *AblI*^{+/+} cells (Figure 6A). Transcriptional microarray analysis suggests that lower levels of H_2O_2 and $\cdot\text{O}_2^-$ in BCR-ABL1 *AblI*^{-/-} cells were probably caused by 15-fold upregulation of glutathione peroxidase 3 (*Gpx3*) responsible for detoxification of H_2O_2 and 1.7-fold downregulation of mitochondrial electron transfer flavoprotein-ubiquinone oxidoreductase (*Etf1dh*) which facilitates production of superoxide.^{38,39} As predicted by previous reports, decrease in ROS levels in BCR-ABL1 *AblI*^{-/-} cells was accompanied by twofold lower oxidative DNA damage measured by 8-oxoG as well as threefold decrease in the number of DSBs quantitated by γ -H2AX when compared with BCR-ABL1 *AblI*^{+/+} cells (Figure 6B-C).^{16,40}

These unexpected findings prompted us to examine whether ABL1 regulated the accumulation of chromosomal aberrations in BCR-ABL1 leukemia cells. BCR-ABL1 *AblI*^{-/-} and BCR-ABL1 *AblI*^{+/+} cells were cultured for 10 weeks followed by spectral karyotype analysis (SKY) to detect acquired chromosomal aberrations. Results from metaphase spreads revealed a nearly twofold increase in chromosome numbers and ~2.5-fold higher number of chromosomal aberrations (whole chromosome and intrachromosomal gains/losses, as well as nonrecurrent chromosomal translocations) in BCR-ABL1 *AblI*^{-/-} cells compared with BCR-ABL1 *AblI*^{+/+} cells (Figure 6D-F), consistent with overall high levels of genomic instability in the former cells.

The absence of ABL1 did not affect cell cycle distribution of BCR-ABL1 cells in response to DNA damage (supplemental Figure 5), but transcriptional microarray analysis revealed that the presence of ABL1 in BCR-ABL1 cells is associated with expression of numerous genes whose products regulate DNA damage response and mitotic spindle assembly checkpoint (Figure 6G-H). The majority of genes downregulated in BCR-ABL1 *AblI*^{-/-} cells in comparison with BCR-ABL1 *AblI*^{+/+} cells are involved in promotion of DNA repair, for example, *Ercc5*, *Fancc2*, *Polg*, *Exo1*, *Nhej1*, *Rif1*, *Rad18*, *Mre11*, *Msh6*, *Xrcc6*, *Top2b*, and *Atr*. IPA suggested that simultaneous downregulation of *Fancc2*, and *Xrcc6* and *Nhej1* (involved in faithful homologous recombination and relatively faithful classical nonhomologous end-joining [C-NHEJ], respectively) may favor highly unfaithful PARP1-mediated alternative NHEJ resulting in facilitated accumulation of additional chromosomal aberrations in BCR-ABL1 *AblI*^{-/-} cells (supplemental Figure 6).⁴¹

Genes involved in kinetochore/spindle/centrosome regulation (*Pcid*, *Csnk2a2*, *Zwint*, *Plk2*, *Nek1*) and sister chromatid segregation (*Smc1a*) are downregulated in BCR-ABL1 *AblI*^{-/-} cells compared with BCR-ABL1 *AblI*^{+/+} cells, which may be responsible for abundant aneuploidy in the former cells.

Activation of native ABL1 kinase enhances the efficacy of TKIs in leukemias expressing oncogenic ABL1 kinase mutants

Inhibition of intracellular ABL1 kinase may require threefold to 10-fold higher concentrations of imatinib than required to effectively inhibit BCR-ABL1 kinase⁴² (supplemental Figure 7). For example, 1 to 2 μM imatinib, the concentration typically achievable in steady state in patients,^{43,44} inhibits cytoplasmic BCR-ABL1, but it is suboptimal for inhibition of nuclear and mitochondrial ABL1.^{45,46} Because ABL1 kinase enhanced the sensitivity of CML cells to imatinib,¹⁴ we hypothesized that stimulation of residual ABL1 activity may enhance the effect of TKIs in CML-CP treatment.

ABL1 allosteric activator DPH has been reported to stimulate ABL1 kinase by displacing the myristate moiety from the myristoyl-binding site, thereby preventing the autoinhibitory conformation of ABL1.⁴⁷ To determine whether DPH was able to induce and/or maintain the activity of ABL1 kinase under imatinib treatment, ABL1 was overexpressed in Phoenix cells followed by treatment with 10 μM DPH, 1 μM imatinib, or a combination of the 2 agents. DPH induced approximately eightfold and fourfold increase of phospho-Y245-ABL1, indicative of ABL1-kinase activation, in nuclear and cytoplasmic fractions, respectively (Figure 7A). Moreover, ~50% to 70% of DPH-mediated activation of ABL1 kinase persisted in the presence of imatinib.

To determine whether ABL1 kinase could be a therapeutic target, we examined the effect of imatinib and/or DPH on BCR-ABL1 *AblI*^{+/+} and BCR-ABL1 *AblI*^{-/-} cells. We found that treatment with 0.125 μM imatinib in combination with 10 μM DPH for 3 consecutive days reduced the number of BCR-ABL1 *AblI*^{+/+} leukemia cells when compared with single agents (Figure 7B). In contrast, BCR-ABL1 *AblI*^{-/-} cells were not sensitive to the combination treatment thereby confirming the ABL1-specific effect of DPH. In addition, the combination of 1 μM imatinib and 10 μM DPH reduced clonogenic growth of CML-CP Lin⁻CD34⁺ cells by more than twofold in comparison with either agent alone, at the same time normal counterparts were not affected by the treatments (Figure 7C). Moreover, BCR-ABL1–positive B-ALL primary human xenograft cells and those harboring imatinib-resistant BCR-ABL1 (T315I) mutation were extremely sensitive (>10-fold) to the combination of imatinib (1 μM) + DPH (10 μM) and ponatinib (12.5 nM) + DPH, respectively, when compared with single-agent treated cells (Figure 7D-E).

Leukemia cells expressing TEL-ABL1 and NUP214-ABL1 oncogenic kinases were also more sensitive (more than twofold) to the combination of imatinib + DPH (Figure 7F-G).

Discussion

We showed here that ABL1 exerts a tumor suppressor function in CML-CP by restricting the expansion of LSCs, inhibiting clonogenic activity of LSCs/LPCs, promoting myeloid differentiation and apoptosis, and reducing genomic instability. In concordance, loss of ABL1 facilitated development of highly malignant CML-BP–like disease in mice inoculated with BCR-ABL1 *AblI*^{-/-} BMCs in contrast to CML-CP–like disease that arose from BCR-ABL1–transformed *AblI*^{+/+} cells.

The tumor suppressor function of ABL1 contrasting the oncogenic function of BCR-ABL1 is supported by reports that BCR-ABL1 and ABL1 can exert opposite effects on a variety of cellular functions.⁴⁸ We have shown that BCR-ABL1 kinase, which localizes exclusively to the cytoplasm,⁴⁹ protects leukemic cells from genotoxic stress, and facilitates genomic instability by promoting unfaithful DNA repair and deregulation of mitotic spindle.⁵⁰⁻⁵⁵ Conversely, ABL1 which is targeted to the nucleus upon DNA damage can inhibit or stimulate DNA repair and stabilize mitotic spindle.^{37,56-61} BCR-ABL1 inhibits apoptosis,⁶² conversely nuclear and mitochondrial ABL1 kinase may facilitate apoptosis or necrosis.^{28,31-33,63} Activation of the nuclear ABL1 inhibits cell proliferation but cytoplasmic BCR-ABL1 stimulates cell cycle progression.^{64,65} These striking differences between ABL1 and BCR-ABL1 functions may explain the dramatic changes in proliferation, apoptosis, and genomic instability of BCR-ABL1 leukemic cells upon the loss of ABL1.

Remarkably, even though ABL1 did not affect myeloid differentiation of HSCs,^{5,6} and BCR-ABL1 seemed to promote myeloid differentiation,²⁶ loss of ABL1 in BCR-ABL1 leukemic cells resulted in dramatic arrest of myeloid differentiation. Similarly, although ABL1 does not regulate the number of HSCs, BCR-ABL1 promotes rather mild expansion of LSCs in CML-CP^{4,66} (and present study); nonetheless, rapid expansion of LSCs was observed in the absence of ABL1. It is plausible to speculate that a combined effect of enhanced proliferation and differentiation arrest resulted in rapid expansion of BCR-ABL1 *Abi1*^{-/-} LSCs.

The opposite effects of BCR-ABL1 and ABL1 may result from differences in their substrate preferences; in addition, their intracellular substrate repertoire may differ due to modification of kinase-substrate interactions and/or intracellular localization.^{67,68} Accordingly, the exclusively cytoplasmic localization of BCR-ABL1 may allow it to escape the modulatory effect of intranuclear proteins such as retinoblastoma (Rb) tumor suppressor which negatively regulates ABL1.⁶⁹ Moreover, BCR-ABL1 demonstrated more abundant binding and modification of actin microfilaments than ABL1, which plays an important role in leukemogenesis.⁷⁰

Considering their opposite, pro- and antioncogenic properties, the BCR-ABL1-to-ABL1 ratio may play a key role in shaping the ultimate malignant features of the BCR-ABL1-transformed cells. It has been reported that CML-CP to CML-BP progression is associated with hypermethylation of the ABL1 promoter embedded in the translocation, which may be accompanied by clonal loss of expression of ABL1 transcribed from nontranslocated chromosome 9.^{13,14,71-73} That would tip the balance toward BCR-ABL1 oncogene in individual clonogenic cells, which in conjunction with decreased sensitivity to imatinib¹⁴ might promote malignant progression. Moreover, patients undergoing imatinib treatment with yet undetected clones carrying BCR-ABL1 kinase mutations may be at greater risk of clonal evolution, eventually leading to CML-BP because even partial inhibition of ABL1 kinase (especially in patients treated with higher doses of imatinib) in the presence of intact BCR-ABL1 kinase mutant (eg, T315I) would promote chromosomal instability (supplemental Figure 8).

The fact that ABL1 kinase not only opposes BCR-ABL1 (present study) but also sensitizes CML cells to TKIs¹⁴ suggests that ABL1 activity could be explored therapeutically. The standard clinical dose of imatinib (400 mg per day) corresponds to trough plasma levels of ~1 to 2 μ M which effectively inhibited BCR-ABL1,^{43,44} whereas ABL1 kinase required 3 to 10 μ M of the drug to suppress its activity^{45,46} (and this work). The tumor suppressor function of the intact ABL1 creates therapeutic opportunity for a combination of TKI and ABL1 activator.

We showed here that allosteric activation of normal ABL1 kinase activity by DPH “hypersensitized” leukemic cells expressing oncogenic variants of ABL1 to TKIs in vitro. The beneficial antileukemia effect of imatinib + DPH was detected only in leukemia cells expressing ABL1, which supports the notion that DPH exerted its activity by activating ABL1, but not by displacing HSP90 from BCR-ABL1.⁷⁴

Optimization of DPH or other ABL1 activators may generate new drugs useful for development of novel therapeutic modalities against tumors expressing oncogenic mutants of ABL1.^{75,76} For example, combination of TKI and allosteric ABL1 activator may enhance the therapeutic effect of TKIs in a cohort of CML-CP “poor responders” (no major cytogenetic response in 12 months, BCR-ABL1 transcript levels >10% after 3 months) and also in a majority of more aggressive BCR-ABL1-positive B-ALLs, which display unfavorable prognosis on TKIs.^{77,78}

In summary, we showed here that the proto-oncogene ABL1 acts as a tumor suppressor in CML-CP cells expressing its oncogenic variant, BCR-ABL1. Moreover, analysis of the CGAP Mitelman database revealed that actual del(9q34) or deletions encompassing this region resulting in loss of ABL1 have been detected in other hematologic malignancies (see supplemental Table 1), thus suggesting that ABL1 may play a tumor suppressor role not only in CML. On the other hand, normal ABL1 kinase could be targeted to enhance the therapeutic effect of TKIs in leukemias driven by BCR-ABL1 and other oncogenic forms of ABL1 kinase. It is possible that the enhanced cytotoxic effect of TKI in the context of activation of ABL1 may not be limited to tumors expressing oncogenic forms of ABL1 but it could be a more general paradigm as supported by our findings of a similar effect in leukemia cells harboring the FLT3-ITD mutation when treated with the FLT3 inhibitor AC220 in combination with DPH (supplemental Figure 8).

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Authorship

Contribution: Y.D. performed in vitro and ex vivo experiments for stemness, proliferation, differentiation, DNA damage response, DPH treatment, and microarray analysis, and wrote the first draft of the manuscript; M.K. performed in vitro and in vivo experiments for proliferation and in vitro experiments for stemness, myeloid differentiation, apoptosis, and DNA damage response; G.H. performed in vivo experiments; M.N.-S. assisted with experiments using primary cells; K.K., D.R., M.A.W., and B.G. ran histopathological analyses and differentiation tests; E.B.-G. assisted with flow cytometry data analysis; C.R. performed the SKY assay; S.C.-R., P.V., T. Stoklosa, and M.M. provided patient samples and primary

leukemia xenografts, and performed CGAP Mitelman database analysis; O.H. provided NUP214-ABL1-transformed cells; H.v.d.K. performed the experiments in supplemental Figure 1; and T. Skorski conceived the idea, supervised the project, and wrote the final version of the manuscript.

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