Regulation of the Interferon regulatory factor-8 (IRF-8) Tumor Suppressor Gene by the Signal Transducer and Activator of Transcription 5 (STAT5) Transcription Factor in Chronic Myeloid Leukemia*

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Background: The IRF-8 tumor suppressor gene is down-regulated in chronic myeloid leukemia (CML).

Results: The STAT5 transcription factor regulates IRF-8 expression in CML cells.

Conclusion: In CML, oncogene-mediated activation of STAT5 is responsible for suppressing the IRF-8 gene.

Significance: An oncogene driven STAT5-IRF-8 signaling axis regulates CML biology and may be implicated in other types of myeloid malignancies.

Tyrosine kinase inhibitors such as imatinib can effectively target the BCR-ABL oncoprotein in a majority of patients with chronic myeloid leukemia (CML). Unfortunately, some patients are resistant primarily to imatinib and others develop drug resistance, prompting interest in the discovery of new drug targets. Although much of this resistance can be explained by the presence of mutations within the tyrosine kinase domain of BCR-ABL, such mutations are not universally identified. Interferon regulatory factor-8 (IRF-8) is a transcription factor that is essential for myelopoiesis. Depressed IRF-8 levels are observed in a majority of CML patients and Ifr-8−/− mice exhibit a CML-like disease. The underlying mechanisms of IRF-8 loss in CML are unknown. We hypothesized that BCR-ABL suppresses transcription of IRF-8 through STAT5, a proximal BCR-ABL target. Treatment of primary cells from newly diagnosed CML patients in chronic phase as well as BCR-ABL+ cell lines with imatinib increased IRF-8 transcription. Furthermore, IRF-8 expression in cell line models was necessary for imatinib-induced anti-tumor responses. We have demonstrated that IRF-8 is a direct target of STAT5 and that silencing of STAT5 induced IRF-8 expression. Conversely, activating STAT5 suppressed IRF-8 transcription. Finally, we showed that STAT5 blockade using a recently discovered antagonist increased IRF-8 expression in patient samples. These data reveal a previously unrecognized BCR-ABL-STAT5-IRF-8 network, which widens the repertoire of potentially new anti-CML targets.

A precise molecular understanding of the pathogenesis of both hematologic and non-hematologic neoplastic diseases has become critical for the development of novel drug therapies that exhibit greater target specificity and less systemic toxicity than conventional chemotherapeutics. Chronic myeloid leukemia (CML) is a prototypic example of successful application of that paradigm through the development of tyrosine kinase inhibitors (TKI) that target the BCR-ABL chimeric fusion protein (1).

BCR-ABL is the causative basis of 99% of all CML cases and functions as a constitutively active tyrosine kinase with unregulated signaling potential, ultimately leading to the aberrant proliferative and anti-apoptotic myeloid phenotype characteristic of CML (2, 3). Because the BCR-ABL chimeric fusion protein is critical for CML development, it has become an attractive target for TKI-based therapy, most notably the first generation TKI, imatinib mesylate. However, despite the considerable success of TKI-based therapy, both primary and secondary drug resistance remain a problem (4). Further elucidation of the BCR-ABL signaling pathway may lead to the identification of major therapeutic resistance mechanisms and enable the development of new anti-CML treatments.

One potential mechanism of resistance to TKI results from the constitutive activation of the STAT5 (signal transducer and activator of transcription 5) transcription factor, a well known proximal target of BCR-ABL signaling that may override drug efficacy (5–7). Under normal physiologic conditions, the activation (phosphorylation) state of STAT5, as with other STAT family members, is tightly regulated in response to cytokine signaling (8). In CML, however, STAT5 is constitutively active in response to oncogenic BCR-ABL signaling, resulting in impaired regulation of target genes (e.g. Bcl-XL, c-Myc, and Mcl and cyclin D1/D2) that are critical for cellular survival, prolif-
eration, and death (9). These perturbations are instrumental to tumorigenesis and maintenance of the leukemic phenotype; multiple studies have demonstrated that STAT5 activity is necessary for development of BCR-ABL⁺ myeloproliferative disorders (10–14).

In a separate body of work, another transcription factor known as interferon regulatory factor-8 (IRF-8) has been demonstrated to be markedly depressed in the marrow of CML patients, particularly in those with more advanced disease (15). IRF-8 has long been regarded as a myeloid essential transcription factor governing myeloid lineage commitment (16). Loss of IRF-8 also leads to the development of a myeloproliferative disease resembling human CML (17–19). Moreover, ectopic reintroduction of IRF-8 expression antagonized BCR-ABL-induced CML in vivo in mouse models (20, 21). Interestingly, IRF-8 levels were rapidly restored in patients who achieved complete cytogenetic remission in response to IFN-α-based therapy (22). Collectively, these observations implicate IRF-8 as a tumor suppressor gene for leukemogenesis in CML. Despite such compelling evidence supporting a pivotal role for IRF-8 in CML, it remains unknown how IRF-8 fits into the mechanism of BCR-ABL-induced CML. We hypothesized that BCR-ABL and IRF-8 are connected via STAT5 activation. The rationale to pursue STAT5 as the bridge between BCR-ABL and IRF-8 is strengthened by studies showing that STAT5 represses IRF-8 transcription in murine models of dendritic cell development (23) and myeloid-derived suppressor cell biology (24).

In this report, our data support a new model whereby BCR-ABL induces STAT5 activation, which enables STAT5 to partner directly with IRF-8 to repress its transcription, thereby losing its potential tumor suppressor capability. Our data also identify IRF-8 as a previously unrecognized target of STAT5 in leukemia, which adds to our broader understanding of the BCR-ABL signaling pathway for potential clinical exploitation. From a fundamental standpoint, our results provide a novel explanation for the longstanding conundrum of why IRF-8 levels are absent or strongly depressed in patients with CML.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Human Samples**—The BCR-ABL⁺ (Philadelphia chromosome) cell lines K562 and KU812, originally derived from patients with blast-phase CML (ATCC, Manassas, VA), were maintained in an RPMI-based medium supplemented with 10% fetal calf serum. 32Dp210 cells were kindly provided by Dr. David Frank (Dana Farber Cancer Institute). RAW264.7 cells were obtained from ATCC and were maintained in RPMI-based culture medium. Unfractionated bone marrow cells from healthy donors and patients with chronic phase CML at diagnosis were obtained through the tissue repository at Roswell Park Cancer Institute under Internal Review Board-approved protocols. All *in vitro* assays were performed in RPMI-based culture medium.

**PCR Analyses**—Total RNA was isolated using RNeasy Mini kits (Qiagen; Valencia, CA) according to the manufacturer’s instructions. cDNA was synthesized using the iScript RT-PCR system (Bio-Rad). The cDNA was then used as the template for PCR amplification of the indicated murine genes in a PTC-200 thermal cycler (MJ Research, Waltham, MA) under the following standard conditions: 94 °C for 2 min, 30 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min) and 72 °C for 10 min. The following human primer sets were used: IRF-8, 5’-TGGCTTGATCGACGCGATTGACAGT-3’ (forward) and 5’-AAGGGATCTCGGAACATCTGCTTCTTG-3’ (reverse); STAT5A, 5’-CACAGATCAAGGAGTTGGTC-3’ (forward) and 5’-CCATTGGTCGCGTAA-3’ (reverse); STAT5B, 5’-GATTCTCAAGGAAAGAATGTT-3’ (forward) and 5’-TGTTGCTCCAGATCGAAGG-3’ (reverse); β-actin, 5’-ATGGATGATGATATGCCGCGC-3’ (forward) and 5’-CTAGAAGATCCATGGGTTGACG-3’ (reverse); and GAPDH, 5’-AGGGCCCTCCCATAGCTTCC-3’ (forward) and 5’-AGGGCCCATCCACAGCTTCC-3’ (reverse).

In addition, the following mouse primers were used: IRF-8, 5’-CGTGGAGACAGCGATTGCTG-3’ (forward) and 5’-GCTGAATGTTGCTGATAGGC-3’ (reverse); and GAPDH, 5’-CATCACCACATTCTCCGGAGACGG-3’ (forward) and 5’-ACGGACACATTGGGTTAGG-3’ (reverse). PCR reactions were separated on a 1% agarose gel, and the images were captured with the Chemidoc Imaging System (Bio-Rad). Quantitative PCR reactions were conducted on an ABI PRISM 7900HT Sequence Detection System (Applied Biosciences, Carlsbad, CA) using RT² SYBR Green Master Mix (Qiagen). The validated primer sets listed above were also used for quantitative PCR analysis.

**IRF-8 and STAT5 Knockdown Studies**—K562 cells were stably transfected with the following shRNA plasmids, which also contain the gene encoding GFP: pshRNA-h7SKgz-control (ATACGCACTAAACACATCAA) and pshRNA-h7SKgz-hIRF8 (AGCCTTCTTGAGGACGTAT). Each shRNA sequence was custom-designed using siRNA Wizard (InvivoGen, San Diego, CA). The shRNA-control plasmids contained a scrambled, nontargeting sequence. Sequences were cloned into psRNA-h7SKgz plasmids by InvivoGen. Cells were transfected with shRNA plasmids using Lipofectamine 2000 reagent (Invitrogen). Transfected cells were then selected and maintained in culture medium containing 200 μg/ml of zeocin (InvivoGen). GFP⁺-expressing tumor cells were sorted shortly after antibiotic selection. The level of IRF-8 knockdown was determined by quantitative PCR using the IRF-8 primers listed previously.

Separately, K562 cells were transiently transfected with STAT5-specific (STAT5A and STAT5B, Santa Cruz Biotechnology, Dallas, TX) or control siRNA (2 μg/sample) using Lipofectamine 2000. Expression assays were conducted 24 h following transfection. The levels of STAT5A and STAT5B knockdown were verified by quantitative PCR using the STAT5 primers listed previously.

**Luciferase Reporter Assay**—A human IRF-8 reporter vector was generated through cloning a fragment of the human IRF-8 promoter (−1320 to −1) into the pGL3-Basic luciferase reporter vector (Promega, Fitchburg, WI). The human IRF-8 promoter fragment was generated through PCR amplification of genomic DNA using the following primers: 5’-AAAAGCTAGCAAAACTCTCATATTCTTCTT-3’ (forward) and 5’-AAAAACCTCGAGCGAGCTGCGCAGCCT-3’ (reverse). Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instruc-
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Inhibitors. Protein concentrations were measured using the BCA assay kit (Thermo Scientific), and 40 μg of protein/sample was used for gel electrophoresis. After transfer, membranes were incubated overnight with antibodies directed against IRF-8 (1:800 dilution, D20D8; Cell Signaling), total STAT5 (1:500 dilution, C-17; Santa Cruz Biotechnology), or total STAT3 (1:500, C-20; Santa Cruz Biotechnology), or actin (1:1000, Sigma-Aldrich). After incubation with the primary antibody, membranes were blotted with HRP-conjugated anti-rabbit secondary antibody (1:2500; Promega). Bands were visualized using the Super Signal Western detection kit (Thermo Scientific).

Chromatin Immuno precipitation (ChIP)—Chromatin was prepared from untreated K562 cells (5 × 10^6) using the EZ ChiP kit (Millipore, Burlington, MA), following the manufacturer’s instructions. Protein-bound chromatin was immunoprecipitated using either normal rabbit IgG (1:50 dilution, 2729, Cell Signaling) or phospho-STAT5 antibody (1:50 dilution, 9135, Cell Signaling). After protein digestion and reverse cross-linking, PCR reactions were conducted on the purified chromatin using a PTC-200 thermal cycler (MJ Research) under the conditions defined previously: 94 °C for 2 min, 35 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min), and 72 °C for 10 min. Human IRF-8 promoter enrichment was analyzed using the following primer set: from −235 to −80 (relative to the transcription start site); 5'-TGACCCCCAGGTGTGAGGAG-3' (forward) and 5'-CCGACCAATAGCGTCAGC-3' (reverse). The PCR products were separated on a 1% agarose gel, and the images were captured with the Chemidoc Imaging System (Bio-Rad).

Transient Silencing with siRNA—One million K562 cells were plated in triplicate in 24-well plates and transfected with 1 μg of siRNA (STAT5 siRNA, sc-29495; Crk-L siRNA, sc-35114) and incubated for 40 h post-transfection. Knockdown was confirmed with Western blot for STAT5 and RT-PCR for Crk-L siRNA with nested PCR primers Crk-L (h)-PR: sc-35114-PR.

Statistical Analysis—When comparing two groups, unpaired t tests were used to validate statistical significance, whereby p < 0.05 was considered to be significant. All bar graphs are presented as mean ± S.E.

RESULTS

Imatinib Induces IRF-8 Expression in Primary CML Cells—To test our hypothesis, we used imatinib as a pharmacologic tool to interrogate the link from BCR-ABL to IRF-8 in primary CML samples. We quantified IRF-8 mRNA levels in unfractionated bone marrow cells obtained at diagnosis from patients with chronic phase CML (Table 1). To test the interaction between BCR-ABL activity and IRF-8 expression, we treated primary CML samples with 1 μM imatinib for 24 h and measured IRF-8 mRNA levels. In six of eight patient samples, we observed a significant increase in IRF-8 mRNA levels in samples treated with imatinib compared with vehicle (Fig. 1A). Imatinib did not affect IRF-8 mRNA levels in healthy bone marrow cells, ruling out the possibility that the observed increases were due to effects of treatment on the non-neoplastic cells of the CML preparation (Fig. 1B).
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**TABLE 1**
Clinical characteristics of primary patient samples

| Patient No. | Age | Gender | Sokal score |
|------------|-----|--------|-------------|
| 1          | 64  | F      | 0.9 (Intermediate) |
| 2          | 41  | M      | 0.99 (Intermediate) |
| 3          | 53  | M      | 0.74 (Low)     |
| 4          | 54  | F      | 0.72 (Low)     |
| 5          | 62  | M      | 0.8 (Intermediate) |
| 6          | 79  | F      | 0.95 (Intermediate) |
| 7          | 61  | M      | 0.72 (Low)     |
| 8          | 67  | M      | 0.83 (Intermediate) |

In agreement with the data obtained from patient samples, treatment of human K562 and KU812 CML cell lines or mouse 32D cells stably transfected with BCR-ABL oncogene (32Dp210) with imatinib increased IRF-8 mRNA levels compared with treatment with vehicle (Fig. 1C). Together, these data reveal a functional interaction between BCR-ABL activity and IRF-8 transcription in primary CML cells and cell lines.

**FIGURE 1.** Imatinib induces IRF-8 expression in primary CML samples. A, IRF-8 mRNA levels in primary CML cells obtained from eight patients at diagnosis treated with 1 μM imatinib in vitro for 24 h (n = 3 per sample; *, p < 0.05). For clinical information connected to the samples, see Table 1. B, IRF-8 mRNA levels in primary healthy bone marrow (left) and representative CML (right) cells treated with 1 μM imatinib for 24 h (n = 3; *, p < 0.001). IRF-8 mRNA levels, relative to GAPDH mRNA levels, were quantified by real-time RT-PCR. Data were normalized to reference control. C, representative IRF-8 mRNA levels in human K562 (left), KU812 (middle), or murine 32Dp210 cells were treated with 1 μM imatinib (+) or vehicle control (−) for various time points. For all panels, data are reported as mean ± S.E. of the indicated number of samples (*, p < 0.05).

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**IRF-8 Mediates the Response to Imatinib**—To test whether re-expression of IRF-8 after imatinib treatment regulated the survival of CML cells, we utilized shRNA technology to stably silence IRF-8 expression in K562 cells (IRF-8-shRNA). We confirmed stable silencing of IRF-8 as IRF-8 mRNA levels were reduced by >85% compared with K562 cells transfected with a scramble shRNA vector (Fig. 2A). To test the effect of enforced suppression of IRF-8 on response to imatinib, we measured cell proliferation using [3H]thymidine incorporation as readout. Although imatinib treatment inhibited the proliferation of both K562 populations in dose-dependent fashion, it is important to note that K562 cells silenced for IRF-8 were significantly less susceptible to the inhibitory effects of imatinib compared with the controls (Fig. 2B). Differences in proliferation could also reflect or correlate with differences in cell death response to imatinib. Therefore, we analyzed the effects of imatinib treatment on cell death by measuring propidium iodide staining (Fig. 2C). There was no difference in cell death between IRF-8-shRNA and control cells in the absence of imatinib. However, after 48 h of imatinib treatment, stable silencing of IRF-8 led to a significant decrease in the percentage of propidium iodide+ cells compared with the control cells (Fig. 2D). These data suggest that re-expression of IRF-8 contributes to the antitumor effects of TKIs such as imatinib.

**STAT5 Represses IRF-8 Transcription in CML Cells**—Although our data demonstrate a causal link between BCR-ABL signaling and IRF-8 transcription, they do not indicate how the two events are bridged. Based on studies establishing that STAT5 as a proximal target of BCR-ABL and that STAT5 inhibits transcription of IRF-8 in other myeloid systems, we hypothesized that BCR-ABL-mediated activation of STAT5 results in suppression of IRF-8 transcription in CML cells (5, 6).

To test the effect of inhibiting STAT5 activity on IRF-8 expression, we transduced CML cell lines with siRNA that targeted STAT5. Transfection of K562 cells with STAT5 siRNA significantly suppressed expression of both STAT5A and STAT5B genes (Fig. 3A), resulting in decreased levels of active phosphorylated STAT5A protein and total STAT5A compared with the scramble control (Fig. 3B). Silencing of STAT5 in K562 cells resulted in an increased level of IRF-8 protein (Fig. 3C). This increase was also observed at the transcriptional level as silencing of STAT5 in K562 cells and 32Dp210 cells significantly increased in IRF-8 mRNA levels (Fig. 3D), implicating STAT5 as a repressor of IRF-8 transcription. To test whether other proximal targets of BCR-ABL regulated IRF-8 transcription, we silenced the Crk-like protein gene (CRKL), which is directly phosphorylated by BCR-ABL and is necessary for BCR-ABL-mediated transformation (28–30). However, silencing of CRKL in K562 cells did not significantly increase IRF-8 mRNA levels, suggesting that regulation of IRF-8 expression is not a ubiquitous consequence of other members of the BCR-ABL signaling pathway.

We then tested whether enforced activation of STAT5 activity was sufficient to suppress IRF-8 transcription. Using the mouse RAW264.7 macrophage cell line as a model of endogenous Irf-8 transcription, we transduced these cells with a retroviral vector that expresses a constitutively active form of Stat5a (Stat5aS711F) (27). Constitutive activation of Stat5a decreased transcription of Irf-8 and Irf-8 protein levels compared with...
vector control (Fig. 4, A and B), indicating that activation of STAT5A are sufficient to repress Irf-8 transcription. To determine whether this effect was specific to Stat5a, we transduced RAW264.7 cells with a vector that expresses constitutively active Stat3, another STAT family member activated in CML cells (31, 32). Constitutive activation of Stat3 in RAW264.7 cells led to a decrease in Irf-8 mRNA levels compared with the vector control cells. These data support our previous studies demonstrating that Stat3 suppresses Irf-8 transcription in myeloid-derived suppressor cells. Together, these data suggested that decreased levels of Irf-8 in CML cells are due to increased activation of multiple members of the STAT family.

STAT5 Regulates IRF-8 Promoter Activity in CML Cells—The human IRF-8 promoter is known to have at least one well defined palindromic motif within positions −175 to −155 for STAT binding, including STAT5 (5′-TTCTCGGA-3′) (Fig. 5A) (23). To test whether STAT5 regulates transcription through the IRF-8 promoter we performed ChIP assays on K562 cells. Immunoprecipitation with a phospho-STAT5-specific antibody, but not an isotype-matched control antibody, resulted in the appearance of a PCR product reflecting the expected IRF-8 promoter fragment. This PCR product spanned the identified STAT5 binding site and did not include any other potential sites (Fig. 5, A and B). Thus, STAT5 directly binds to the IRF-8 promoter in CML cells, suggesting that STAT5 inhibits IRF-8 transcription through a direct interaction with the IRF-8 promoter. To test a direct role for STAT5 in the regulation of IRF-8 transcription, we transfected K562 cells with a reporter construct in which the human IRF-8 promoter drives
the expression of a luciferase reporter (IRF-8-luc). We found that silencing STAT5 in IRF-8-luc cells significantly increased luciferase levels compared with scramble siRNA control, indicating that STAT5-mediated repression of IRF-8 transcription was regulated via the IRF-8 promoter (Fig. 5C).

To determine whether STAT5 binding to the IRF-8 promoter required binding to the putative STAT5 binding element, we returned to our RAW264.7 cell line model in which we had developed both wild-type and mutant IRF-8 reporter constructs reflecting an alteration the STAT binding element (25). As with the human construct, the murine construct contains the canonical TTC\textsubscript{3}GAA sequence (26, 33). Using site-directed mutagenesis, the critical TTC sequence was replaced with AAA. We reasoned that if STAT5 is a negative regulator of IRF-8 transcription and this effect is due to a direct binding interaction between STAT5 and that particular element, then corruption of that site should abolish STAT5-mediated downregulation of IRF-8 reporter activity. For proof-of-concept, we first generated vector control or constitutively active Stat5a-expressing RAW264.7 cell populations and then transiently transfected each with either the wild-type or mutant Irf-8 reporter construct. We found that transfection of Stat5a-ex-
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Pressing RAW264.7 cells with the wild-type construct led to significant reduction in Irf-8 reporter activity compared with the vector control cells (Fig. 5D, left panel). In contrast, transfection of Stat5α-expressing RAW264.7 cells with the mutant construct did not lead to a reduction in Irf-8 reporter activity compared with the vector control cells (Fig. 5D, right panel), suggesting that Stat5α down-regulated Irf-8 transcription via that particular STAT binding element.

Pharmacologic Suppression of STAT5 Activity Induces IRF-8 Expression in CML Cells—Nelson et al. (34) demonstrated that treatment of CML cells with pimozide resulted in suppression of STAT5 activity, but not other STAT family members. Using this agent, we tested whether pharmacologic suppression of STAT5 induced IRF-8 transcription in K562 cells and primary CML samples. In agreement with these studies, pimozide treatment suppressed phosphorylation of STAT5α in K562 cells (Fig. 6A). Similar to imatinib, pimozide treatment resulted in reactivation of IRF-8 expression in K562 cells (Fig. 6B). We then directly compared the effect of pimozide on IRF-8 protein levels with interferon-γ (IFN-γ), a known activator of IRF-8 transcription, and imatinib. Treatment of K562 cells or 32Dp210 cells with all three reagents resulted in increased amounts of IRF-8 protein, corresponding with the increase in IRF-8 mRNA levels (Fig. 6C). Because pimozide has anti-leukemia properties, we determined whether suppression of IRF-8 resulted in decreased sensitivity to pimozide. Similar to the effect on cell death following imatinib, stable silencing of IRF-8 resulted in a significant decrease in the percentage of propidium iodide+ cells following pimozide treatment compared with the vector control (Fig. 6, D and E).

We then tested whether pimozide restored IRF-8 expression in primary CML samples obtained at diagnosis. In four of five primary CML samples tested, we observed significant increases in IRF-8 mRNA levels following pimozide treatment, indicating that inhibition of STAT5 is sufficient to enable expression of IRF-8 in primary CML cells (Fig. 7).

DISCUSSION

The IRF-8 transcription factor has long been appreciated as a tumor suppressor in CML. Animal models first revealed a causal connection between IRF-8 expression and myeloid leukemia (17–19). Irf-8−/− mice develop a myeloproliferative disorder that eventually evolves into a lethal blast crisis, highly reminiscent of human CML. IRF-8 expression was depressed in the majority of patients presenting with chronic phase CML and restored to normal levels in those patients that responded to interferon-α therapy (15). These data suggest that the status of IRF-8 in CML is critical to leukemic development, progression, and response to therapy. However, heretofore, the mechanism by which repression of IRF-8 occurs in CML has remained unknown. In this study, we present the first evidence for a model in which BCR-ABL inhibits IRF-8 expression through activation of STAT5.

This model is supported by our data demonstrating that treatment of CML cell lines and primary patient cells with imatinib increases IRF-8 mRNA levels, indicating that inhibition of BCR-ABL is sufficient to induce IRF-8 transcription. Further support is provided by our observations that specifically targeting STAT5 activity using genetic or pharmacologic approaches results in increased expression of IRF-8 in CML cell lines and primary samples. This mechanism is not dependent on BCR-ABL, as inhibiting STAT5 activity was sufficient to induce IRF-8 transcription in the presence of functional BCR-ABL and constitutive activation of STAT5α was sufficient to inhibit IRF-8 transcription in a macrophage cell line. Thus, STAT5-mediated suppression of IRF-8 may be a conserved mechanism across myeloid lineage cells as activation of STAT5 in murine dendritic cells or myeloid-derived suppressor cell progenitors represses IRF-8 expression (23, 24).

Our data point to a functional role of a BCR-ABL-STAT5-IRF-8 signaling axis in regulating the proliferation and survival of CML cells following exposure to TKIs. Pharmacologic targeting of the BCR-ABL-STAT5 axis by imatinib or pimozide, respectively, resulted in decreased survival. However, silencing IRF-8 in K562 cells, thereby preventing re-expression of IRF-8, significantly increased proliferation and survival despite imatinib or pimozide exposure. These data are in agreement with previous studies suggesting that genetic-based approaches to enforce expression of IRF-8 in BCR-ABL+ cell lines can result in increased imatinib sensitivity (20, 35).

These data also provide additional support for the model in which reduction in IRF-8 mRNA levels promotes growth and survival of BCR-ABL+ cells. IRF-8 likely suppresses growth and survival of leukemia cells through several mechanisms. In murine models, IRF-8 inhibits BCR-ABL+ cell proliferation by inducing expression of genes such as Cdkn4b, Blimp-1, and Mets-1 (36, 37). IRF-8 also promotes apoptosis by repressing transcription of anti-apoptotic genes such as BCL2 and ASAH1 (21, 38). In addition, IRF-8 suppresses WNT/β-catenin signaling, which has been implicated in the development of CML (39, 40).

Our findings indicate that in CML, BCR-ABL-mediated repression of IRF-8 results from a direct effect of STAT5 on the IRF-8 promoter as determined by ChIP and promoter reporter
assays. We and others (23, 24) identified a similar mechanism in which GM-CSF repressed IRF-8 transcription by inducing STAT5 to directly bind to the IRF-8 promoter. Our data, which demonstrates that activation of STAT3 also suppresses IRF-8 transcription, suggests that additional mechanisms besides STAT5 phosphorylation are responsible for the inhibition of IRF-8 expression in CML. This suppression is not a general consequence of BCR-ABL activity, as down-regulation of CRKL has no effect on IRF-8 expression. Vidovic et al. (41) reported that the WT1 (Wilms tumor gene 1) tumor suppressor inhibits IRF-8 transcription in a BCR-ABL-dependent manner. An interaction between WT1 and STAT3 has been reported; it is possible that WT1 and STAT5 could similarly act to regulate IRF-8 expression (42). Alternatively, Hu et al. (38) have demonstrated that the IRF-8 promoter is methylated in CML cell lines and primary blasts, resulting in decreased expression. Thus,
epigenetic-mediated mechanisms may combine with constitutive STAT5 activation to repress IRF-8 transcription. This hypothesis may explain why agents that target DNA methylation have shown some efficacy in combination with imatinib (or other TKIs) and further suggest that novel inhibitors of STAT5 might be of value to restore IRF-8 expression.

In summary, we demonstrate the existence of a novel STAT5-IRF-8 axis that regulates the growth and survival of BCR-ABL+ leukemia cells, providing a new avenue for clinical exploitation. This is particularly important in cases of CML resistance to therapeutic agents that act primarily at the level of BCR-ABL signaling, including both first and subsequent generation TKIs. Agents that directly inhibit STAT5 or activate IRF-8 transcription may provide an alternative means to bypass such resistance mechanisms. Our finding that pimozide treatment, recently characterized as a STAT5 antagonist, results in IRF-8 expression in primary patient samples showcases the potential impact on CML biology in a BCR-ABL-independent manner (34). Furthermore, given the prominent role of IRF-8 and STAT5 in hematologic malignancies in addition to CML such as BCR-ABL+ B-ALL or AML, this mechanism may play a broader role in the leukemogenesis (15, 43–46). Altogether,
our data strengthen the contention that IRF-8 is a leukemia suppressor, with important clinical implications for prognostic or therapeutic purposes.

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