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Sp1/NFκB/HDAC/*miR-29b* Regulatory Network in KIT-Driven Myeloid Leukemia

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SUMMARY

The biologic and clinical significance of *KIT* overexpression that associates with *KIT* gain-of-function mutations occurring in subsets of acute myeloid leukemia (AML) (i.e., core binding factor AML) is unknown. Here, we show that *KIT* mutations lead to *MYC*-dependent *miR-29b* repression and increased levels of the *miR-29b* target Sp1 in KIT-driven leukemia. Sp1 enhances its own expression by participating in a NFκB/HDAC complex that further represses *miR-29b* transcription. Upregulated Sp1 then binds NFκB and transactivates *KIT*. Therefore, activated KIT ultimately induces its own transcription. Our results provide evidence that the mechanisms of Sp1/NFκB/HDAC/*miR-29b*-dependent *KIT* overexpression contribute to leukemia growth and can be successfully targeted by pharmacological disruption of the Sp1/NFκB/HDAC complex or synthetic *miR-29b* treatment in KIT-driven AML.

INTRODUCTION

The *KIT* gene encodes a 145 kDa transmembrane protein that is a member of the type III receptor tyrosine kinase (RTK) family (Yarden et al., 1987), regulates cell survival, proliferation or differentiation (Schlessinger, 2000), and participates in normal mechanisms of hematopoiesis, melanogenesis, and gametogenesis. KIT protein expression is modulated by a variety of mechanisms including microRNAs (miRNAs) (Felli et al., 2005) and/or proteolytic degradation (Masson et al., 2006) and is subjected to covalent posttranslational modifications, which influence its tyrosine kinase activity through interaction with a variety of factors including KIT ligand (also known as stem cell factor), tyrosine phosphatases (Kozlowski et al., 1998), protein kinase C, and calcium ionophores (Miyazawa et al., 1994; Yee et al., 1993).

KIT is overexpressed and/or mutated in several human neoplasms, including gastrointestinal stromal tumors (GISTs), germ cell tumors, and hematologic malignancies (Ikeda et al., 1991). In acute myeloid leukemia (AML), while *KIT* expression is detectable in the majority of the cases (Ikeda et al., 1991), gain-of-function mutations resulting in constitutive tyrosine kinase activity appear to be restricted to core binding factor (CBF) disease [(8;21) or inv(16) or the respective molecular equivalent *RUNX1/RUNX1T1*- or *CBFB/MYH11*-positive AML],

Significance

KIT encodes a tyrosine kinase receptor that activates downstream pathways leading to cell proliferation and survival. Overexpression of mutated or wild-type *KIT* alleles occurs in specific subsets of AML and predicts poor outcome, thereby supporting a critical role of high levels of the KIT protein in leukemogenesis. Here we report deregulation of a protein-microRNA network, Sp1/NFκB/HDAC/*miR-29b*, that results in *KIT* overexpression in KIT-driven leukemia. We also show that this network is targetable by proteasome, NFκB, Sp1, or HDAC inhibitors or ectopic *miR-29b* expression. These compounds provide antileukemic activity by decreasing *KIT* expression through *miR-29b*-dependent Sp1 downregulation, and represent promising therapeutic approaches to disrupt *KIT* expression and efficiently override aberrant KIT activity in KIT-driven AML.

in which these mutations associate with unfavorable outcome (Paschka et al., 2006).

Tyrosine kinase (TK) inhibitors (e.g., imatinib, dasatinib or PKC412 [midostaurin]) have been shown to suppress aberrant activity of KIT mutants and delay tumor growth (Heinrich et al., 2002; Growney et al., 2005). However, clinical response to these compounds depends mostly on the nature of *KIT* mutations (Heinrich et al., 2002). For example, *KIT* mutations in codon 822 are sensitive to imatinib, whereas mutations in codon 816 are not and can be targeted successfully with midostaurin or dasatinib. Therefore, to take fully clinical advantage of the therapeutic approach with inhibitors, the type of *KIT* mutation needs to be identified at the time of initial diagnosis. Even if this strategy is adopted, however, the sensitivity of a distinct *KIT* mutation to an optimally chosen TK inhibitor is likely to decrease over time due to acquisition of secondary *KIT* mutations (Gajiwala et al., 2009) that mediate resistance (Heinrich et al., 2008). These observations justify investigation of novel strategies to effectively target all *KIT* mutations and improve the likelihood of inducing durable clinical responses in *KIT*-driven malignancies. Flavopiridol and *KIT* siRNA have been shown to downmodulate *KIT* transcription and induce apoptosis in GIST cells (Sambol et al., 2006). Therefore, direct targeting of *KIT* expression may represent a valuable approach to overcome aberrant KIT enzymatic activity and circumvent the drawbacks of TK inhibitor therapies in AML. This strategy, however, can be effectively developed and implemented only if the regulatory mechanisms controlling the expression of both the wild-type and mutated *KIT* alleles in myeloid cells are elucidated.

The overarching goal of the present study is to characterize the molecular pathways that control aberrant expression of both wild-type and mutated KIT alleles in AML and devise molecular targeting strategies to downregulate KIT and, in turn, attain significant and durable antileukemic activity in KIT-driven leukemia.

RESULTS

KIT Overexpression in AML

Aberrant KIT protein activity plays a pivotal role in human malignancies. Although *KIT* expression is relatively common in blasts from all AML subtypes, activating *KIT* mutations appear to be restricted to CBF AML, where they predict poor outcome (Paschka et al., 2006). In CBF AML, the *KIT* gene also appears to be overexpressed. In a cohort of Cancer and Leukemia Group B (CALGB) patients, we showed that *RUNX1/RUNXT1*-positive patients with *KIT* mutation (KIT^{mut}) or wild-type (KIT^{wt}) have higher *KIT* levels compared with patients with cytogenetically normal (CN) AML (Figure 1A). Interestingly, *KIT* overexpression adversely impacts outcome, and *RUNX1/RUNXT1*-positive patients with higher *KIT* levels had a significantly shorter survival ($p = 0.04$; see Figure S1A available online). Among AML cell lines, higher levels of *KIT* expression are also found in CBF AML cell lines, i.e., *RUNX1/RUNXT1*-positive and KIT^{mut} Kasumi-1 and SKNO-1 and *CBFB/MYH11*-positive and KIT^{wt} ME-1, when compared with non-CBF cell lines (Figure 1B and Figure S1B). Thus, we hypothesized that in distinct molecular subsets of AML like CBF AML, the KIT protein is aberrantly activated and upregulated. We also hypothesized that KIT overexpression

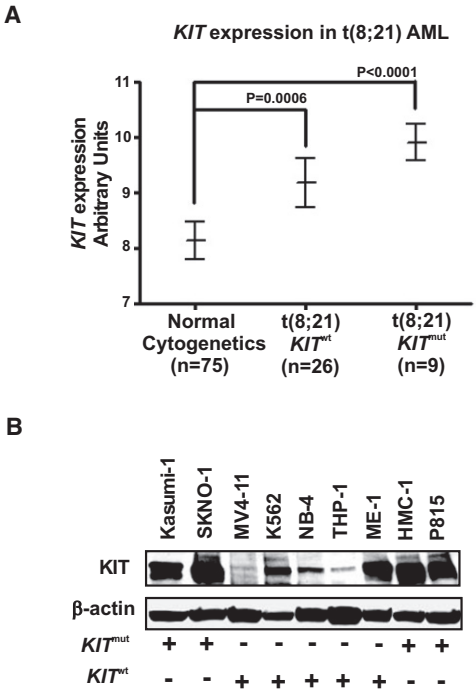


Figure 1. *KIT* Expression in AML Patients and Cell Lines

(A) *KIT* expression in bone marrow from Cancer and Leukemia Group B AML patients.

(B) KIT protein expression in various AML or mastocytosis (HMC-1) cell lines. + or − indicates presence or absence of KIT^{mut} or KIT^{wt} alleles. Data are representative of three independent experiments. (See also Figure S1).

itself contributes to leukemogenesis and therefore should be therapeutically targeted in KIT-driven AML. In order to prove these hypotheses, however, the mechanisms that govern *KIT* expression and its leukemogenic role in *KIT*-driven leukemia need to be fully elucidated.

Sp1/NFκB Modulates *KIT* Expression in AML

To start unraveling the regulatory mechanisms of *KIT* expression in AML, we examined the *KIT* promoter region for transcription factor binding sites, and identified binding sites for both Sp1 and NFκB in a 1kb region spanning the human *KIT* gene promoter. Because we and others have recently shown that transactivation of certain oncogenes (e.g., *DNMT1*) involved in leukemogenesis requires physical interaction of the transcription factors Sp1 and NFκB (Liu et al., 2008; Hirano et al., 1998), we reasoned that the Sp1/NFκB complex is likely to be also involved in modulation of *KIT* expression in *KIT*-driven leukemia cells.

To support this hypothesis, we performed electrophoretic mobility-shift assays (EMSA) using probes spanning the Sp1/NFκB binding sites (XN2 probe) on the *KIT* promoter or consensus binding elements for Sp1 (Sp1C) or NFκB (NFκBC) on nuclear extracts from Kasumi-1 cells. These cells were selected because they harbor mutated and overexpressed *KIT* (Figure 1B). The DNA-protein complexes attained with the XN2 probe comigrated with those attained with the Sp1C and NFκBC probes, supporting enrichment of both Sp1 and NFκB on the *KIT* promoter (Figure 2A, lanes 2, 5, and 8). These data were

confirmed by chromatin immunoprecipitation (ChIP) showing Sp1 and NFκB enrichment on the *KIT* promoter (Figure 2B). Higher levels of Sp1 enrichment on the *KIT* promoter were observed in Kasumi-1 cells that harbor overexpressed KIT^{mut} compared with AML lines (MV4-11 and THP-1) carrying lower levels of KIT^{wt} (Figure 2B).

To further assess the biological role of Sp1 and NFκB on *KIT* expression, we applied loss- and gain-of-function approaches in Kasumi-1 cells. First we showed that ectopic expression (Figure 2C, left panel) or siRNA knockout (Figure 2C, right panel), respectively, reduced and increased Sp1 and NFκB(p65) enrichment on the *KIT* promoter, and resulted, respectively, in KIT downregulation or upregulation in Kasumi-1 cells (Figure 2D). The role of Sp1 in *KIT* gene transcription was further elucidated by treating Kasumi-1 cells or AML patient primary blasts with mithramycin A, a previously reported Sp1 inhibitor (Ray et al., 1989). Mithramycin A exposure led to decrease in KIT RNA transcription and protein expression (Figure 2E) in both Kasumi-1 and patient primary cells and time- and dose-dependent inhibition of Kasumi-1 cell proliferation (Figure S2). With regard to NFκB function, exposure to the NFκB inhibitor bay11-7082 decreased Sp1 and KIT expression (Figure 2F).

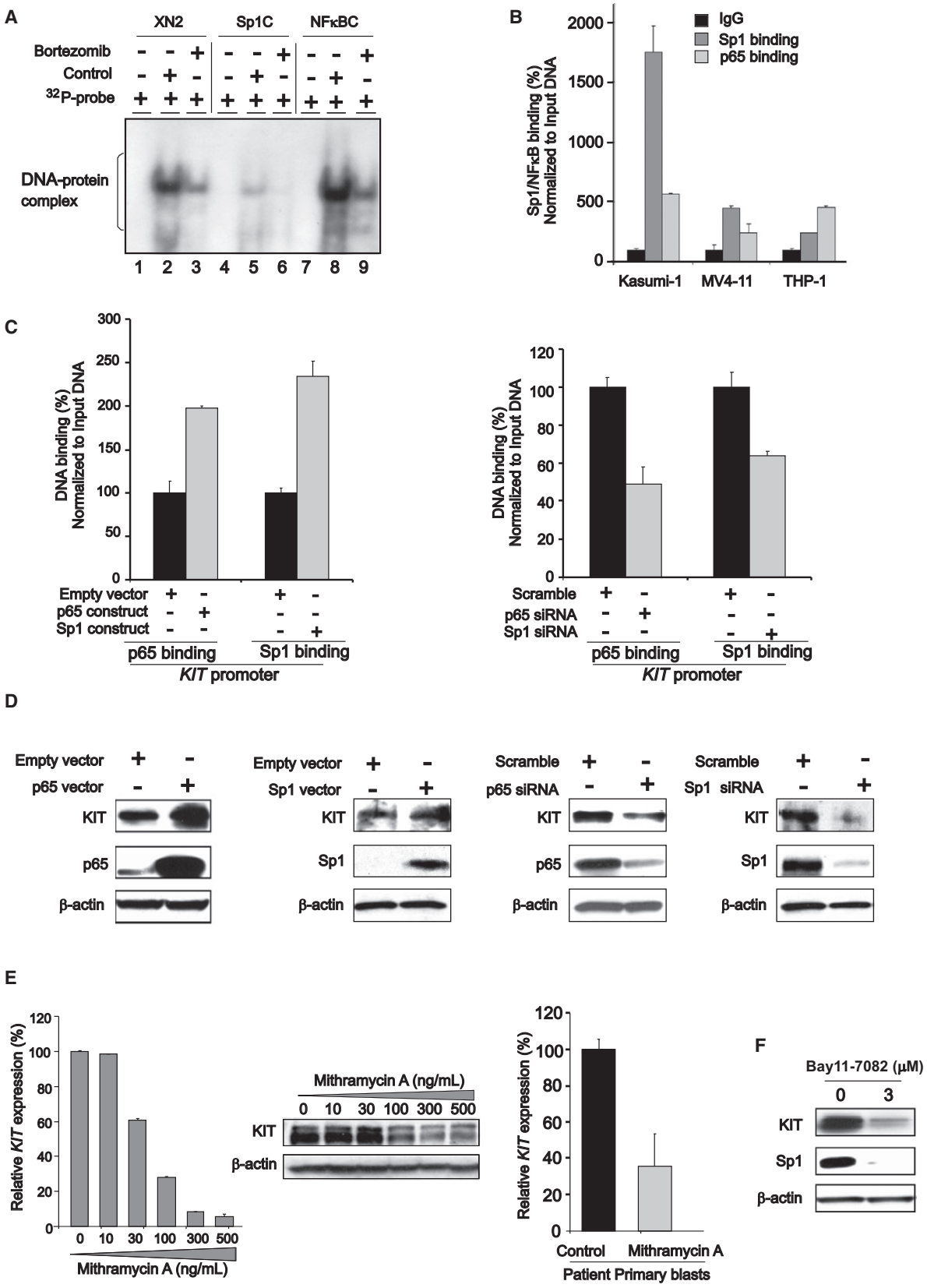
Sp1 expression and functions are, in part, regulated via the 26S proteasome, a common pathway controlling the degradation of a plethora of other survival factors (Karin et al., 2004; Pagano et al., 1995). Activation of NFκB is also controlled by the 26S proteasome (Bargou et al., 1997; Mori et al., 2000). We have also previously reported that the 26S proteasome inhibitor bortezomib interferes with Sp1/NFκB activity (Liu et al., 2008). To further establish the regulatory role of the Sp1/NFκB complex in *KIT* expression, we treated Kasumi-1 cells with bortezomib. The pharmacologic activity of bortezomib was then demonstrated by accumulation of polyubiquitinated proteins indicating adequate proteasome inhibition, concurrent increase in expression of the *Noxa* and *p21* genes, and *miR-29b* occurring prior to any evidence of obvious cytotoxicity (Figures S3A–S3D and S3G). In agreement with recent reports (Hideshima et al., 2009), we also observed that bortezomib resulted in NFκBp65 and IKBα phosphorylation (Figure S3E), thereby suggesting possible activation of the NFκB canonical pathway in Kasumi-1 cells. Concurrent with these changes, however, we also evidenced increase in Sp1 ubiquitination, more diffused Sp1 nucleus/cytoplasm localization, and most importantly disruption of the Sp1 and NFκB physical interaction (Figures S3E and S3F). The latter was likely to abrogate Sp1/NFκB gene transactivating activity thereby leading to bortezomib-induced dose- and time-dependent reduction in *KIT* expression (Figures 3A and 3B; see also Figure S3H) as observed in Kasumi-1 cells and confirmed in primary blasts from three *RUNX1/RUNX1T1*-positive and KIT^{mut} AML patients diagnosed at our institution (Figure 3C). Moreover, we found that bortezomib not only induced KIT downregulation, but also KIT dephosphorylation (Figure 3B) and inhibition of KIT-dependent downstream signaling effectors (Figure 3D). Decreased protein expression and phosphorylation of tyrosine (tyr) or serine (ser) residues of STAT1 (tyr701), STAT3 (tyr705), AKT (ser473), and ERK (tyr204) were observed in Kasumi-1 cells upon exposure to bortezomib (Figure 3E). Hence, these results support a critical role of both Sp1 and NFκB on *KIT* expression and in turn on KIT aberrant kinase activity in leukemia.

MiR-29b Modulates *KIT* Expression by Targeting *Sp1* through an Autoregulatory Loop

Sp1 is a bona fide target of *miR-29b* (Garzon et al., 2009). The clinical relevance of this finding was supported here by the negative linear correlation between Sp1 and *miR-29b* expression levels in *RUNX1/RUNX1T1*–positive AML patients (Spearman's correlation coefficient −0.6; p = 0.016; Figure S4A). Consistent with these results, these patients have high *Sp1/miR-29b* ratio (Figure 4A).

Given that miRNAs are frequently involved in feedback loops where they target the same factors that regulate their expression (Tsang et al., 2007) and Sp1 participates in *KIT* transactivation, we then hypothesized a microcircuitry mechanism where Sp1 represses *miR-29b* transcription and this in turn increases Sp1 at levels sufficient to participate with NFκB in *KIT* transcriptional upregulation. Consistent with our hypothesis, forced *miR-29b* expression in Kasumi-1 cells led to KIT protein downregulation, while decreased *miR-29b* expression by anta*go*miR-29b led to upregulation of KIT (Figure 4B). Furthermore, exposure of Kasumi-1 cells to mithramycin A, bay11-7082 or bortezomib, that interfere respectively with Sp1, NFκB or Sp1/NFκB activities, resulted not only in KIT downregulation (Figures 2E and F; Figure 3A and B), but also in increased *miR-29b* expression (Figure 4C) and in turn downregulation of the *miR-29b* target Sp1 (Figure 4D). Collectively these data suggest that *miR-29b* participates in modulating KIT level by regulating expression of Sp1 and its participation in transcriptional regulation complexes with NFκB. The clinical relevance of the *miR-29b* in *KIT*-driven AML was supported by the observation that *RUNX1/RUNX1T1*–positive patients, who showed worse survival when expressing higher *KIT* levels, tended also to have worse outcome when expressing lower *miR-29* levels (Figure S4B).

Next we focused on dissecting the mechanisms of *miR-29b* transcriptional regulation. We identified three Sp1 and one NFκB binding sites within a 1 kb span of DNA upstream from the 5′ end of the primary transcript of *miR-29b* on human chromosome 7 (using the software package available at www.gene-regulation.com) (Figure 5A). To determine whether a functional interaction occurred between Sp1/NFκB and the *miR-29b* upstream regulatory sequence, we initially performed EMSA assays using probes (see Supplemental Information) spanning the −125/−75 *miR-29b* sequence in K562 cells. These cells were selected because they have high levels of Sp1 while expressing low levels of endogenous *miR-29b* (Garzon et al., 2009). As shown in Figure 5B (left panel, lane 2), the 29BNFκBSp1 probe containing both Sp1 and NFκB binding sites yielded two major complexes (indicated as C1 and C2), suggesting that both Sp1 and NFκB interact with elements of the *miR-29b* enhancer region. The specificity of the protein-DNA binding complexes was demonstrated by their abrogation of binding in the presence of 100-fold excess of unlabeled probes (29BNFκBSp1, Sp1C, or NFκBC containing both or single Sp1/NFκB binding site) (Figure 5B, left panel, lanes 3, 4, and 5), whereas the same-fold excess of an irrelevant oligonucleotide (TFIIAC) containing the TFIIA binding site failed to change the profile of these complexes (Figure 5B, left panel, lane 6). Interestingly, the unlabeled Sp1C probe preferentially decreased the C2 complex, whereas the unlabeled NFκBC probe decreased the C1 complex and eliminated the C2 complex. Similarly, incubation of


A
XN2
Sp1C
NFκBC
Bortezomib
Control
32P-probe
DNA-protein complex
1 2 3 4 5 6 7 8 9
B
IgG
Sp1 binding
p65 binding
Sp1/NFκB binding (%) Normalized to Input DNA
Kasumi-1
MV4-11
THP-1
C
DNA binding (%) Normalized to Input DNA
Empty vector
p65 construct
Sp1 construct
p65 binding
Sp1 binding
KIT promoter
Scramble
p65 siRNA
Sp1 siRNA
D
Empty vector
p65 vector
Sp1 vector
Scramble
p65 siRNA
Sp1 siRNA
KIT
p65
Sp1
β-actin
E
Relative KIT expression (%)
Mithramycin A (ng/mL)
0 10 30 100 300 500
Control
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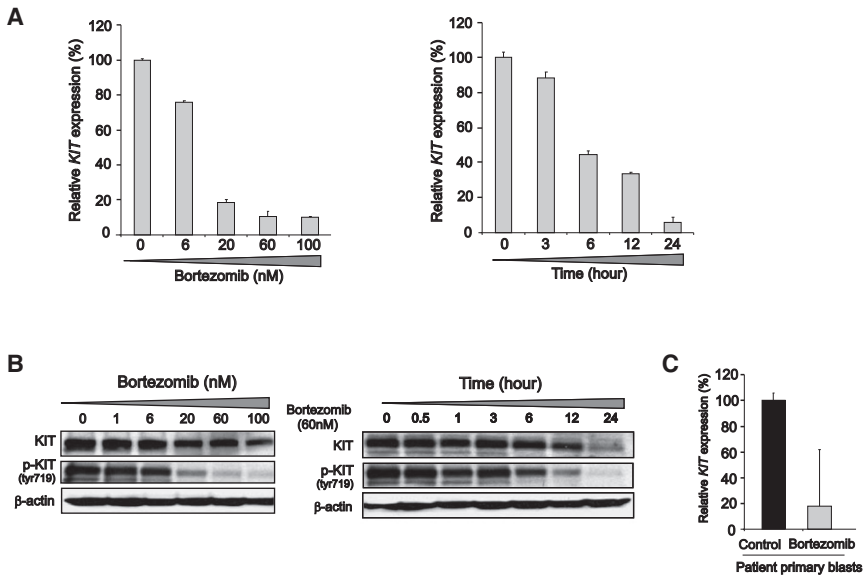
Patient Primary blasts
F
Bay11-7082 (μM)
0 3



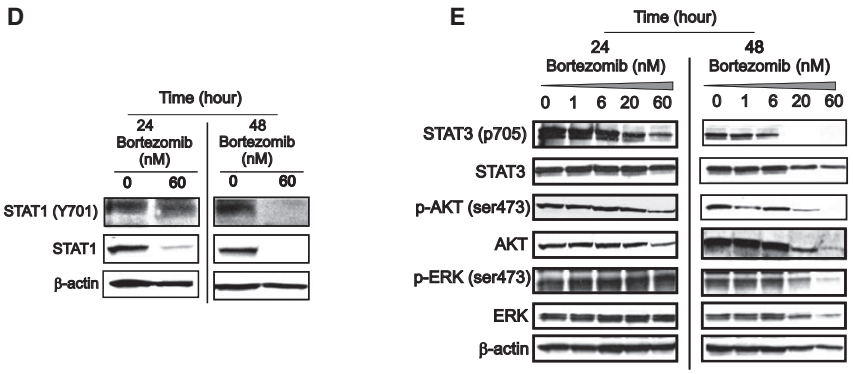


Figure 3. Proteasome Inhibition by Bortezomib Impairs KIT Expression and Its Downstream Signaling Pathway

(A and B) Dose-dependent (left) and time-dependent (right) reduction of *KIT* RNA and protein expression and KIT protein phosphorylation in tyrosine (tyr) 719 residue in Kasumi-1 cells incubated with bortezomib (mean ± SEM).

(C) Inhibitory effect of bortezomib on *KIT* mRNA expression was evaluated using qRT-PCR in AML blasts from three patients with mutated KIT^{mut} t(8;21) AML treated with 60 nM bortezomib for 24 hr (mean and standard deviation [± SD]).

(D and E) Immunoblotting analysis demonstrated the downregulation of KIT downstream effectors such as STAT1 (D), STAT3, AKT, and ERK (E) in Kasumi-1 cells treated with bortezomib. p, phosphorylated. Data are representative of three independent experiments. (See also Figure S3).

extracts with Sp1 antibody decreased the C1 complex and eliminated the C2 complex, whereas antibody to NFκBp65 decreased the intensity of both complexes (Figure 5B, right panel). These data suggest that the C1 and C2 complexes contained both Sp1 and NFκBp65, probably with different stoichiometry; C1 is likely to contain less Sp1. Similar results were attained in Kasumi-1 cells (data not shown).

Next, we used gain- and loss-of-function assays to show that forced expression of Sp1 or NFκB(p65) reduced *miR-29b* in MV4-11, an AML cell line with relatively high endogenous *miR-29b* levels (Garzon et al., 2009), or Kasumi-1 cells (Figure 5C, left and middle panels). Conversely, Sp1 or NFκB(p65) knockdown by siRNAs resulted in *miR-29b* upregulation in K562 cells that have barely detectable levels of endogenous *miR-29b* (Garzon et al., 2009) (Figure 5C, right panel). Consistent with these results, bortezomib treatment reduced the binding of Sp1/NFκB complex to *miR-29b* regulatory elements (Figure 5D), thereby resulting in *miR-29b* re-expression (Figure 4C) and Sp1 reduction (Figure 4D). Notably, ectopic *miR-29b* expression disrupted Sp1 binding to the *miR-29b* enhancer region (Figure 5E) through abrogation of Sp1 protein and disruption of Sp1/NFκB DNA binding as confirmed by antibody supershift (Figure S4C), thereby closing the *miR-29b*/Sp1 autoregulatory loop. Interestingly, ectopic expression of a synthetic mature *miR-29b* in K562 cells resulted in an increase of the endogenous *miR-29b* precursor (Figure 5F), thereby further supporting *miR-29b* as an active participant in its own transcriptional regulation.

Figure 2. The Regulatory Role of Sp1/NFκB in *KIT* Expression

(A) Sp1/NFκB complex is present on *KIT* promoter. EMSA was performed with nuclear extracts from Kasumi-1 cells incubated with ^{32}P-labeled double-stranded oligonucleotides containing Sp1/NFκB binding elements on the *KIT* promoter region from nucleotides −102/−82 (XN2) or Sp1 consensus binding sites (Sp1C) or NFκB consensus binding sites (NFκBC). Lanes 1, 4, and 7, free ^{32}P-labeled probes; lanes 2, 5, and 8, control (untreated) cells; lanes 3, 6, and 9, bortezomib-treated cells.

(B) Chromatin immunoprecipitation (ChIP) assays to demonstrate Sp1/NFκB on *KIT* gene promoter in KIT^{mut} Kasumi-1 and KIT^{wt} MV4-11 and THP1 cells (mean and standard error of the mean [± SEM]).

(C) ChIP assays to show Sp1/NFκB enrichment on *KIT* promoter in Kasumi-1 cells transfected with NFκB or Sp1 overexpression vector (left panel) or siRNAs (right panel) (mean ± SEM).

(D) Sp1, NFκB, and KIT protein expression in Kasumi-1 cells transfected with corresponding overexpression vector (left panel) or siRNA (right panel).

(E) Sp1 inhibition by mithramycin A impaired KIT RNA transcription and protein expression in Kasumi-1 cells (left and middle panels) or patient primary blasts (right panel) (mean ± SEM).

(F) NFκB inhibitor bay11-7082 (3 μM) decreased *KIT* expression in Kasumi-1 cells. Data are representative of three independent experiments. (See also Figure S2).

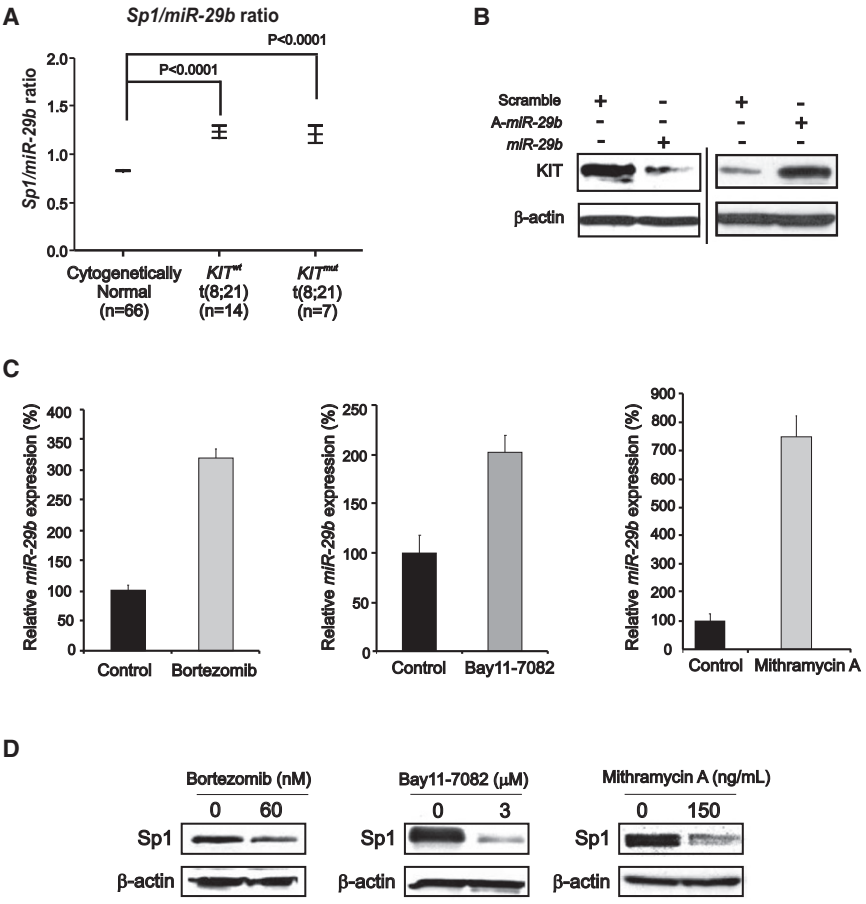


Figure 4. Role of *miR-29b* in *KIT* Expression Regulation

(A) *Sp1/miR-29b* expression ratio (measured by microarray) in bone marrow samples from Cancer and Leukemia Group B *RUNX1/RUNX1T1*-positive patients with cytogenetically normal AML and KIT^{mut} or KIT^{wt}.

(B) Changes in *miR-29b* expression and KIT protein levels in Kasumi-1 cells transfected with *miR-29b* or antago*miR-29b (A-miR-29b)* for 72 hr.

(C) Upregulation of *miR-29b* in Kasumi-1 cells treated with bortezomib (60 nM) or bay11-7082 (3 μM) or mithramycin A (150 ng/ml) for 6 hr. qRT-PCR analysis of *miR-29b*, normalized by U44, was performed (mean ± SEM).

(D) Immunoblotting analysis showing reduction of Sp1 protein in Kasumi-1 cells treated with bortezomib or bay11-7082 or mithramycin A. Data are representative of three independent experiments.

Histone Deacetylases Contribute to the Repressor Activity of Sp1/NFκB on *miR-29b*

Having shown that Sp1/NFκB acts as a repressive complex for *miR-29b* and as an activating complex for *KIT* expression, next we asked whether other factors could participate in conferring differentiating regulatory functions to this complex. Although Sp1/NFκB is involved in the regulation of DNA hypermethylation (Liu et al., 2008), we observed only few CpG islands and no obvious DNA methylation of the 5′ putative regulatory region of *miR-29b* in either AML patient samples or cell lines with low expression of this miRNA (G.M., unpublished data). Therefore, we postulated that epigenetic mechanisms causing chromatin changes other than DNA hypermethylation could be involved in silencing *miR-29b*. A number of previous studies showed that Sp1/NFκB physically interacts with histone deacetylases (HDACs) 1 and 3 to repress target gene transcription (Doetzlhofer et al., 1999). Therefore, in order to test whether HDAC1 and 3 associate with Sp1/NFκB to repress *miR-29b* expression, we incubated ^{32}P-labeled probes designed from the *KIT* promoter or *miR-29b* regulatory regions with recombinant NFκBp50/p65 proteins to form a DNA-protein complex (Figure 6A, left panel, lanes 2–10). Recombinant Sp1 (Figure 6A, left panel, lanes 3 or 8), HDAC1 (Figure 6A, left panel, lanes 4 or 9) or HDAC3 (Figure 6A, left panel, lanes 5 or 10) proteins were then added. No obvious alterations of the DNA-protein complex were observed in the *KIT* promoter indicating that HDAC1 and 3 (Figure 6A, lanes 4 and 5) were not part of the Sp1/NFκB complex. In contrast, in the *miR-29b* regulatory sequence, we observed delayed and more intense bands after the addition of recombinant HDACs (Figure 6A, left panel, lanes 9 and 10) (indicated by arrow) supporting the interaction of HDAC1 and 3 and Sp1/NFκB within the *miR-29b* regulatory sequence. The enrichment of HDACs and Sp1/NFκB on the *miR-29b* regulatory sequences was further confirmed by ChIP (Figure 6A, right panel). Preferential HDAC binding on *miR-29b* with respect to the *KIT* promoter was also confirmed in Kasumi-1 cells (Figure 6B).

The biological function of HDACs in *miR-29b* regulation was further supported by the observation that HDAC1 siRNA knockout or ectopic expression resulted respectively in higher and lower *miR-29b* expression (Figure 6C). Accordingly, treatment with the HDAC inhibitor OSU-HDAC42 (Sargeant et al., 2008) resulted in an increase of *miR-29b* transcription (Figure 6D) with concurrent reduction of both Sp1 and KIT RNA and protein expression (Figure 6E). Similar results were attained with another HDAC inhibitor, MS275 (Figure S5). Conversely, ectopic HDAC1 expression resulted in Sp1 and KIT upregulation (Figure 6E). Consistent with these data, we also observed that HDAC inhibitors induced a relative decrease of HDAC1 and 3 enrichment and increase in histone acetylation in the *miR-29b* enhancer region (Figure 6F, left panel). The decreased binding of HDACs on the *miR-29b* enhancer region was likely due to the disruption of the Sp1/HDAC physical interaction by the HDAC inhibitors (Figure 6F, right panel).

KIT Autoregulatory Loop

Because *KIT* expression levels are relatively high in cells harboring gain-of-function mutations, we next examined whether aberrant KIT activation may feed back to regulate its own transcription through the Sp1/NFκB/*miR-29b* network. Previous studies reported that KIT^{mut} induces wnt pathway signaling and

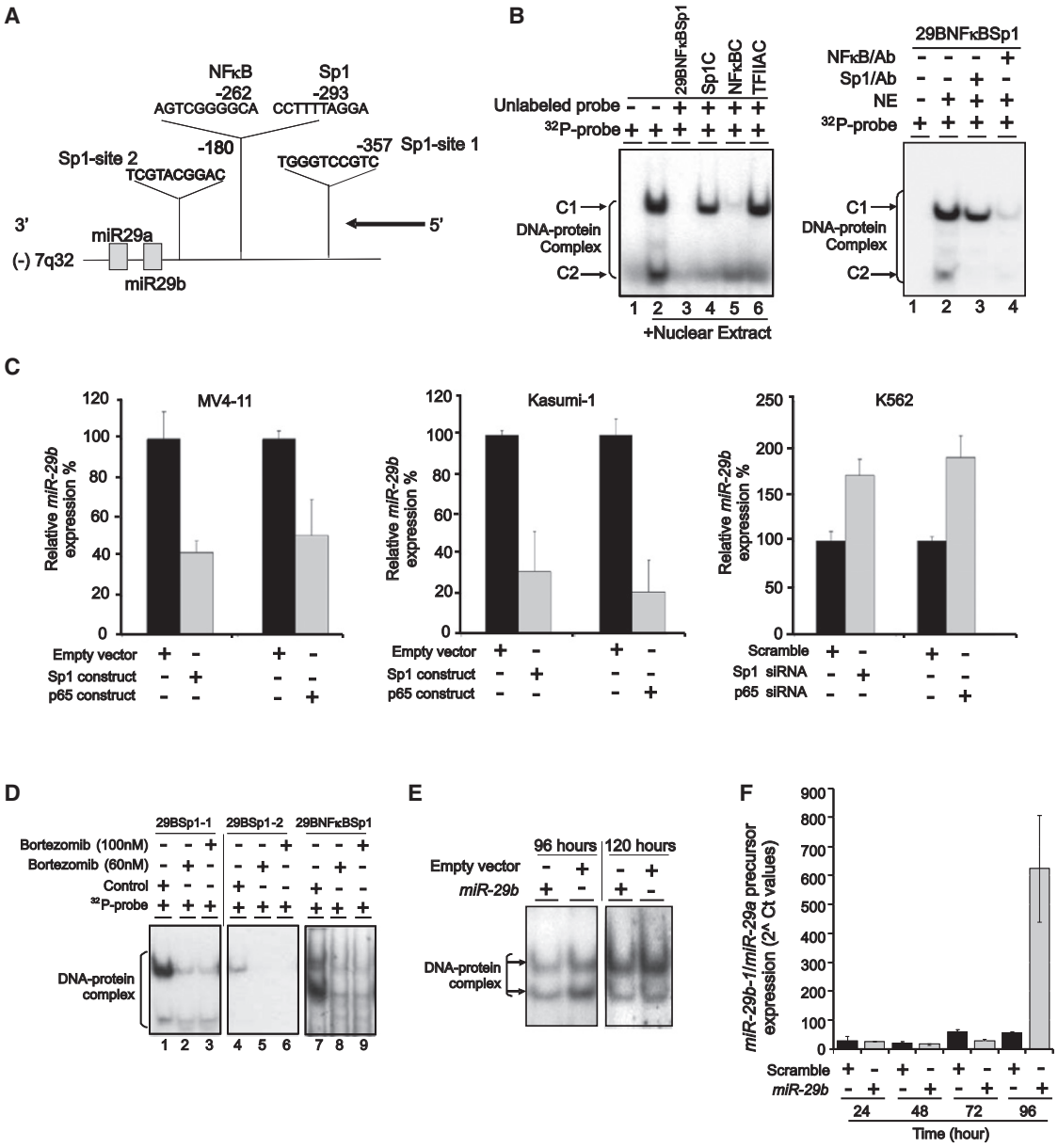


Figure 5. Regulation of *miR-29b* Transcription

(A) Schematic diagram showing the location of Sp1 and NFκB binding sites on *miR-29b*-1 regulatory region on chromosome 7.

(B) EMSA demonstrated that Sp1/NFκB complex was present on the *miR-29b* regulatory region. Kasumi-1 nuclear extract incubated with ^{32}P-29bNFκBSp1 probe containing NFκB and Sp1 binding sites yielded two DNA-protein complexes C1 and C2 (lane 2). The specificity of DNA binding was demonstrated by the abolishment or reduction of both complexes with excess (100x) unlabeled 29BNFκBSp1 (lane 3) or Sp1 consensus binding site (Sp1C, lane 4) or NFκB consensus binding site (NFκBC, lane 5) probes, but not with an irrelevant competitor probe that contains the TFIIA binding site (lane 6). The presence of NFκB and Sp1 in the DNA-protein complexes was demonstrated by antibody supershift assay (right panel).

(C) Changes in Sp1, NFκB, and *miR-29b* levels in MV4-11, Kasumi-1 or K562 cell lines transfected with Sp1 or NFκB overexpression vector or siRNA (mean ± SEM).

(D) EMSA showed that bortezomib treatment diminished the binding of Sp1/NFκB complex to *miR-29b* regulatory region in Kasumi-1 cells. Control, untreated cells.

(E and F) *miR-29b* regulated its own transcription. Ectopic *miR-29b* expression dissociated Sp1 binding from its own regulatory region by EMSA (E) and synthetic mature *miR-29b* enhanced endogenous *miR-29b* precursor level (F) following 96 hr from initial treatment (mean ± SEM). Data (B–F) are representative of three independent experiments. (See also Figure S4).

MYC expression (Tickenbrock et al., 2008). The latter, in turn, was shown to downregulate *miR-29b* expression (Chang et al., 2008). Therefore, we postulated that KIT protein activity would drive aberrant *KIT* gene expression by inducing *MYC*-dependent *miR-29b* downregulation. We validated our hypothesis by showing that treatment with KIT ligand resulted in increase in *KIT* and

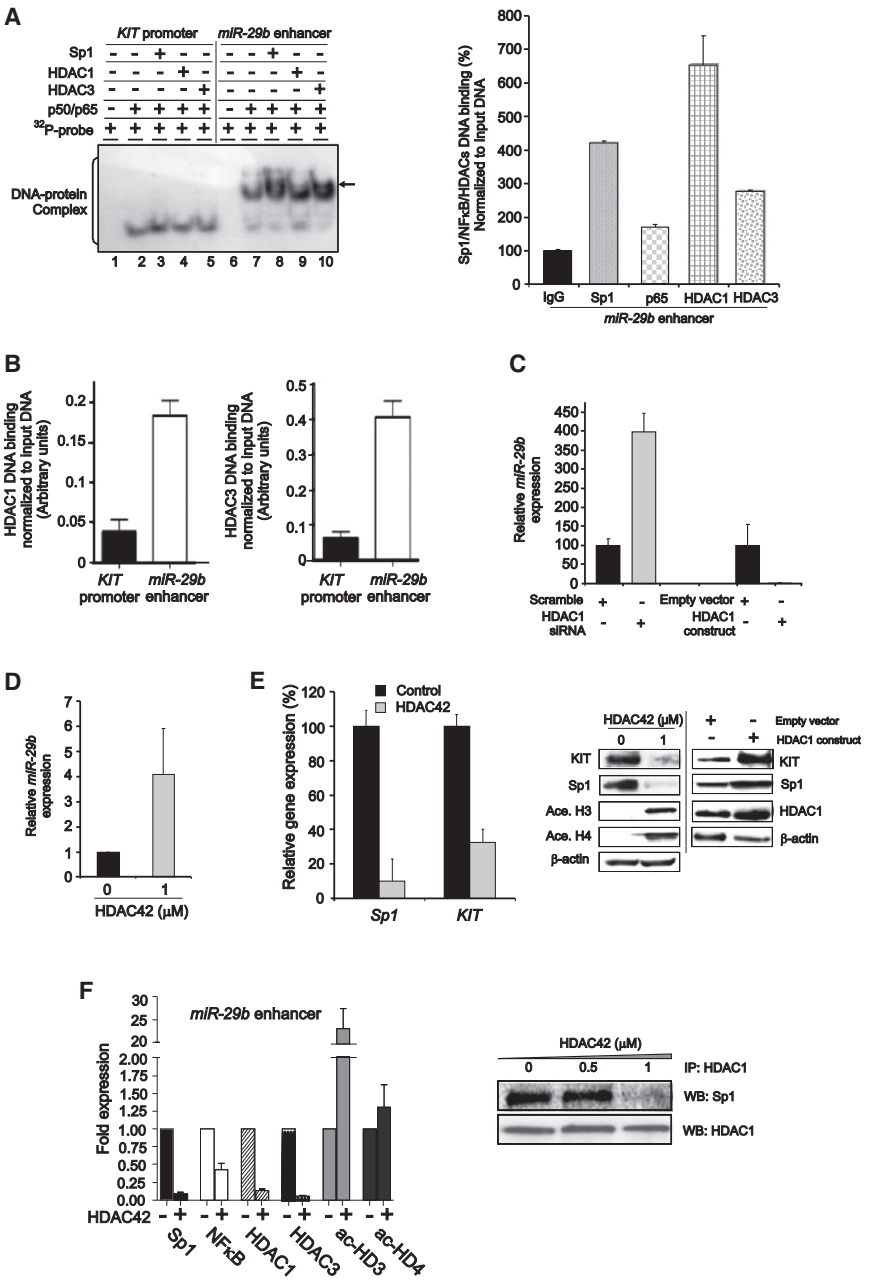


Figure 6. HDACs Participate in the Sp1/NFκB Complex to Inhibit *miR-29b* Expression

(A) Using recombinant proteins, EMSA (left panel) demonstrated the association of HDACs with Sp1/NFκB on *miR-29b* regulatory region, which was confirmed by ChIP (right panel) (mean ± SEM). ^{32}P-labeled double-stranded oligonucleotides containing Sp1 and NFκB consensus sites from *KIT* promoter or *miR-29b* regulatory regions were incubated with recombinant proteins NFκBp50 and p65, and supplemented with recombinant proteins Sp1 (lanes 3 and 8), HDAC1 (lanes 4 or 9), or HDAC3 (lanes 5 or 10). Additional complexes seen only with *miR-29b* probe were indicated with arrow. In right panel, ChIP assays showed that Sp1/NFκB/HDACs were enriched on *miR-29b* enhancer.

(B) ChIP assays showed that HDAC1 and HDAC3 had higher DNA binding affinity on *miR-29b* than *KIT* regulatory element (mean ± SEM).

(C) *miR-29b* transcription inversely related to the level of HDAC1 in Kasumi-1 cells transfected with HDAC1 siRNA or overexpression construct (mean ± SEM).

(D) HDAC inhibitor (HDAC42) enhanced *miR-29b* transcription determined by qRT-PCR (mean ± SEM).

(E) HDAC inhibition by HDAC42 concurrently reduced *Sp1* and *KIT* RNA (left panel) (mean ± SEM) or protein (middle panel) expression in Kasumi-1 cells. Conversely, HDAC1 overexpression increased Sp1 and KIT level (right panel).

(F) HDAC inhibition by HDAC42 abrogated Sp1/NFκB/HDAC repressor complex. In left panel, ChIP assays demonstrated that the disruption of Sp1/NFκB/HDAC complex and the accumulation of acetylated histone H3 (ac-HD3) and H4 (ac-HD4) on *miR-29b* regulatory region (mean ± SEM). In right panel, coimmunoprecipitation showed that HDAC42 disrupted Sp1/HDAC1 interaction. Data are representative of three independent experiments. (See also Figure S5).

MYC and decrease in *miR-29b* in THP-1 cells, which, when unstimulated, express relatively low KIT levels and higher *miR-29b* levels (Figure 7A). Similarly, overexpression of *KIT*^{mut} or *KIT*^{wt} in THP-1 cells resulted in *MYC* upregulation and *miR-29b* downregulation (Figure 7B). Finally, ectopic expression of *MYC* resulted in downregulation of *miR-29b* (Figure 7C, left panel) and upregulation of the *miR-29b* target *Sp1* (Figure 7C, middle panel), thereby resulting in higher levels of KIT expression in Kasumi-1 and MV4-11 cells (Figure 7C, right panel).

For further determining the biologic role of KIT protein abundance, *KIT* expression was knocked out by siRNA in Kasumi-1 cells. We observed *miR-29b* upregulation and Sp1 downregulation (Figure 7D), decrease of the Sp1/NFκB complex binding to the *KIT* promoter as demonstrated by EMSA assays (Figure 7E) and significant antileukemic activity in Kasumi-1 cells (Figures S6A–S6E). Finally, we demonstrated that bortezomib treatment also led to a decrease in MYC protein expression (Figure 7F). Altogether, these results support that MYC-induced *miR-29b* downregulation, occurring upon activation of the KIT protein in leukemia cells, leads to the *KIT* gene overexpression through the Sp1/NFκB/HDAC/*miR-29b* network. A summary diagram that outlines the above regulatory network is described in Figure 7G.

Treatment with Bortezomib Suppresses In Vivo *KIT*-Driven Leukemogenesis

Having demonstrated the relevance of the Sp1/NFκB/*miR-29b* feedback loop on *KIT* regulation, we next tested whether this loop represented a potentially viable therapeutic target to

overcome KIT-driven leukemia in vivo. We cloned D816V KIT^{mut} or KIT^{wt} into pBABE-puro retroviral vector and stably expressed these constructs in the FDC-P1 cell line, a murine nontumorigenic diploid cell line derived from myeloid precursors. In in vitro studies, we observed that overexpression of either KIT^{mut} or KIT^{wt} promoted cell proliferation determined by clonogenic assay, albeit more pronounced effects were attained with KIT^{mut} (Figure S7A). In order to investigate the leukemic role of KIT protein in vivo, FDC-P1/KIT^{mut} cells (5×10^6/mouse) were then engrafted into NOD/SCID mice, which developed significant splenomegaly (Figure 8A) and died from a leukemia-like illness within 4 weeks. In contrast, no evidence of disease was observed in empty-vector transfected FDC-P1 parental cells. Western blot confirmed KIT expression in the enlarged spleen of FDC-P1/KIT^{mut} engrafted mice (Figure 8A).

Additional in vivo experiments were performed to demonstrate the potential therapeutic relevance of KIT downregulation. We selected bortezomib among the different compounds that we showed to interfere with the Sp1/NFκB/*miR-29b* regulatory loop, as this compound targets Sp1/NFκB complex, upregulates *miR-29b* and is an FDA-approved anticancer drug. Sp1/NFκB binding sites were found by computational methods (http://www.cbrc.jp/research/db/TFSEARCH.html) in the promoter region of the pBABE vector carrying KIT^{mut} and used to transfect FDC-P1 cells (not shown). In vitro, bortezomib treatment inhibited proliferation (Figure 8B, left panel) and decreased clonogenic activity (Figure 8B, right panel) of FDC-P1/KIT^{mut} cells. These effects were associated with Sp1 and KIT protein downregulation, KIT protein hypophosphorylation, and *miR-29b* upregulation (Figure 8C). In contrast, forced Sp1 or NFκB(p65) expression enhanced mutated *KIT* (Figure 8D, left panel) and ectopic *miR-29b* expression inhibited colony-forming ability in FDC-P1/KIT^{mut} cells (Figure 8D, right panel). These findings therefore supported the relevance of the Sp1/NFκB/*miR-29b* regulatory complex to *KIT* expression and the pharmacologic activity of bortezomib in FDC-P1/KIT^{mut} cells, thereby validating FDC-P1/KIT^{mut} engrafted mice as a suitable in vivo model for KIT^{mut}-driven leukemia. Similar results were also achieved in FDC-P1/KIT^{wt} cells exposed to bortezomib (Figures S7B and S7C).

NOD/SCID mice engrafted with FDC-P1/KIT^{mut} cells were then treated with one dose bortezomib (1 mg/kg/dose) and sacrificed 48 hr later. We observed that bortezomib abrogated *KIT* mRNA transcription and protein expression and increased *miR-29b* expression in vivo (Figure 8E). The role of *miR-29b* upregulation as a potential key step in the therapeutic response of KIT-driven leukemia to bortezomib was further supported by a decreased engraftment efficiency of FDC-P1/KIT^{mut} cells transfected with synthetic *miR-29b*. The size and weight of murine FDC-P1/KIT^{mut} tumors from cells pretreated with *miR-29b* was significantly lower than those of cells pretreated with vehicle alone or scrambled miRNA when measured at day 21 after engraftment (Figure 8F and Figure S7D).

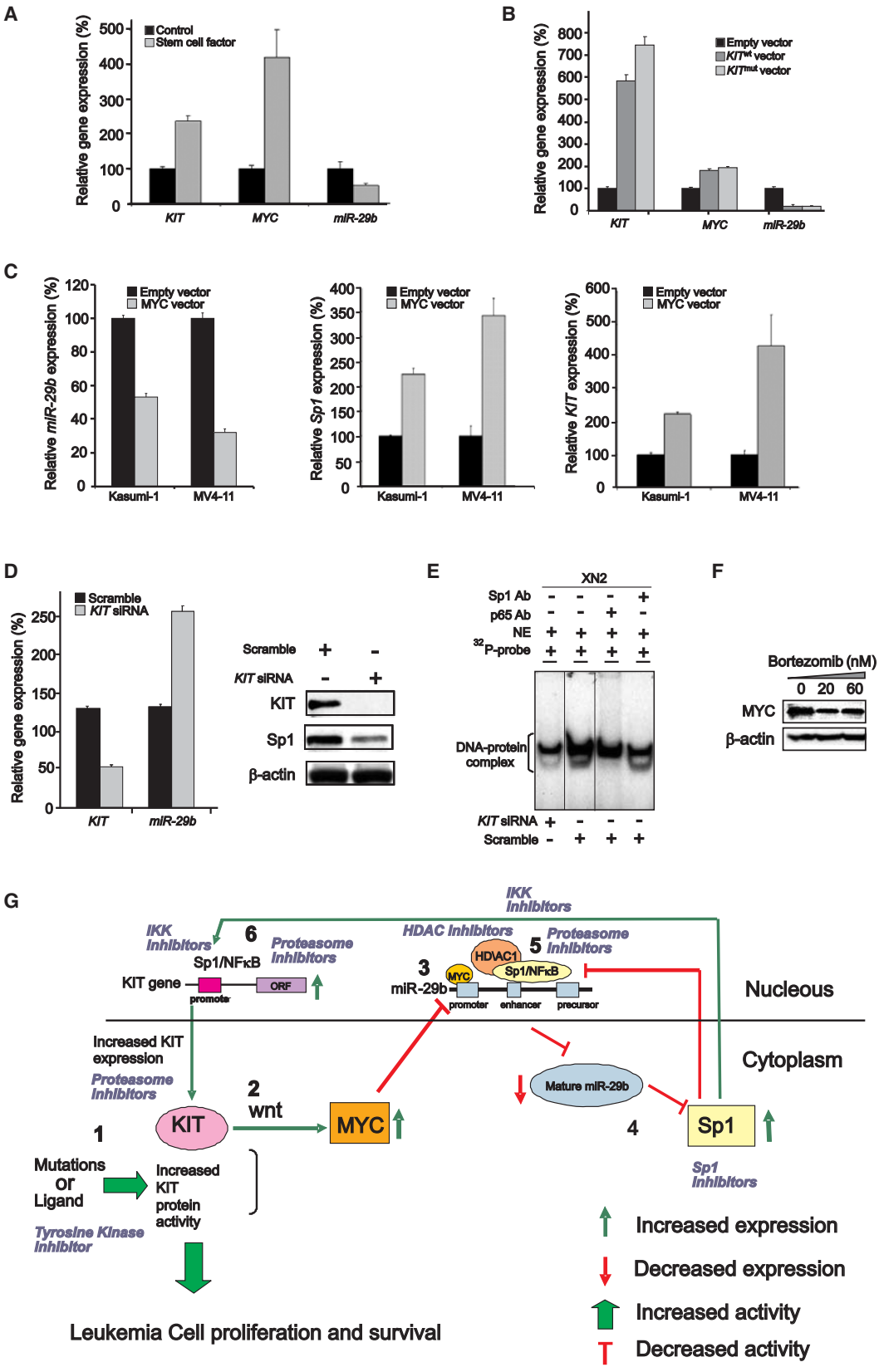
Next, FDC-P1/KIT^{mut}-engrafted mice were treated with 1 mg/kg bortezomib once or twice weekly for 3 weeks, starting at day 21 after engraftment (n = 5 mice/group), and then followed longitudinally. Animals treated with bortezomib demonstrated significantly longer periods of survivals than vehicle-treated controls (Figure 8G). Vehicle-treated FDC-P1/KIT^{mut} engrafted mice exhibited massive splenomegaly, whereas spleen size and weight of the bortezomib-treated animals were similar to those of age-matched controls (Figure 8H). Cytospins of bone marrow cells and histopathology of spleen and liver sections from FDC-P1/KIT^{mut}-engrafted mice treated with vehicle showed extensive infiltration of blast cells. In contrast, cytospins of bone marrow cells and histopathology of spleen and liver from the bortezomib-treated leukemic mice were similar to that of the age-matched control groups (Figure 8I).

To validate these in vivo data in a model where *KIT* expression is controlled via an endogenous promoter, we next established murine xenografts with the human mastocytosis HMC-1 cell line carrying KIT^{mut}. These cells were sensitive in vitro to bortezomib treatment which induced *miR-29b* upregulation, and Sp1 and KIT downregulation (Figures S7E and S7F). NOD/SCID mice engrafted with 1×10^7 HMC-1 cells subcutaneously received intratumor administration of 1 mg/kg bortezomib twice a week for 2 weeks starting from when the tumor size approached 20 mm^3. Significant decrease in tumor size was observed in bortezomib-treated mice when compared with vehicle-treated controls (Figure 8J). Similarly, bortezomib was therapeutically advantageous in mice engrafted with ME-1 cells overexpressing KIT^{wt} (Figure S7G). Collectively, these results indicate that KIT overexpression significantly contributes to malignant cell proliferation, and targeting KIT abundance through the miRNA-protein network represents a promising therapeutic approach to overcome KIT-driven leukemia.

DISCUSSION

Previous studies revealed that certain human cancers including AML are characterized by aberrant KIT tyrosine kinase activity (Beadling et al., 2008; Went et al., 2004). To date, much effort has been focused on targeting aberrantly activated KIT mutants using TK inhibitors. Although treatment with these compounds can induce clinical responses in both solid tumors and hematologic malignancies harboring *KIT* mutations (Heinrich et al., 2008), this strategy is complicated by the needs for adjustment of therapy based on individual *KIT* genotypes and early onset of treatment resistance due to acquired secondary mutations or/and KIT overexpression. Here we show that aberrantly activated KIT protein itself may drive upregulation of the *KIT* gene, and high *KIT* expression is an important contributor to malignant cell proliferation and aggressive disease. Our findings therefore support the rationale for therapeutic targeting of KIT abundance to overcome aberrant KIT activity and induce significant antileukemic effects. The current study was designed to investigate mechanisms that regulate *KIT* expression, so that treatment strategies attacking directly *KIT* gene deregulators in leukemia can be developed to circumvent the drawbacks encountered with TK inhibitor therapy. Our investigation indeed led to the identification of a Sp1/NFκB/HDAC/*miR-29b* network that deregulates *KIT* gene transcription, that impacts leukemogenesis, and that is targetable pharmacologically.

Previous investigations reported that *miR-221/222* directly target *KIT* expression (Felli et al., 2005). Here, we provide the first evidence of an indirect but pivotal role of *miR-29b* in modulating *KIT* expression in KIT^{mut} leukemia. By using computational analyses we found lack of *miR-29b* binding sites in *KIT* mRNA 3′UTR.


A
Relative gene expression (%)
Control
Stem cell factor
KIT
MYC
miR-29b
B
Relative gene expression (%)
Empty vector
KITwt vector
KITmut vector
KIT
MYC
miR-29b
C
Relative miR-29b expression (%)
Empty vector
MYC vector
Kasumi-1
MV4-11
Relative Sp1 expression (%)
Relative KIT expression (%)
D
Relative gene expression (%)
Scramble
KIT siRNA
Scramble
KIT siRNA
KIT
Sp1
β-actin
E
XN2
Sp1 Ab
p65 Ab
NE
32P-probe
DNA-protein complex
KIT siRNA
Scramble
F
Bortezomib (nM)
0 20 60
MYC
β-actin
G
IKK Inhibitors
HDAC Inhibitors
Proteasome Inhibitors
Sp1/NFκB
KIT gene
ORF
promoter
HDAC1
MYC
miR-29b
promoter
enhancer
precursor
Nucleous
Cytoplasm
Increased KIT expression
Mature miR-29b
KIT
wnt
MYC
Sp1
Mutations or Ligand
Increased KIT protein activity
Tyrosine Kinase Inhibitor
Sp1 Inhibitors
Leukemia Cell proliferation and survival
Increased expression
Decreased expression
Increased activity
Decreased activity

However, treatment with ectopic *miR-29b* or compounds that led to increase in endogenous *miR-29b* resulted in *KIT* downregulation. We showed that this was due to an indirect effect on *KIT* expression mediated by a *miR-29b*/Sp1 mutual feedback loop. Sp1, a transactivator of the *KIT* gene, binds to the *miR-29b* regulatory elements and represses *miR-29b* expression, whereas *miR-29b* blocks Sp1 translation and in turn upregulates its own transcription. NFκB, a transcription factor that is in part modulated by the 26S proteasome system and is constitutively activated in AML, physically interacts with Sp1 to regulate *miR-29b* and *KIT* expression. HDACs confer transcription repressing activity to the Sp1/NFκB complex binding the *miR-29b* regulatory elements in leukemia cells, but do not participate in the Sp1/NFκB complex that binds and transactivates the *KIT* promoter. Thus, when *miR-29b* is aberrantly suppressed by a Sp1/NFκB/HDAC complex in KIT^{mut} leukemia, KIT^{mut} becomes upregulated, thereby contributing to malignant proliferation. But what is the primary event deregulating this miRNA-protein network? We showed that gain-of-function mutations or aberrant ligand-dependent activation of the KIT protein in leukemia cells lead to constitutive *MYC* upregulation, which is likely to produce the initial step for decreasing *miR-29b* below a threshold that results in Sp1 increase, aberrantly high levels of Sp1/NFκB/HDAC activity, and ultimately KIT upregulation. The latter perpetuates autoregulatory loops that minimize *miR-29b* expression and maximize KIT expression and activation in leukemia cells.

Pharmacologic intervention with synthetic *miR-29b* oligonucleotides or compounds that inhibit proteasome (bortezomib), NFκB (bay11-7082), Sp1 (mithramycin A), and HDACs (HDAC42), targets the Sp1/NFκB/HDAC complex in leukemia cells, and sequentially results in endogenous *miR-29b* upregulation, *Sp1* downregulation, disruption of the Sp1/NFκB complexes, and inhibition of the *KIT* gene. The net results are *KIT* downregulation, inhibition of aberrant TK activity, and arrest of leukemia growth. The pivotal role of *miR-29b* in this miRNA/protein network is supported by upregulation or downregulation of *KIT* expression in response to repression of endogenous *miR-29b* or forced expression of ectopic *miR-29b*, respectively. This was further confirmed by showing that ectopic *miR-29b* expression inhibited the colony-forming ability and in vivo growth of KIT-driven leukemia cells (FDC-P1/KIT^{mut} cells).

Sp1 and NFκB are ubiquitous transcription factors and are overexpressed in human malignancies. We and others demonstrated that Sp1 physically interacts with NFκB to enhance target gene transactivation (Hirano et al., 1998; Liu et al., 2008). Here, we showed that, like *miR-29b*, these two factors are located at a central position within a regulatory network controlling *KIT* expression. The proteasome inhibitor bortezomib, which effectively interferes with the activity of Sp1/NFκB complex at concentrations (i.e., 60 nM) that are achievable in patients treated at the recommended dose of the drug (Quinn et al., 2009), was then chosen to test the therapeutic relevance of targeting *KIT* expression in KIT^{mut} leukemia. The intended in vivo target for this compound was the Sp1/NFκB/HDAC/*miR-29b* network. Our data indeed indicated that bortezomib disrupts both Sp1/NFκB and Sp1/NFκB/HDAC complexes, thereby resulting in *miR-29b* upregulation, Sp1 downregulation, and inhibition of the *KIT* gene transactivation. These events ultimately result in strong antileukemic activity and improved survival in NOD/SCID mice that were engrafted with FDC-P1/KIT^{mut} cells. Similar results were also attained in mice xenografted with malignant cells overexpressing *KIT* under the control of an endogenous promoter. Thus, bortezomib appears to be a potentially effective treatment for *KIT*-driven leukemia, despite that it is not predicted by computer-modeling to bind to the same KIT enzymatic pocket where interaction with PKC412, imatinib, or other tyrosine kinase inhibitor small molecules occurs (not shown).

In conclusion, our investigation has identified a critical regulatory Sp1/NFκB/HDAC/*miR-29b* network that modulates *KIT* expression. We show that aberrant activation of *KIT* results in MYC-dependent *miR-29b* downregulation and an increase in Sp1 expression. The latter interacts with NFκB and HDACs to further inhibit *miR-29b* expression, and with NFκB alone to transactivate *KIT*. Because of the central role of Sp1/NFκB complex in mechanisms of *KIT* dysregulation, proteasome inhibition appears particularly advantageous to target therapeutically this network. Similar pharmacologic effects can be also achieved through inhibition of NFκB (by bay11-7082), Sp1 (by mithramycin A), HDAC1/3 (by HDAC42), or addition of *miR-29b*. Notably, our previous reports show that *miR-29b* controls the expression of DNA methyltransferases and restores epigenetically silenced gene expression and cell differentiation patterns in AML blasts displaying DNA hypermethylation (Liu et al., 2008; Garzon et al., 2009). Therefore, therapeutic targeting of the Sp1/NFκB/HDAC/*miR-29b* network may lead to control not only of *KIT*, but also of other aberrantly expressed oncogenes (i.e., DNMTs) that, though not directly involved in regulation of *KIT* expression, may play an equally relevant role in leukemogenesis. Importantly, many of the pharmacologic agents that we have used to target *KIT* expression are already in the clinic. Thus, we believe that an attractive aspect of our study points to the possibility of rapidly translating our findings into clinical

Figure 7. Role of Activated KIT in *KIT* Gene Transcription

(A) Treatment with KIT ligand (stem cell factor) induced *KIT* and *MYC* upregulation and *miR-29b* downregulation in THP-1 cells harboring KIT^{wt} (mean ± SEM).
(B) Overexpression of KIT^{mut} or KIT^{wt} increased *MYC* expression and decreased *miR-29b* downregulation in THP-1 cells (mean ± SEM).
(C) *MYC* overexpression increased *KIT* and *Sp1* transcription and decreased *miR-29b* expression in KIT^{mut} Kasumi-1 and KIT^{wt} MV4-11 cells (mean ± SEM).
(D) *KIT* knockout by siRNA enhanced *miR-29b* expression leading to Sp1 downregulation in Kasumi-1 cells transfected with *KIT* siRNA (mean ± SEM).
(E) EMSA demonstrated that siRNA-induced KIT knockout decreased Sp1/NFκB binding affinity on its own promoter. The presence of NFκB and Sp1 in the DNA-protein complexes was demonstrated by the abolishment or reduction of complexes with antibody supershift assay. Note, the inserted lines indicate the reposition of the gel.
(F) MYC protein expression is suppressed in Kasumi-1 cells treated with bortezomib for 24 hr.
(G) Summary diagram describes the Sp1/NFκB/HDAC/*miR-29b* network that regulates *KIT* expression. Indicated are also sites of potential therapeutic interventions within the network that may result in the inhibition of KIT expression thereby its activity. Data (A-F) are representative of three independent experiments. (See also Figure S6).

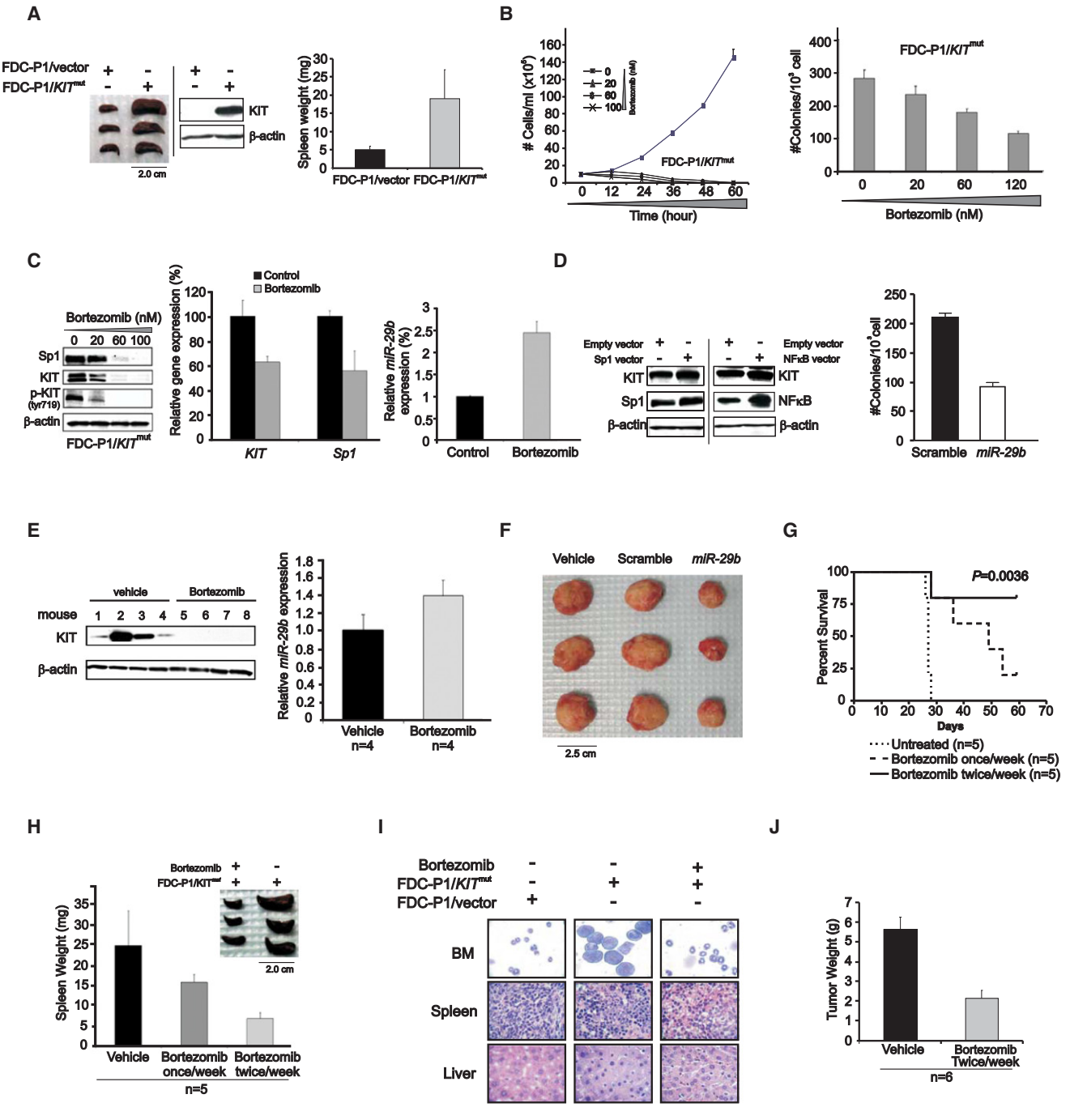


Figure 8. In Vivo Activity of Bortezomib on *KIT*^{mut}-Driven Leukemia

(A) Mice engrafted with FDC-P1/*KIT*^{mut} cells developed leukemia-like disease with enlarged spleens. Shown on the left are spleens from mice injected with FDC-P1/*KIT*^{mut} cells. As shown in the middle, immunoblotting indicated the presence of human KIT expression in the spleen from the mice engrafted with FDC-P1/*KIT*^{mut} cells, but not in FDC-P1/vector only cells. Shown on the right is a graph of spleen weight (mean ± SD).

(B) Bortezomib inhibited proliferation (left panel) and colonogenic activity (right panel) in FDC-P1/*KIT*^{mut} cells (mean ± SEM).

(C) Bortezomib treatment decreased Sp1 and KIT protein (left panel) and RNA (middle panel) expression and increased *miR-29b* level (right panel) (mean ± SEM) in FDC-P1/*KIT*^{mut} cells.

(D) Forced Sp1 and NFκB expression in FDC-P1/*KIT*^{mut} cells increased KIT level (left panel) and ectopic *miR-29b* expression inhibited the colonogenic activity in FDC-P1/*KIT*^{mut} cells (right panel) (mean ± SD).

(E) KIT protein expression (left panel) was decreased and *miR-29b* transcription was increased (right panel) (mean ± SEM) in FDC-P1/*KIT*^{mut} cell engrafted mice 48 hr following in vivo treatment with bortezomib.

(F) Ectopic *miR-29b* expression significantly inhibits tumor growth in mice engrafted with FDC-P1/*KIT*^{mut} cells transfected with synthetic *miR-29b*.

trials targeting molecular subsets of AML in which the Sp1/NFκB/*miR-29b* network appears to play a central role for oncogene expression.

EXPERIMENTAL PROCEDURES

Plasmids and Cell Lines

Construction of the human Sp1 in EBV/retroviral hybrid vector and cell culture (Kasumi-1, K562, MV4-11, THP-1) were done as previously reported (Liu et al., 2008). *KIT* expression plasmids (*KIT*^{mut} and *KIT*^{wt}) were constructed by inserting the *KIT* gene sequence into pBABE-puro retroviral vector. pCMV-p65 expressing NFκB (p65) and pcDNA3-Flag-HDAC1 expressing HDAC1 (Taunton et al., 1996) were also used. Retroviral infection to establish FDC-P1 cell line stably expressing *KIT*^{mut} or *KIT*^{wt} was performed as previously reported (Neviani et al., 2007).

Cells were treated with the following reagents (concentrations, times, and schedules indicated in Results): bortezomib (Millennium Pharmaceuticals Inc., Cambridge, MA), MS275, mithramycin A, decitabine, and PKC412 (Sigma-Aldrich, St Louis, MO), HDAC-OSU 42 (HDAC42) (OSU, Columbus, OH) (Sargeant et al., 2008), or bay11-7082 (Abcam Inc., Cambridge, MA).

Patient Samples

Mononuclear cells (MNC) from pretreatment BM samples with > 70% of blasts from AML patients with t(8;21) were obtained from the OSU Leukemia Tissue Bank. All patients signed the informed consent for the OSU 1997C0194 protocol to store and use their leukemia tissue for discovery studies. The OSU 1997C0194 protocol was approved by the OSU Cancer Institutional Review Board (IRB) Committee.

Gene Expression in AML Patients

KIT, *Sp1*, and *miR-29b* expression levels were measured in RNA samples of BM MNC from CBF and CN AML patients enrolled on CALGB treatment studies 8525, 9621, and 19808, using the Affymetrix U133 Plus 2.0 GeneChips (*KIT* and *Sp1*) (Affymetrix, Santa Clara, CA) and OSU microRNA microarray chip as previously reported (Radmacher et al., 2006; Marcucci et al., 2008). For the gene expression microarrays, summary measures of the expression levels were computed for each probe set using the robust multichip average method, which incorporates quantile normalization of arrays (Irizarry et al., 2003). For the microRNA expression microarrays, summary measures of expression levels were computed for each probe using quantile normalization, making an adjustment for array batch (Rao et al., 2008). Samples for analyses were obtained from patients who were enrolled on CALGB clinical studies and signed an informed consent for CALGB 20202 to store and use their leukemia tissue for molecular characterization of AML. The CALGB 20202 protocol was locally approved by the OSU Cancer IRB Committee.

Transient Transfection, Immunoprecipitation, and Western Blotting

On-target^{plus} Smart pool SiRNA for *Sp1*, *NFκBp65*, *KIT*, and *HDAC1* were purchased from Thermo Fisher Scientific (Waltham, MA). Precursor *miR-29b* was from Applied Biosystems (Foster City, CA). Antago-*miR-29b* was from Exiqon, Inc (Woburn, MA). SiRNA, miRNA oligos, or plasmid constructs were introduced into leukemia cell lines by Nucleofector Kit (Lonza Walkerrsville Inc, Walkersville, MD) according to the manufacturer's instruction. The immunoprecipitation and western blots were preformed as previously described (Liu et al., 2008). The antibodies used were: Sp1, total KIT, p-tyrosine, p-ERK (tyr 204) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA); phospho-KIT (tyr719), phospho-p65 (Ser536), phospho-IKBα (Ser32), phospho-Stat3 (Tyr705), phospho-Stat1 (tyr701), phospho-Stat5 (ser694), phosphor-Akt, total Akt, total Erk, total Stat1, total Stat3, and total Stat5 (Cell Signaling Technology, Danvers, MA); ubiquitin (Millipore, Billerica, MA).

Electrophoretic Mobility-Shift Assays

EMSA with nuclear extracts and ^{32}P-labeled probes were performed as described (Hong et al., 2003; Liu et al., 2008). The primers for *KIT* and *miR-29b* promoter are listed in Supplemental Information. Recombinant proteins, NFκB(p50), and Sp1 (Promega, Madison, WI), NFκB(p65), and HDAC1 and HDAC3 (Caymanchem, Ann Arbor, MI) were purchased.

Real-Time RT-PCR

For normalized expression of *KIT, MYC* and *Sp1*, qRT-PCR was performed as described elsewhere (Marcucci et al., 2005). For miRNA expression, qRT-PCR was carried out by TaqMan MicroRNA Assays (Applied Biosystems) according to the manufacturer's protocol and normalized by U44/48 (for human) or Sno202 (for mouse) levels. Expression of the target genes were measured using the ΔCT approach.

Chromatin Immunoprecipitation

ChIP assays were performed using the EZ ChIP Assay Kit (Millipore) according to the manufacturer's standard protocol. DNA was quantified using qRT-PCR with SYBR green incorporation (Applied Biosystems). The antibodies used were: anti-acetyl-histone H4, acetyl-histone H3, HDAC1, HDAC3, Sp1, and NFκB(p65) (Millipore). The primers specific for *KIT* gene promoter or *miR-29b* enhancer are listed in Supplemental Information.

Leukemogenesis in NOD/SCID Mice

Four to six-week-old NOD/SCID (The Jackson Laboratory, Bar Harbor, ME) were intravenously injected through the tail vein with 5 × 10^6 cells of FDC-P1 cells harboring D816V *KIT*^{mut}. After engraftment, cell-injected mice (n = 5) were intravenously treated with 1 mg/kg bortezomib via tail-vein in 0.2 ml saline solution once or twice a week. Longitudinal follow-up to assess survival was conducted and the trial was terminated 8.5 weeks after injection. Mice injected with FDC-P1/*KIT*^{mut} cells (n = 5) and injected with saline solution only served as controls. The effect of bortezomib on targets (*KIT* and *miR-29b*) was tested in vivo in FDC-P1/*KIT*^{mut}-engrafted mice (n = 4) treated with 1 mg/kg bortezomib and assessed for *KIT* and *miR-29b* expression 48 hr following drug administration. Following euthanasia, isolated spleens were grounded, and the red blood cells were lysed to attain single MNCs utilized for immunoblotting and qRT-PCR assays. For pathological examination, tissue sections from liver and spleen were fixed on formalin, embedded in paraffin blocks, and H&E stained. The effect of a synthetic *miR-29b* engraftment ability of FDC-P1/*KIT*^{mut} was tested by engrafting FDC-P1/*KIT*^{mut} cells transfected with *miR-29b*, scrambled miRNA, or vehicle. The transfection efficiency of the miRNA compounds was approximately 50%–60% as evaluated by concurrent transfection of a plasmid expressing GFP.

Finally, NOD/SCID mice were also injected with 1 × 10^7 HMC-1 or ME-1 cells subcutaneously. When tumor size approached approximately 20 mm^3, the animals received 1 mg/kg bortezomib or vehicle alone twice a week (intravenous bolus) for 2 weeks. The experiments were terminated in two weeks after drug administration. All animal studies were performed in accordance with OSU institutional guidelines for animal care and under approved protocols (OSU 2007A0149 and 2008A0027) by the OSU Institutional Animal Care and Use Committee.

Statistical Analysis

Statistical analyses relative to microarray gene and microRNA expression data were performed by the CALGB Statistical Center.

(G) Bortezomib administered at the dose of 1 mg/kg once a week or twice weekly increased survival duration in mice engrafted with FDC-P1/*KIT*^{mut} cells compared with untreated FDC-P1/*KIT*^{mut} cell engrafted controls.
(H) Spleens from FDC-P1/*KIT*^{mut} cell engrafted mice untreated versus bortezomib-treated (mean ± SD).
(I) May-Grumwald/Giemsa staining of BM cells and H&E staining of sections from spleen and liver of FDC-P1/*KIT*^{mut} cell engrafted mice untreated and bortezomib-treated. FDC-P1/empty vector cell engrafted mice were also used as control.
(J) Tumor growth was inhibited in mice engrafted with HMC-1 cell after the administration of bortezomib (mean ± SD). Data are representative of three independent experiments. (See also Figure S7).

ACCESSION NUMBERS

The microarray data discussed in this study have been deposited in the EBI ArrayExpress database and are accessible at http://www.ebi.ac.uk/microarray-as/ae/ under Array Express accession number: E-TABM-945.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online doi:10.1016/j.ccr.2010.03.008.

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