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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.

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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.

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 et al, 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 [t(8;21) or inv(16) or the respective molecular equivalent *RUNX1/RUNX1T1*- or *CBFB/MYH11*-positive AML], where 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 the *KIT* mutations 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. While *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 appears to be also 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 impacts adversely on outcome and *RUNX1/RUNXT1*-positive patients with higher *KIT* levels had a significantly shorter survival (*P*=.04; Supplemental Figure S1A). 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 Supplemental 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 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. As 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, electrophoretic mobility-shift assays (EMSA) were performed 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 co-migrated 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 level of Sp1 enrichment on the *KIT* promoter was 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, loss- and gain-of function approaches were applied in Kasumi-1 cells. First we showed that ectopic expression (Figure 2C, left panel) or siRNA knock-out (Figure 2C, right panel) respectively reduced and increased Sp1 and NFκB(p65) enrichment on the *KIT* promoter, and resulted respectively in KIT down- or up-regulation 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 (Supplemental 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 the Sp1/NFκB activity (Liu et al., 2008). To further establish the regulatory role of the Sp1/NFκB complex in *KIT* expression, Kasumi-1 cells were then treated 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 (Supplemental Figure S3A, B, C, D and G). In agreement with recent reports (Hideshima et al., 2009), we also observed that bortezomib resulted in NFκBp65 and IKBα phosphorylation (Supplemental 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 (Supplemental Figure S3E and F). The latter was likely to abrogate Sp1/NFκB gene transactivating activity thereby leading to bortezomib-induced dose- and time-dependent reduction in *KIT* expression (Figure 3A and B; 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 Coefficient Correlation .6; *P*=.016; Supplemental 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 microcircuity 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 antago*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 (Supplemental 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 software

package @ 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 supplementary material) spanning the -125/-75 *miR-29b* sequence in K562 cells. These cells were selected because they have high level 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), while 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, while the unlabeled NFκBC probe decreased the C1 complex and eliminated the C2 complex. Similarly, incubation of extracts with Sp1 antibodies 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 (Supplemental 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 *miR-29b* endogenous precursor (Figure 5F), thereby further supporting *miR-29b* as an active participant in its own transcriptional regulation.

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. While 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 (Marcucci-unpublished results). 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 to 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 (Supplemental 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

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

To further determine 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 (Supplemental Figure S6 A-E). 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 non-tumorigenic 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} (Supplemental 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 (Supplemental Figure S7B and C).

NOD/SCID mice engrafted with FDC-P1/KIT^{mut} cells were then treated with one dose bortezomib (1mg/kg/dose) and sacrificed 48 hours 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} tumor 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 Supplemental Figure S7D).

Next, FDC-P1/KIT^{mut}–engrafted mice were treated with 1mg/kg of bortezomib once or twice weekly for three 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 (Supplemental Figure S7E and F). NOD/SCID mice engrafted with 10^7 of HMC-1 cells subcutaneously received intratumor administration of 1 mg/kg bortezomib twice/week for two weeks starting from when the tumor size approached $20mm^3$. Significant decrease in tumor size was observed in bortezomib-treated mice when compared to vehicle-treated controls (Figure 8J). Similarly, bortezomib was therapeutically advantageous in mice engrafted with ME-1 cells overexpressing KIT^{wt} (Supplemental 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 draw-backs 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, impacts leukemogenesis and 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. However, treatment with ectopic *miR-29b* or compounds that led to increase in endogenous *miR-29b* resulted in *KIT* down-regulation. 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* promoter and represses *miR-29b* expression, while *miR-29b* blocks Sp1 translation and in turn up-regulates 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 level 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* up-regulation, *Sp1* downregulation, disruption of the Sp1/NFκB complexes and inhibition of the *KIT* gene. The net results are *KIT* down-regulation, inhibition of aberrant TK activity and arrest of leukemia growth. The pivotal role of *miR-29b* in this miRNA/protein network is supported by up-regulation 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 over-expressed 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 effectively interferes with the activity of Sp1/NFκB complex at concentrations (i.e., 60nM) that are achievable in patients treated at the recommended dose of the drug (Quinn et al., 2009) and 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 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, while 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 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. All microarray data has been submitted to ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) and can be found under the accession number E-TABM-945.

Transient transfection, immunoprecipitation and Western blot

On-target^{plus} Smart pool SiRNA for *Sp1*, *NF Bp65*, *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)

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 were listed in supplementary material. 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 (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)

ChIP assays were performed using the EZ ChIP Assay Kit (Millipore, Billerica, MA) 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 were listed in supplementary material.

Leukemogenesis in NOD/SCID mice

Four to six-week-old NOD/SCID (The Jackson Laboratory, Bar Harbor, ME) were i.v. 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 i.v. treated with 1mg/kg of bortezomib via tail-vein in 0.2 ml of 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 1mg/kg of bortezomib and assessed for *KIT* and *miR-29b* expression 48 hours 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 10^7 HMC-1 or ME-1 cells subcutaneously. When tumor size approached approximately $20mm^3$, the animals received 1mg/kg of bortezomib or vehicle alone twice a week (intravenous bolus) for two 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.

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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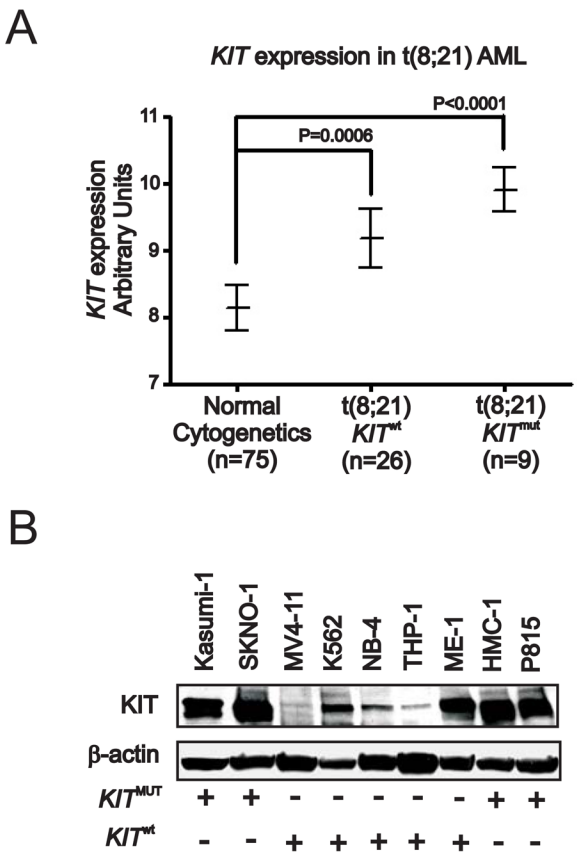

Figure 1. *KIT* expression in AML patients and cell lines

(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 − indicate presence or absence of KIT^{mut} or KIT^{wt} alleles. Data are representatives of three independent experiments. (See also Figure S1)

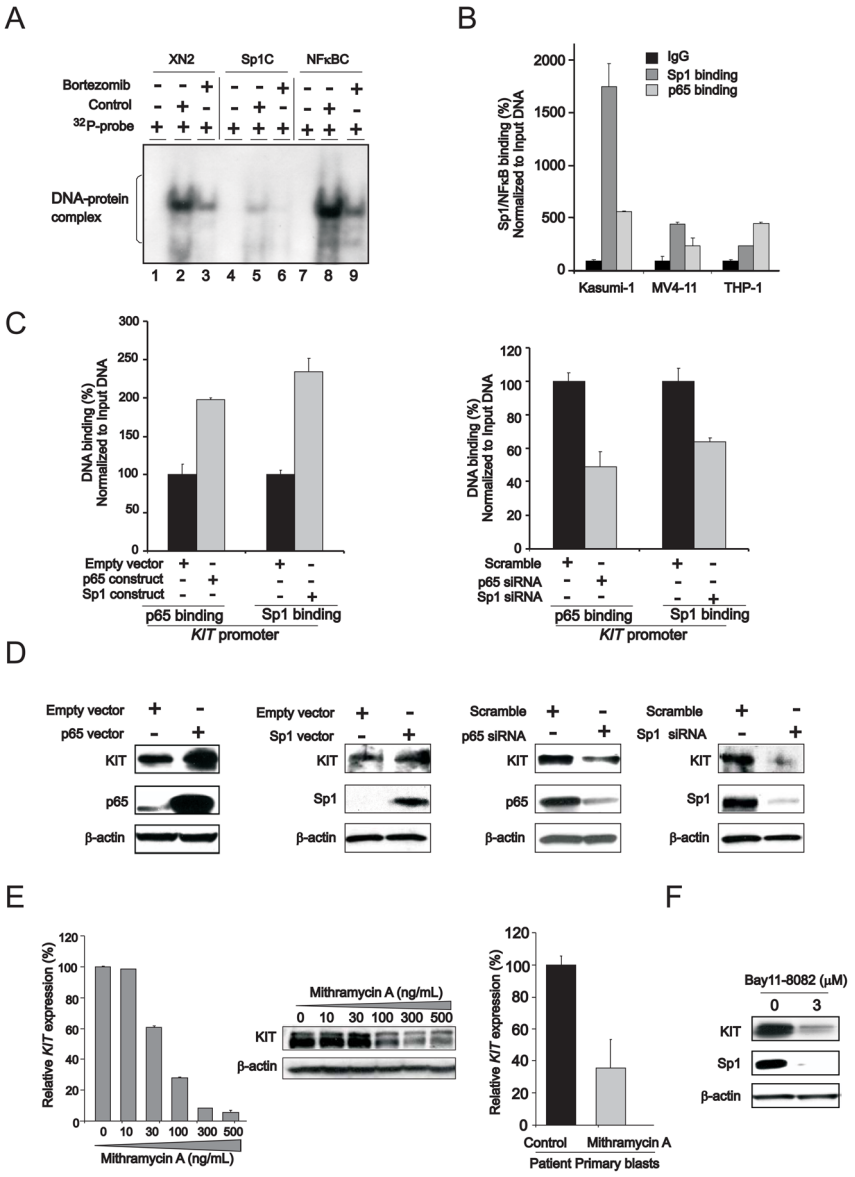

Figure 2. The regulatory role of Sp1/NFκB in *KIT* expression

(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 ± 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 representatives of three independent experiments. (See also Figure S2)

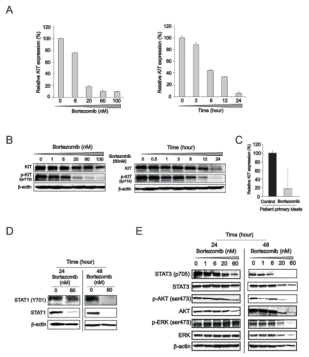

Figure 3. Proteasome inhibition by bortezomib impairs KIT expression and its downstream signaling pathway

(A and B) Dose- (left) and time- (right) dependent 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 of bortezomib for 24 hours (mean ± SD).

(D and E) Immunoblotting analysis demonstrated the down-regulation of KIT downstream effectors such as STAT1 (D), STAT3, AKT and ERK (E) in Kasumi-1 cells treated with bortezomib. p, phosphorylated.

Data are representatives of three independent experiments. (See also Figure S3)

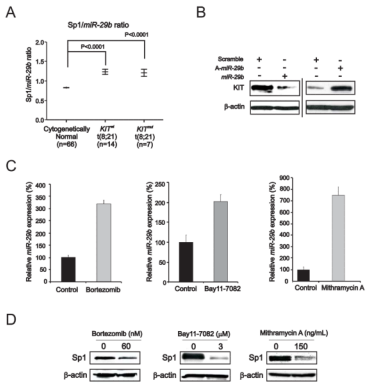

Figure 4. Role of *miR-29b* in *KIT* expression regulation

(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 hours.

(C) Up-regulation 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 hours. 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 representatives of three independent experiments.

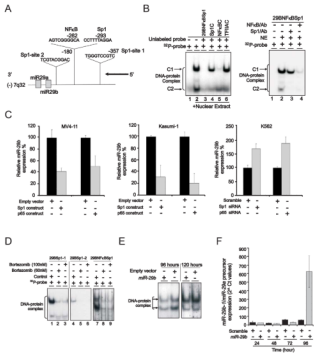

Figure 5. Regulation of *miR-29b* transcription

(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 hours from initial treatment (mean ± SEM).

Data (B–F) are representatives of three independent experiments. (See also Figure S4)

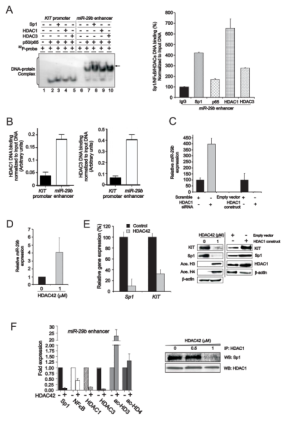

Figure 6. HDACs participate in the Sp1/NFκB complex to inhibit *miR-29b* expression

(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 3 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 everexpression 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, co-immunoprecipitation showed that HDAC42 disrupted Sp1/HDAC1 interaction.

Data are representatives of three independent experiments. (See also Figure S5)

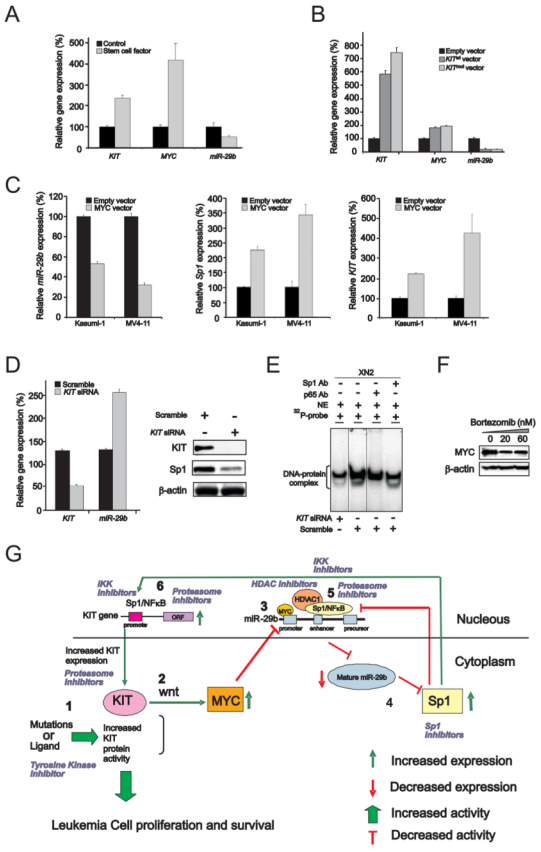

Figure 7. Role of activated KIT in *KIT* gene transcription

(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 hours.

(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 representatives of three independent experiments. (See also Figure S6)

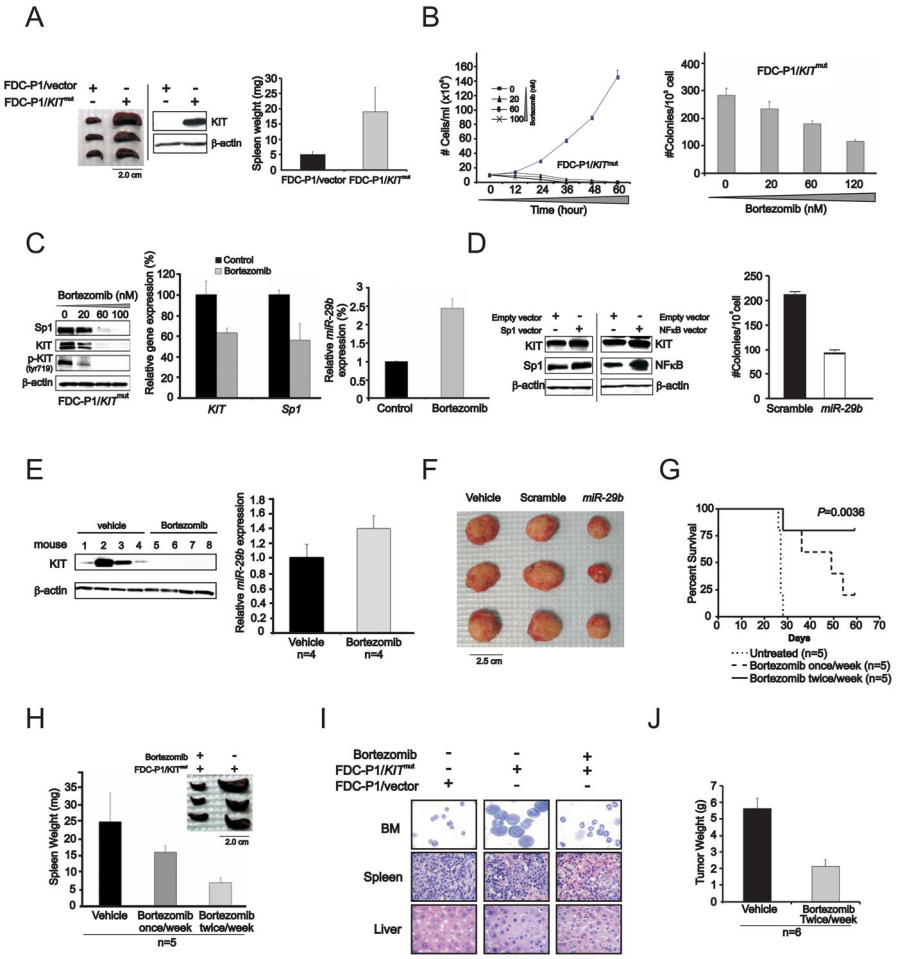

Figure 8. ***In vivo*** **activity of bortezomib on** ***KIT*** $^{\mathbf{mut}}$**-driven leukemia**

(A) Mice engrafted with FDC-P1/*KIT*^{mut} cells developed leukemia-like disease with enlarged spleens. Left: spleens from mice injected with FDC-P1/*KIT*^{mut} cells; 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. Right: 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 hours 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*.

(G) Bortezomib administered at the dose of 1mg/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 representatives of three independent experiments. (See also Figure S7)