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Cancer Research

Activation-Induced Cytidine Deaminase Accelerates Clonal Evolution in *BCR-ABL1*–Driven B-Cell Lineage Acute Lymphoblastic Leukemia

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Abstract

Activation-induced cytidine deaminase (AID) is required for somatic hypermutation and immunoglobulin (Ig) class switch recombination in germinal center (GC) B cells. Occasionally, AID can target non-Ig genes and thereby promote GC B-cell lymphomagenesis. We recently showed that the oncogenic BCR-ABL1 kinase induces aberrant expression of AID in pre-B acute lymphoblastic leukemia (ALL) and lymphoid chronic myelogenous leukemia blast crisis. To elucidate the biological significance of aberrant AID expression, we studied loss of AID function in a murine model of *BCR-ABL1* ALL. Mice transplanted with *BCR-ABL1*–transduced $AID^{-/-}$ bone marrow had prolonged survival compared with mice transplanted with leukemia cells generated from $AID^{+/+}$ bone marrow. Consistent with a causative role of AID in genetic instability, $AID^{-/-}$ leukemia had a lower frequency of amplifications and deletions and a lower frequency of mutations in non-Ig genes, including *Pax5* and *Rhoh* compared with $AID^{+/+}$ leukemias. $AID^{-/-}$ and $AID^{+/+}$ ALL cells showed a markedly distinct gene expression pattern, and $AID^{-/-}$ ALL cells failed to downregulate a number of tumor-suppressor genes including *Rhoh, Cdkn1a* (p21), and *Blnk* (SLP65). We conclude that AID accelerates clonal evolution in *BCR-ABL1* ALL by enhancing genetic instability and aberrant somatic hypermutation, and by negative regulation of tumor-suppressor genes. *Cancer Res; 70(19); 7411–20. ©2010 AACR.*

Introduction

Activation-induced cytidine deaminase protein (AID) is essential for somatic hypermutation (SMH) and class switch recombination (CSR) in germinal center (GC) B cells (1, 2). AID introduces point mutations by converting cytidine into uridine followed by UNG1-mediated base excision repair (3). Although mutations are largely confined to immunoglobulin (Ig) variable region genes, AID occasionally targets non-Ig genes, including *BCL6, PIM1, BTG1, RHOH*, and *PAX5* (4). Hypermutation of non-Ig gene targets has been reported to occur in >50% of GC-derived diffuse large B-cell lymphomas (DLBCL; refs. 5, 6). In agreement with a role for AID in lymphomagenesis at the GC B-cell stage, mice deficient in AID fail to develop GC-derived *BCL6*-dependent lymphoma (7). Likewise, AID enzymatic activity is required for the acquisition of *Myc-IgH* translocations (8), which drive malignant transformation in Burkitt lymphoma and DLBCL. In addition, a recent work, which analyzed a database of more than 1,700 breakpoints, suggests that AID can synergize with the RAG1 and RAG2 enzymes to initiate chromosomal translocations at the pro-B/pre-B cell stage that are found in a wide range of B-cell malignancies (9).

The *BCR-ABL1* oncogene is found in a subset of patients with acute lymphoblastic leukemia (ALL) carrying the so-called Philadelphia chromosome. This translocation is the most common cytogenetic abnormality in adults, with ALL occurring in ~25% of adult patients and ~3% of children with ALL (10). *BCR-ABL1* defines a high-risk group and as such, patients receive intensive chemotherapy in combination with a tyrosine kinase inhibitor and are considered for bone marrow transplant. A recent study showed dramatically improved outcome for children with *BCR-ABL1* ALL when they were treated with a combination of high-dose tyrosine kinase inhibitors and intensive chemotherapy (11). *BCR-ABL1* ALL arises from pre-B cells, a compartment that normally does not express AID. We and others recently reported aberrant expression of AID in *BCR-ABL1*–positive ALL (12, 13). In addition, our group recently showed that overexpression of AID promotes lymphoid blast crisis transformation in *BCR-ABL1* chronic myeloid leukemia (CML), an entity that seems clinically similar to *de novo BCR-ABL1* ALL but carries multiple distinct molecular characteristics (14, 15). These studies collectively suggest a major role of AID in the clonal evolution of *BCR-ABL1*–driven leukemias. To formally test this hypothesis in a genetic experiment, we investigated the functional significance of aberrant AID expression in *BCR-ABL1* ALL in a loss-of-function study.

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Materials and Methods

Mice, retroviral transductions, and bone marrow transplants

Six- to 8-week-old BALB/c mice were purchased from The Jackson Laboratory and maintained in our animal care facility under standard conditions. A BALB/c AID$^{-/-}$ breeding pair was generously provided by Dr. Michel C. Nussenzweig (Rockefeller University, New York, NY). AID$^{-/-}$ mice were bred and maintained under standard conditions. AID$^{-/-}$ status was confirmed for all transplant donor mice by reverse transcriptase-PCR (RT-PCR) for *Aid* (Supplementary Table S3). All experiments involving mice were reviewed and approved by the Institutional Animal Care and Use Committee. Retroviral transductions and bone marrow transplants were performed as described in Supplementary Table S3 (16).

Tyrosine kinase inhibitor treatment

Imatinib and nilotinib were provided by Novartis. Treatments with imatinib began 4 days post transplant by p.o. gavage with a dose of 200 mg/kg/d divided twice daily for a total of 30 days. In experiments using nilotinib, a 75 mg/kg/ dose was administered p.o. every 3 days beginning 4 days post transplant and continuing until 60 days post transplant.

Agilent comparative genomic hybridization analysis

DNA was extracted from CD19 purified leukemia test samples using PureLink Genomic DNA Purification (Invitrogen). CD19-positive lymphocyte DNA obtained from three male BALB/c mice was pooled and used as a reference. CD19 purification was done using MACS microbead technology (Miltenyi Biotec). DNA was labeled and hybridized to the Agilent Mouse Genome Comparative Genomic Hybridization (CGH) 244A chip according to the manufacturer's protocol (MOgene LC). Data were analyzed with the use of DNA Analytics 4.0 software (Agilent Technologies). Aberration detection method 2 with centralization and fuzzy zero correction was used to define aberrant intervals. Default filter settings were applied.

Gene expression analysis

Total RNA was extracted from leukemia cells using RNeasy columns (Qiagen). Microarray was performed on mouse genome 430 2.0 arrays according to the manufacturer's instructions (Affymetrix). Analysis was done with Partek Genomic Suite software using one-way ANOVA statistical analysis with a false discovery rate (FDR) of <0.05 to detect differentially expressed genes ($P < 0.0026$). RT-PCR verification was performed using primers and conditions as described in Supplementary Table S3.

Western blot

Monoclonal antibody against p53 (IC12) was purchased from Cell Signaling Technologies. Polyclonal antibody against β-actin was purchased from Abcam. Cells were lysed in the presence of protease inhibitors, and the supernatant was run on NuPage 4% to 12% gradient Bis-Tris gels (Invitrogen). Protein was then transferred to a polyvinylidene difluoride membrane and blocked with 5% nonfat milk before overnight incubation with primary antibodies at 4°C. Horseradish peroxidase–conjugated secondary antibodies followed by incubation with substrate were used to detect protein bands.

Flow cytometry

Leukemia cells were resuspended in PBS and preincubated with anti-CD16/CD32 Fc-block (BD Pharmingen). Aliquots were stained for 15 minutes at 4°C with phycoerythrin-Cy5–conjugated monoclonal antibodies specific for murine Gr-1 and CD19, fluorescein isothiocyanate–conjugated monoclonal antibody specific for murine CD19 or c-Kit, allophycocyanin-conjugated monoclonal antibody specific for murine CD44, or the appropriate isotype control antibodies. All antibodies were purchased from BD Pharmingen. Cells were washed and resuspended in propidium iodide solution (50 μg/mL propidium iodide in PBS) for subsequent analysis using FACScan (BD Biosciences).

MTT assay

Cells were plated at a concentration of 2×10^5 per well in 96-well plates with a volume of 100 μL medium and incubated overnight at 37°C. The following day, imatinib or SU6656 was added at the indicated concentrations in a total volume of 50 μL and plates were kept at 37°C for 72 hours. Cell proliferation and viability were then determined using the TACS MTT Assay system according to the manufacturer's instructions (R&D Systems).

Results

AID expression shortens leukemia latency in a mouse model of BCR-ABL1 leukemia

Our previous studies evaluated the role of AID in CML blast crisis progression in AID overexpression experiments (14). To measure the contribution of aberrant AID expression to the clonal evolution of p190 *BCR-ABL1* ALL in a genetic experiment, we compared the course of disease of AID$^{+/+}$ and AID$^{-/-}$ *BCR-ABL1* ALL using a classic murine bone marrow transplant model (ref. 17; Supplementary Fig. S1). In this model, syngeneic lethally irradiated mice receiving AID$^{-/-}$ or AID$^{+/+}$ bone marrow cells transduced with a retrovirus carrying the *BCR-ABL1*^{p190} oncogene develop CD19$^+$ pre-B-cell leukemia within 3 weeks (Supplementary Fig. S2; Fig. 1A; Table 1). AID mRNA expression was present by RT-PCR in *BCR-ABL1* ALL cells and interleukin-4/lipopolysaccharide–stimulated splenocytes from AID$^{+/+}$ mice but not from AID$^{-/-}$ mice (Supplementary Fig. S2B). To study potential differences in biological outcome of AID-deficient leukemia, we then evaluated survival in mice transplanted with AID$^{-/-}$ or AID$^{+/+}$ *BCR-ABL1*–transformed bone marrow cells by Kaplan-Meier estimation (Fig. 1; Table 1). Compared with AID$^{+/+}$ leukemia, mice transplanted with AID$^{-/-}$ *BCR-ABL1*–transduced bone marrow had prolonged survival in the primary transplant setting (median 34 days versus 13 days, $P < 0.0001$ by log-rank test). Transduction efficiencies of the retroviral *BCR-ABL1* oncogene were equivalent in both cohorts as shown by quantitative PCR (Supplementary Fig. S2A). A difference in survival was also observed in a secondary

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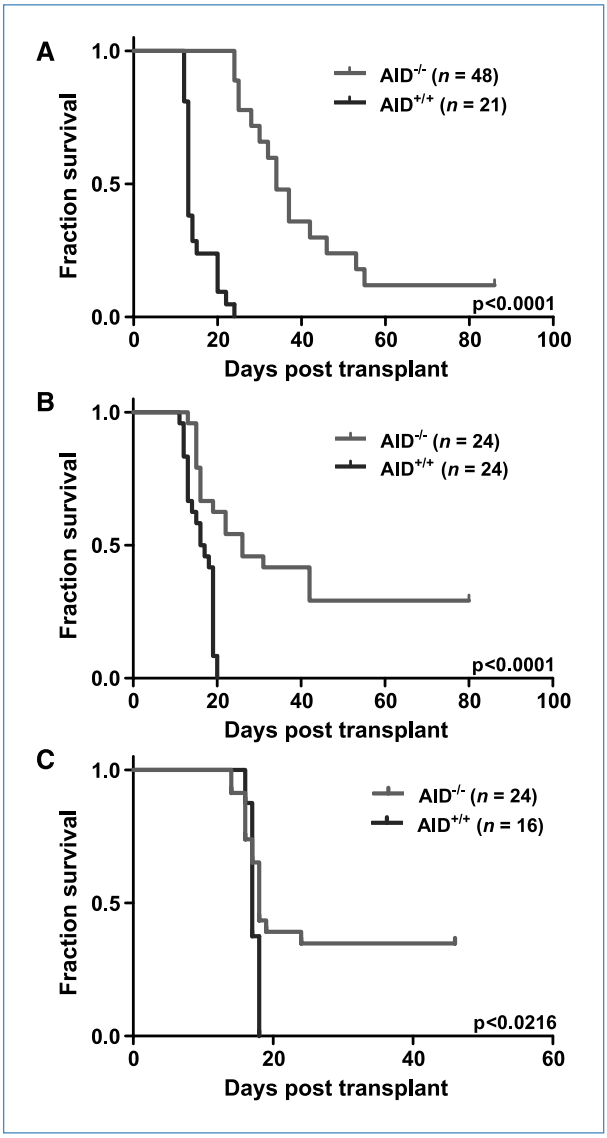

Figure 1. AID deficiency in ALL confers prolonged survival in primary and secondary transplants. A, $AID^{+/+}$ or $AID^{-/-}$ bone marrow was transduced with the *BCR-ABL1* oncogene *in vitro*. Following transduction, 10^6 cells were injected into lethally irradiated BALB/c wild-type recipients (curve compiled from two experiments). B, Kaplan-Meier overall survival of secondary transplantation. Leukemia cells from the spleen and peripheral blood of diseased mice sacrificed in primary transplant experiments were harvested, CD19 purified, and grown *in vitro* for 1 week. Following expansion, 10^4 cells from 6 different $AID^{+/+}$ mice and 10 different $AID^{-/-}$ mice (approximately two recipient mice per clone) were injected into lethally irradiated BALB/c wild-type recipients (curve compiled from four experiments). C, Kaplan-Meier overall survival of tertiary transplantation. Leukemia cells (10^4) from secondary transplants were injected into lethally irradiated BALB/c wild-type recipients (curve compiled from two experiments). Statistical analysis of the serial transplantation experiments is presented in Table 1.

transplant setting, in which leukemia cells from primary recipient mice were recovered from the spleen and purified by $CD19^+$ MACS. From leukemic mice in primary transplant experiments, 10^4 leukemia cells were transplanted together with 10^6 nontransduced bone marrow cells into syngeneic lethally irradiated recipients (median survival 26 days versus 16 days, $P < 0.0001$ by log-rank test; Fig. 1B; Table 1).

In the secondary transplant experiments, we noted a narrowing in the median survival time between cohorts with a corresponding decrease in the hazard ratio (Fig. 1A and B; Table 1). To determine if this trend continued with increasing passage of leukemia cells, we isolated $AID^{-/-}$ leukemia from secondary transplant recipients that died between 25 and 40 days. These were compared with $AID^{+/+}$ leukemia isolated from mice in the primary transplant experiments (Fig. 1C; Table 1). In this tertiary transplant setting, median survival became equivalent in the two cohorts and the hazard ratio decreased further. A one-sample *t* test of the difference between survival times in secondary and tertiary transplants found an average difference of −5.9 days in mice that developed leukemia (SEM 4.05 days, *z* value −1.47, $P = 0.15$; mice failing to develop leukemia were censored in this analysis). Although median survival times narrowed over successive transplants, some clones failed to induce leukemia in both secondary and tertiary settings. The acceleration of disease in sequential transplantations of $AID^{-/-}$ leukemia cells suggests that in that absence of AID, other factors are able to compensate in the development and clonal evolution of disease. A failure of this process to occur, however, leads to extinction of the clone as exemplified by decreased penetrance in secondary and tertiary recipients. We conclude that AID expression accelerates transformation and leukemic outgrowth in this model, leading to a more aggressive disease phenotype.

AID increases the frequency of amplifications and deletions in BCR-ABL1 leukemia

During somatic hypermutation and CSR in GC B cells, deamination of cytosine residues leads to the generation of uracil residues, which is subject to error-prone base excision repair and mismatch repair, leading to either mutations or double-strand DNA breaks (18, 19). Deletion and amplification events require DNA double-strand breaks that can result from ubiquitous environmental (e.g., reactive oxygen intermediates) or endogenous factors (e.g., DNA replication errors as a consequence of a high proliferation rate). In the case of *BCR-ABL1*–driven ALL, two additional mechanisms of clonal evolution can be considered, namely (*a*) aberrant V(D)J recombination owing to deregulated Rag1 and Rag2 activity and (*b*) aberrant hypermutation and class switching, which both depend on the enzymatic activity of AID. Indeed, the majority of deletions at the *IKZF1* (Ikaros) and *CDKN2A* (Arf, Ink4a) loci in *BCR-ABL1* ALL are introduced exactly at recombination signal sequences that are recognized by the Rag1 and Rag2 enzymes during the process of V(D)J recombination (15, 20). On the other hand, in most cases of *BCR-ABL1* ALL, leukemia cells aberrantly express AID (12, 13), which leads to aberrant somatic hypermutation (Fig. 2) and even class switching in some cases (12). Based on these findings, we hypothesize that AID-dependent DNA breaks can result in amplification and deletion events in

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Table 1. Statistical analysis of serial transplantation of $AID^{+/+}$ and $AID^{-/-}$ leukemia cells

Parameter	Primary		Secondary		Tertiary	
	$AID^{+/+}$	$AID^{-/-}$	$AID^{+/+}$	$AID^{-/-}$	$AID^{+/+}$	$AID^{-/-}$
Median survival (d)	13	34	16	26	17	18
No. of mice	21	18	24	24	16	24
Hazard ratio	25.56		5.08		2.92	
P	<0.0001		<0.0001		0.0216	

BCR-ABL1 ALL cells that aberrantly express AID (12, 13). Therefore, we evaluated three $AID^{-/-}$ and three $AID^{+/+}$ leukemia samples from diseased mice for genomic alterations by CGH (Table 2; Supplementary Table S1). $AID^{-/-}$ leukemia had a lower frequency of both amplifications and deletions compared with $AID^{+/+}$ leukemia (17 ± 2 versus 45 ± 7 amplifications, $P = 0.0021$ by Student's *t* test; 11 ± 2 versus 40 ± 7 deletions, $P = 0.0025$ by Student's *t* test). In *BCR-ABL1*^{p190}–transgenic mice, progression of leukemia is accompanied by increasing karyotypic abnormalities (21). The decreased frequency of amplification and deletions in our $AID^{-/-}$ leukemia samples shows that AID indeed contributes to this genomic instability seen in *BCR-ABL1*–driven ALL. This is consistent with recent data in CML, in which copy number alterations that are acquired during lymphoid blast crisis progression are often AID dependent (14).

AID induces aberrant somatic hypermutation in BCR-ABL1–driven ALL

Patients with DLBCL, a GC-derived malignancy that expresses AID, are commonly found to have mutations in non-Ig targets of somatic hypermutation (5). In addition, CML cells often acquire somatic mutations in non-Ig loci (e.g., *BCL6* and *MYC*) during progression into lymphoid blast crisis (14). Introduction of these mutations would be consistent with aberrant expression of AID in lymphoid blast crisis

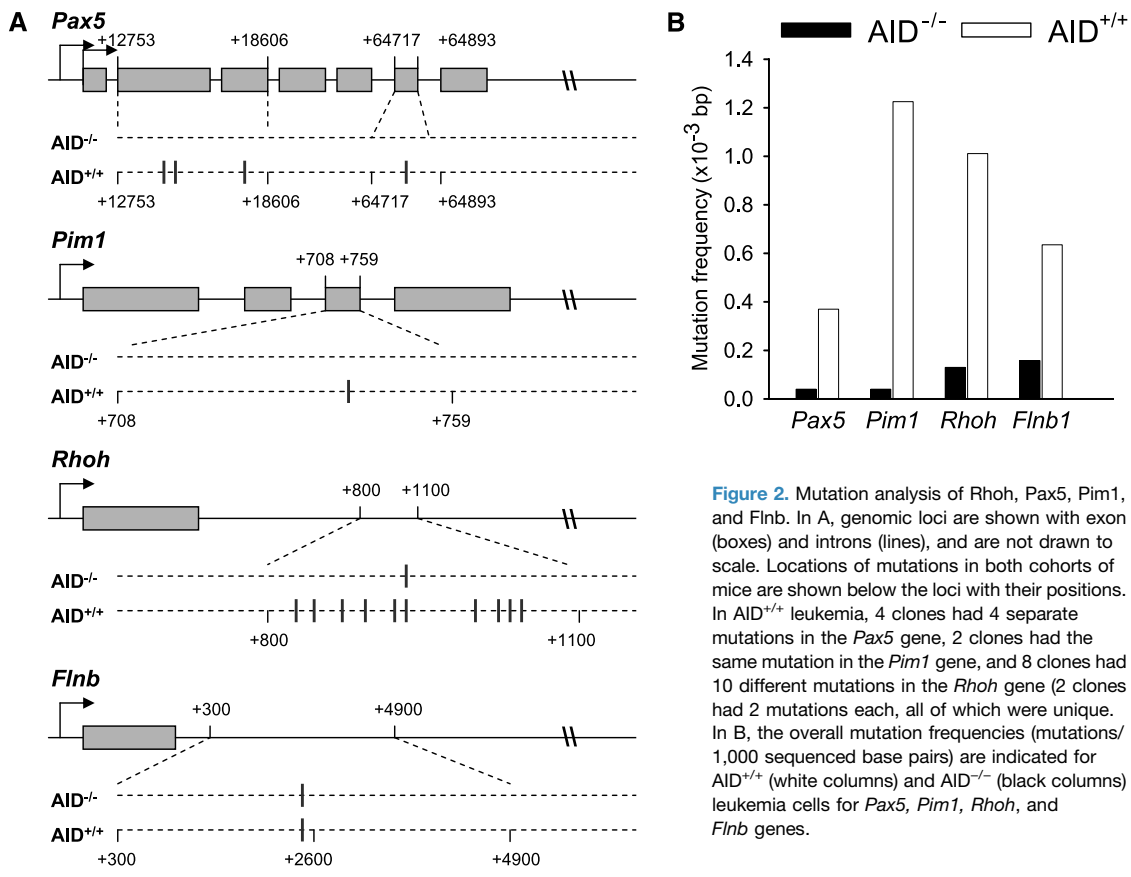

Figure 2. Mutation analysis of Rhoh, Pax5, Pim1, and Flnb. In A, genomic loci are shown with exon (boxes) and introns (lines), and are not drawn to scale. Locations of mutations in both cohorts of mice are shown below the loci with their positions. In $AID^{+/+}$ leukemia, 4 clones had 4 separate mutations in the *Pax5* gene, 2 clones had the same mutation in the *Pim1* gene, and 8 clones had 10 different mutations in the *Rhoh* gene (2 clones had 2 mutations each, all of which were unique. In B, the overall mutation frequencies (mutations/1,000 sequenced base pairs) are indicated for $AID^{+/+}$ (white columns) and $AID^{-/-}$ (black columns) leukemia cells for *Pax5, Pim1, Rhoh*, and *Flnb* genes.

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CML. For this reason, we studied whether somatic mutations of non-Ig genes are also acquired in *BCR-ABL1* ALL and whether acquisition of these mutations is AID dependent. To this end, we performed a comparative mutation analysis of three known AID target genes in $AID^{+/+}$ and $AID^{-/-}$ *BCR-ABL1* ALL cells (*Pax5, Rhoh*, and *Pim1*; Fig. 2). In $AID^{+/+}$ but not $AID^{-/-}$ leukemia cells, we found a significant number of mutations within the first intron of *Rhoh*, similar to patients with DLBCL (5). Also, the frequency of mutations in the *Pax5* and *Pim1* genes was higher in $AID^{+/+}$ leukemia compared with $AID^{-/-}$ leukemia, indicating that AID is active in *BCR-ABL1* ALL cells. In summary with our CGH data, we conclude that AID contributes to genetic lesions in *BCR-ABL1* ALL, including amplifications, deletions, and point mutations.

AID-deficient leukemia has a distinct gene expression profile

Studies in primary human breast tumors have found that genetic lesions often result in far-reaching changes at the gene expression level (22). Additionally, lymphomas driven by deregulated expression of *Myc* and *Bcl6* have distinct gene expression profiles in the presence and absence of AID (7). Therefore, we investigated if the genomic alterations found in $AID^{+/+}$ leukemia result in divergent gene expression patterns in *BCR-ABL1* ALL as observed in *Myc*- and *Bcl6*-driven B-cell lymphomas.

Three $AID^{-/-}$ leukemia samples were compared with three $AID^{+/+}$ samples by Affymetrix gene expression arrays (Fig. 3A; Supplementary Table S2). Consistent with our hypothesis, we found that $AID^{-/-}$ leukemia was largely separate from $AID^{+/+}$ leukemia by a principal component analysis, supporting the concept that genomic alterations led to divergent gene expression patterns. One $AID^{-/-}$ leukemia sample was an outlier in relation to the other two, although it remains more closely related to $AID^{-/-}$ samples than $AID^{+/+}$ leukemia (Fig. 3A). This leukemia persisted to be unique in many of our analyses and is denoted by an asterisk in all of the figures. Using a one-way ANOVA for statistical analysis, we found 2,365 genes that were differentially expressed ($P < 0.0026$; FDR 0.05).

Inactive X-specific transcripts (*Xist*) and *Cd44* were among the genes with the highest gene expression ratio comparing $AID^{+/+}$ and $AID^{-/-}$ leukemias (Supplementary Table S2). These genes were also identified in a recent gene expression comparison between $AID^{+/+}$ and $AID^{-/-}$ *Myc*- and *Myc/Bcl6*-driven B-cell lymphomas (7). When evaluating the top 227 differentially expressed genes, we noted that several of the genes with higher expression levels in $AID^{-/-}$ leukemia have known tumor-suppressor functions (Figs. 3D and 4C), including *Blnk* (SLP65; ref. 23), *Arhgap26* (GRAF; ref. 24), *Rhob* (25), *Cdkn1a* (p21; ref. 26), and *Rhoh* (27). Mutations in the first intron of *Rhoh* as observed in DLBCL and here in *BCR-ABL1* ALL (Fig. 2) have been implicated in downregulation of gene expression (28). The higher expression levels of *Rhoh* in $AID^{-/-}$ leukemia are therefore consistent with our mutation analysis of the *Rhoh* gene (Fig. 2). *Rhoh* is a hematopoietic-specific GTPase that is known to negatively regulate Rac-mediated signaling (27). Mice deficient in Rac GTPases have been shown to have prolonged survival in a murine model of *BCR-ABL1* disease (29). Mimicking Rhoh function by small-molecule inhibition of Rac signaling attenuated leukemic growth in this model, underscoring the importance of Rac/Rhoh interactions in *BCR-ABL1* leukemia (29).

In addition to multiple tumor suppressors, we found lower expression levels of *Cd44* in $AID^{-/-}$ leukemia (Fig. 3). The *Cd44* gene encodes an adhesion molecule that is involved in migration and has been shown to be critical in the homing and engraftment of *BCR-ABL1* leukemia cells (30). In this study, mice receiving $Cd44^{-/-}$ *BCR-ABL1*–transduced bone marrow had prolonged survival compared with $Cd44^{+/+}$ bone marrow, an effect that was overcome by directly injecting cells into the femoral bone marrow of mice. Thus, AID-dependent upregulation of *Cd44* may contribute to the shortened latency of $AID^{+/+}$ compared with $AID^{-/-}$ leukemias and accelerate engraftment. Taken together, we conclude that differential expression patterns of multiple genes, including *Blnk* (SLP65), *Cdkn1a* (p21), *Rhoh*, and *Cd44*, lead to more aggressive disease in $AID^{+/+}$ leukemia.

AID-deficient leukemia fails to downregulate p53

Cdkn1a and *Cd44* are known downstream targets of the p53 tumor suppressor, with *CD44* expression being suppressed by p53 in contrast to *Cdkn1a* expression that is enhanced by p53 (31, 32). Due to their differential expression in $AID^{-/-}$ leukemia, we evaluated other downstream targets of p53 and found higher expression levels of *Pmaip1* (*NOXA*) and *Bax* in $AID^{-/-}$ leukemia as well, consistent with increased p53 levels in $AID^{-/-}$ leukemia (Fig. 4). As p53 mRNA levels

Table 2. Frequency of amplifications and deletions in murine BCR-ABL1 leukemias

Cohort	*n*	Amplifications* (mean ± SD)†	Deletions* (mean ± SD)†	All lesions* (mean ± SD)†
$AID^{-/-}$	3	17.0 ± 2.0 (15–19)	10.7 ± 2.1 (9–13)	27.7 ± 0.6 (27–28)
$AID^{+/+}$	3	45.3 ± 6.7 (38–51)	40.0 ± 7.2 (34–48)	85.3 ± 11.9 (72–95)

*There are significant differences of amplifications ($P = 0.0021$), deletions ($P = 0.0025$), and all lesions ($P = 0.0011$) between $AID^{-/-}$ and $AID^{+/+}$ leukemia as determined by Student's *t* test.
†Ranges are shown in parentheses. Mean values were calculated based on the analysis of three tumor samples from three different mice per cohort.

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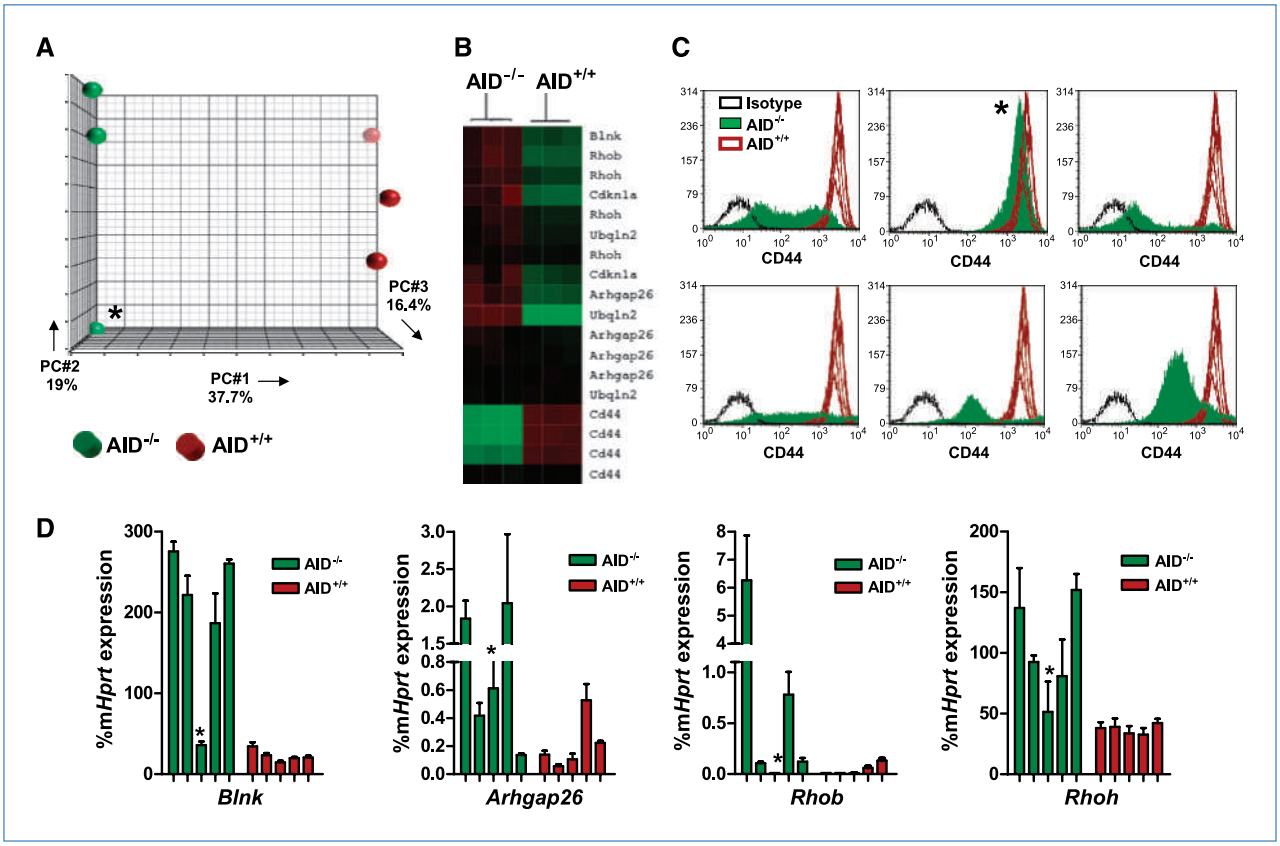

Figure 3. Differential gene expression patterns in AID$^{-/-}$ and AID$^{+/+}$ leukemia. A, principal component analysis. RNA was extracted from leukemia cells harvested from six mice: three mice with AID$^{-/-}$ leukemia and three mice with AID$^{+/+}$ leukemia. The AID$^{-/-}$ outlier is designated by an asterisk in subsequent figures. Samples were hybridized to Affymetrix mouse genome 430 2.0 arrays. Among the genes, 2,365 were found to be significantly differentially expressed (FDR 0.05, $P < 0.0026$). B, heat map of genes chosen for validation by quantitative RT-PCR. C, CD44 expression in AID$^{+/+}$ and AID$^{-/-}$ leukemia. Seven different AID$^{+/+}$ samples comprise the wild-type leukemia histogram (red lines). Shown are six different AID$^{-/-}$ leukemia samples in comparison (solid green overlay) and their isotype controls (dashed black line). D, quantitative real-time RT-PCR of select differentially expressed genes. Data are shown as a percentage of murine *Hprt* gene expression. For each gene, five AID$^{-/-}$ samples and five AID$^{+/+}$ samples from different mice were analyzed.

were similar in AID$^{-/-}$ and AID$^{+/+}$ leukemia by Affymetrix gene array (Fig. 4), we looked at transcript levels of multiple p53 protein stabilizers as well as proteins involved in p53 degradation. The majority of p53-related genes did not have a significant differential expression pattern with the exception of *Ubqln2*, a gene encoding an ubiquitin-like protein that affects *in vivo* protein degradation (Fig. 4). *Ubqln2* has been specifically shown to stabilize the p53 protein (33). In addition to the uniform downregulation of *Ubqln2* in AID$^{+/+}$ leukemia, two of the three AID$^{+/+}$ samples showed downregulation of *Cdkn2a*, a gene that encodes the Arf and Ink4a tumor suppressors. Arf inhibits the Mdm2 protein, a key regulator of p53 degradation, and has been reported to be deleted in ~50% of *BCR-ABL1* ALL cases (33, 34). Consistent with the differential expression of downstream p53 targets, *Cdkn2a* and *Ubqln2*, p53 protein levels were lower in AID$^{+/+}$ leukemia as determined by Western blot analysis (Fig. 4D).

The importance of p53 in progression of multiple tumor types, including *BCR-ABL1* leukemia, is well known (33, 35). Loss of p53 is thought to play an important role in disease progression from chronic phase CML to blast crisis (37). Recent studies have shown a loss of the *CDKN2A* gene in *BCR-ABL1* ALL, which encodes the Mdm2 inhibitor Arf (34). Therefore, functional p53 inactivation by increased protein degradation is an important mechanism in this type of malignancy. p53 represents a DNA damage–response gene, the expression of which is induced by DNA breaks that are acquired in GC B cells undergoing somatic hypermutation and CSR (36). Excessive upregulation of p53 in GC B cells, however, is prevented by the *BCL6* proto-oncogene (37). Outside of GCs, B cells lack *BCL6* expression and hence do not tolerate AID activity, which potentially affords them protection from the deleterious accumulation of genetic lesions (38). AID-mediated activation of the DNA damage response in the absence of sufficient *BCL6* expression would therefore constitute a setting in which loss of p53 (and other DNA damage response genes) would confer a critical selective advantage. In agreement with this hypothesis, p53 has been shown to be involved in the censoring of AID-dependent *Myc-Igh* translocations (39). We propose that

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specific downregulation of p53 in AID$^{+/+}$ but not AID$^{-/-}$ leukemia clones is required to allow AID-dependent genetic lesions to occur and to prevent p53-induced apoptosis in response to AID-dependent DNA damage. Conversely, AID$^{-/-}$ leukemia clones only acquire a relatively small number of genetic lesions (Table 2) and therefore do not require downregulation of p53 to the extent that AID-expressing ALL cells do.

SRC kinase–mediated drug resistance in murine BCR-ABL1 ALL is not AID dependent

To determine if the differences in the clonal evolution of AID$^{-/-}$ and AID$^{+/+}$ leukemia also involve the development of drug resistance, we evaluated their sensitivity to tyrosine kinase inhibition. Fully transformed ALL cells were harvested from mice and subsequently cultured *in vitro* with imatinib, a first-generation *BCR-ABL1* tyrosine kinase inhibitor. We found that AID$^{-/-}$ leukemia cells were more sensitive to treatment than AID$^{+/+}$ leukemia in a standard cell proliferation assay (Fig. 5A). To see if this finding extended to an *in vivo* setting, we treated secondary transplant recipients with either imatinib or the second-generation tyrosine kinase inhibitor nilotinib beginning 4 days post transplant (Fig. 5B and C). Imatinib failed to provide any benefit in either cohort (Fig. 5B); however, nilotinib treatment led to prolonged survival in both AID$^{+/+}$ and AID$^{-/-}$ leukemia (P = 0.003 by Cox regression analysis; Fig. 5C). Both AID$^{-/-}$ and AID$^{+/+}$ cohorts had disease progression in a significant proportion of mice despite treatment with nilotinib (Fig. 5C). We therefore sought to determine if mechanisms of resistance were different.

Previously, we showed that acquired kinase domain mutations represent a major cause of drug resistance in an *in vitro* model of p210 *BCR-ABL1* B lymphoid blast crisis CML. Of note, the vast majority of these mutations are incorporated in an AID-dependent manner (14). Here, we performed a

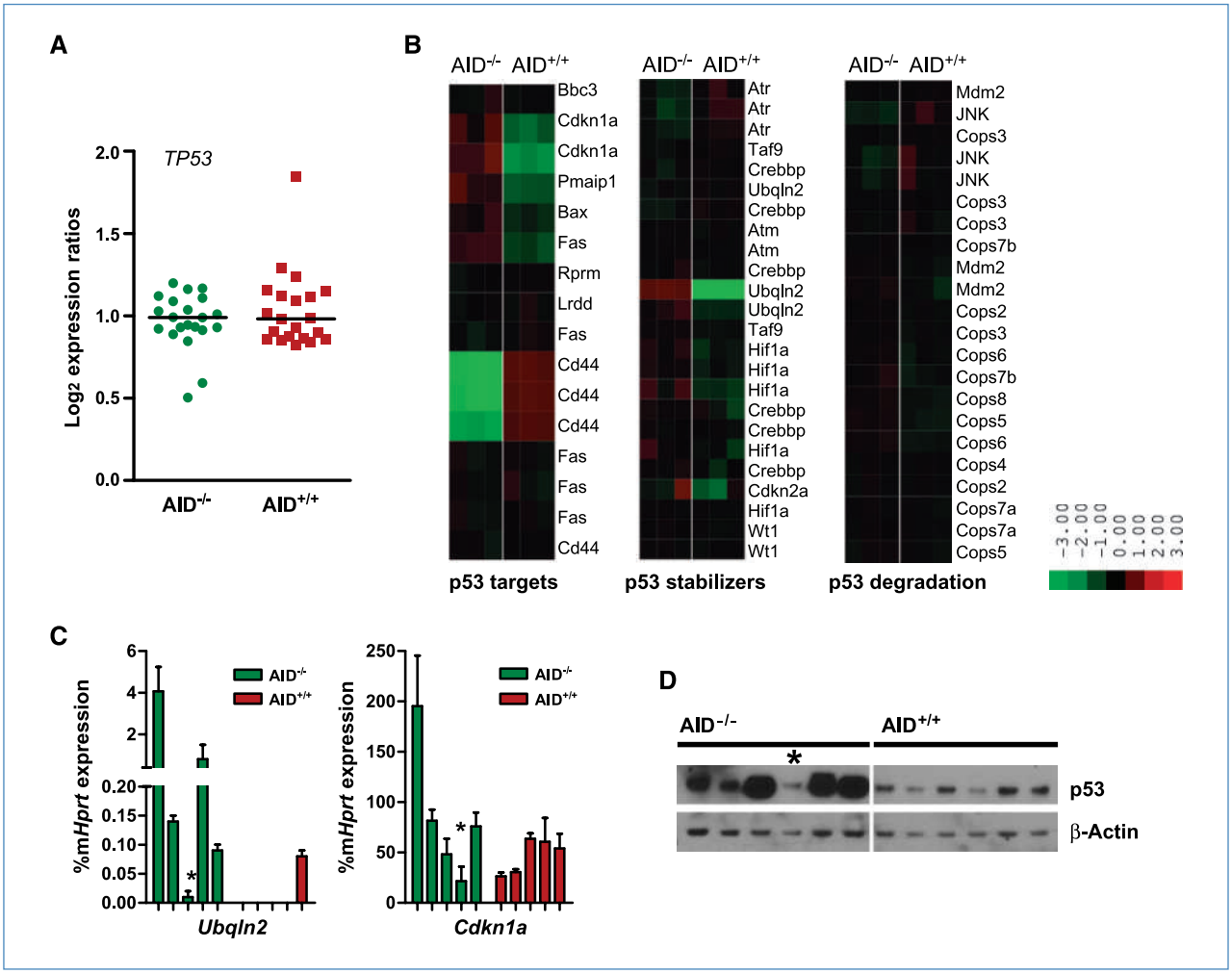

Figure 4. AID$^{-/-}$ leukemia cells fail to downregulate p53. A, $\log_2$ expression ratio for the seven different p53 probe sets in the mouse genome 430 2.0 array for each of the AID$^{+/+}$ (n = 3) and AID$^{-/-}$ (n = 3) samples. B, heat map of downstream p53 targets, molecules involved in p53 stabilization and p53 degradation. C, confirmatory quantitative RT-PCR of *Ubqln2* and *Cdkn1a* mRNA levels in AID$^{-/-}$ mice (percentage of *Hprt* mRNA levels). For each gene, five AID$^{-/-}$ and five AID$^{+/+}$ samples from different mice were analyzed. D, Western blot analysis of p53 protein levels. Proteins from six AID$^{-/-}$ and six AID$^{+/+}$ leukemia samples were extracted and evaluated for p53 expression. *, AID$^{-/-}$ outlier by principal component analysis.

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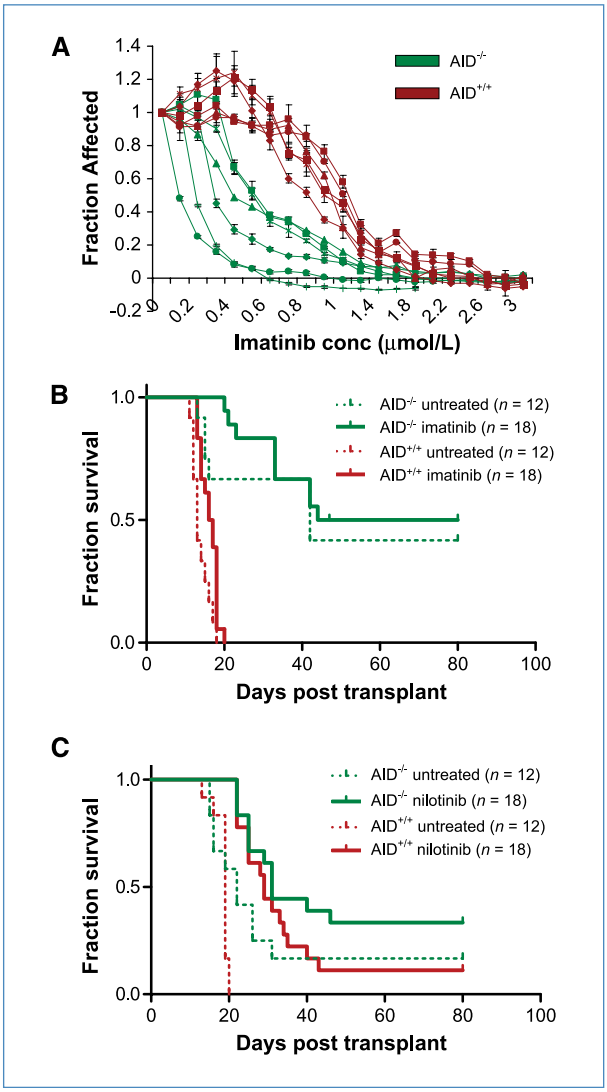

Figure 5. Sensitivity of AID$^{-/-}$ leukemia to tyrosine kinase inhibition. A, leukemia cells were harvested from diseased mice and expanded *in vitro*. Subsequently, cells were plated and treated with increasing concentrations of imatinib as indicated. After 72 hours of incubation, proliferation was measured using a standard MTT assay. Data were compiled from two separate experiments, each experiment with three different AID$^{-/-}$ (green) and AID$^{+/+}$ (red) clones. Each line represents an individual clone. B, secondary transplant recipients were treated with imatinib beginning 4 days post transplant until 30 days post transplant (data compiled from two experiments). C, secondary transplant recipients were treated with nilotinib beginning 4 days post transplant until 60 days post transplant as described in Materials and Methods (data compiled from two experiments).

similar experiment in our p190 *BCR-ABL1* model for *BCR-ABL1* ALL. Three AID$^{-/-}$ and three AID$^{+/+}$ leukemia samples from tyrosine kinase inhibitor–naïve animals were grown *in vitro* with increasing concentrations of imatinib to select for drug-resistant cells. The kinase domain of multiple clones was sequenced from each sample to search for mutations that would confer drug resistance (Supplementary Fig. S3). One AID$^{+/+}$ sample was found to have a clinically relevant mutation in the kinase domain known to lead to drug resistance (L387F; ref. 40). The other samples were found to have a wild-type sequence, suggesting an alternative form of resistance in this experimental setting. Src kinases have been previously shown to play a significant role in this murine model of *BCR-ABL1*–driven leukemia, with an absence of disease when multiple Src kinases are deficient (41). Src-kinase–mediated drug resistance, in which leukemia cells lack kinase domain mutations but have an increased dependence on the Src kinase *LYN*, has also been described in patients (42). We reasoned that there may be differences between AID$^{-/-}$ and AID$^{+/+}$ leukemia cells in their dependence on Src kinases, and thus a difference in the contribution of Src kinases to imatinib resistance. To determine if our imatinib-resistant cells had a greater dependence on Src kinases due to *BCR-ABL1* inhibition, we studied sensitivity to the Src kinase inhibitor SU6656 (43) and compared the response to imatinib-naïve cells (Supplementary Fig. S3). With the exception of one sample, both AID$^{-/-}$ and AID$^{+/+}$ cells resistant to imatinib had an increased sensitivity to Src kinase inhibition when compared with imatinib-naïve cells. We conclude that tyrosine kinase inhibition in both AID$^{-/-}$ and AID$^{+/+}$ leukemia leads to an increased dependency on Src kinases *in vitro*. These data collectively indicate that AID expression in *BCR-ABL1* ALL cells contributes to the acquisition of drug resistance *in vitro* (Fig. 5A). The mechanisms of drug resistance in our experimental system, however, seem to differ from the pathways of drug resistance most commonly encountered in patients, namely *BCR-ABL1* kinase mutations.

Discussion

In a model of *BCR-ABL1*–driven ALL, we have shown that AID expression leads to a more aggressive phenotype, with a shorter median survival time compared with AID-deficient leukemia. In this model, AID$^{+/+}$ leukemia is characterized by an increase in genetic lesions, including amplifications, deletions, and evidence of aberrant somatic hypermutation. We propose that these genomic differences contribute to the divergent gene expression profiles found by gene expression profiling. As proof of principle, we showed that mutations in the first intron of *Rhoh* occur at an increased frequency in AID$^{+/+}$ leukemia consistent with decreased levels of transcript found in this cohort. Surprisingly, gene expression changes between AID$^{+/+}$ and AID$^{-/-}$ leukemia were profoundly different. Given the function of AID as a cytosine deaminase, one would not expect gene expression changes of this magnitude. On the other hand, two recent reports (44, 45) show that AID also functions as a demethylase at CpG islands in a broad range of promoters throughout the genome. Based on these findings, the far-reaching differences in gene expression between AID$^{-/-}$ and AID$^{+/+}$ leukemia cells that we observed here may also reflect changes of the methylation status of numerous promoter regions.

Many of the lesions found in our CGH and gene expression data were similar within AID$^{-/-}$ and AID$^{+/+}$ cohorts; however, there were also differences within the cohorts. In our murine

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model, introduction of the oncogene occurs *in vitro* followed by i.v. injection. The initial transformation thus happens outside of the mouse. Cell division of a few transformed clones may therefore lead to injection of these clones that carry many of the same lesions into several mice. Subsequent clonal evolution *in vivo* can then explain the differences found among the mice within a given cohort.

The significantly increased frequency of genetic lesions found in $AID^{+/+}$ leukemia along with evidence of somatic hypermutation led us to question a possible contribution to tyrosine kinase inhibitor drug resistance. Sequencing of the *BCR-ABL1* gene showed a kinase domain mutation in only one of three resistant $AID^{+/+}$ clones (Supplementary Fig. S3). In a previous study from our group (14), we had shown that AID contributes to the acquisition of *BCR-ABL1* kinase domain mutations as a principal cause of drug resistance. The fact that only one relevant *BCR-ABL1* kinase domain mutation was found in our current experiment may be owing to the fact that leukemia cells acquired resistance to imatinib by shifting their dependence from *BCR-ABL1* to SRC kinases as shown in Supplementary Fig. S3. Autonomous SRC kinase signaling was described previously as a major alternative mechanism of drug resistance in *BCR-ABL1* ALL (43, 45). Our experiments show that imatinib-resistant leukemia cells have shifted their dependence from *BCR-ABL1* to SRC kinase signaling (Supplementary Fig. S3). These findings suggest that activation of SRC kinase signaling rather than acquisition of BCR-ABL1 tyrosine kinase mutations represents the predominant mechanism of drug resistance in our mouse model of *BCR-ABL1* ALL. In human *BCR-ABL1* ALL, *BCR-ABL1* kinase domain mutations are found in ~85% of imatinib-resistant leukemia cases (14, 42). This difference may be owing to the fact that our mouse model of *BCR-ABL1* ALL may not allow sufficient time for *BCR-ABL1* kinase domain mutations to be acquired, or that it favors a resistance phenotype that is immediately available (i.e., SRC kinase signaling). Indeed, injection of *BCR-ABL1*–transformed B-cell precursors into immunodeficient recipient mice leads to lethal disease with a median survival of 13 days (Fig. 1; Table 1), whereas human *BCR-ABL1* ALL is associated with a median disease duration of 26 months (46) or longer (11).

In contrast to $AID^{+/+}$ leukemia cells, their $AID^{-/-}$ counterparts failed to downregulate multiple tumor suppressors at the transcriptional level, including *Blnk* (SLP65), *Cdkn1a* (p21), and *Rhoh*. Leukemia cells deficient in AID also failed to downregulate p53 protein levels as opposed to AID-expressing leukemia. Future studies will explore AID as a potential target for therapeutic approaches to prevent drug resistance and relapse. For instance, AID activity can be suppressed by the cytidine deaminase inhibitor tetrahydrouridine (47), which is currently undergoing a phase I trial in combination with 5-fluoro-2′-deoxycytidine for adults with advanced solid tumors (48). Targeting of AID to hinder further clonal evolution may potentiate current treatment regimens and decrease the incidence of relapse in patients with ALL.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Activation-Induced Cytidine Deaminase Accelerates Clonal Evolution in *BCR-ABL1*–Driven B-Cell Lineage Acute Lymphoblastic Leukemia

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