Bortezomib interferes with C-KIT processing and transforms the t(8;21)-generated fusion proteins into tumor-suppressing fragments in leukemia cells

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The boronic acid dipeptide bortezomib inhibits the chymotrypsin-like activity of the 26S proteasome and shows significant therapeutic efficacy in multiple myeloma. However, recent studies suggest that bortezomib may have more complex mechanisms of action in treating cancer. We report here that the endocytosis and lysosomal degradation of the receptor tyrosine kinase C-KIT are required for bortezomib- but not tyrosine kinase inhibitor imatinib-caused apoptosis of t(8;21) leukemia and gastrointestinal stromal tumor cells, suggesting that C-KIT may recruit an apoptosis initiator. We show that C-KIT binds and phosphorylates heat shock protein 90 β (Hsp90 β), which sequesters apoptotic protease activating factor 1 (Apaf-1). Bortezomib dephosphorylates pHsp90 β and releases Apaf-1. Although the activated caspase-3 is not sufficient to cause marked apoptosis, it cleaves the t(8;21) generated acute myeloid leukemia 1-eight twenty one (AML1-ETO) and AML1-ETO9a fusion proteins, with production of cleavage fragments that perturb the functions of the parental oncoproteins and further contribute to apoptosis. Notably, bortezomib exerts potent therapeutic efficacy in mice bearing AML1-ETO9a-driven leukemia. These data show that C-KIT-pHsp90 β -Apaf-1 cascade is critical for some malignant cells to evade apoptosis, and the clinical therapeutic potentials of bortezomib in C-KIT-driven neoplasms should be further explored.

stem cell factor receptor | internalization | proteolysis | apoptosome

The protooncogene *C-KIT* (or v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog) (1) encodes a class III receptor tyrosine kinase composed of five extracellular Ig-like domains, a transmembrane segment, a juxtamembrane domain, and a split cytoplasmic kinase domain. On binding to its ligand, the stem cell factor (SCF), C-KIT rapidly undergoes dimerization, autophosphorylation (2), and clathrin-mediated internalization (3, 4). Through its downstream signal molecules, including PI3K, Rac-serine/threonine-protein kinase (AKT), ERK, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (SRC), JAK/STAT, and Rat sarcoma (Ras)/Rapidly Accelerated Fibrosarcoma (Raf)/MAPK cascade (5), C-KIT confers survival/proliferative signals to hematopoietic stem cells, mast cells, germ cells, melanocytes, and interstitial cells of Cajal (6). However, how C-KIT is involved in apoptosis remains obscure.

Aberrant expression and gain of function mutations of C-KIT have been reported in human gastrointestinal stromal tumor (GIST) (7) and hematologic malignancies including acute myeloid leukemia (AML) with inversion 16 [inv(16)] or t(8;21)(q22;q22) (8). The t(8;21), which represents the most common chromosomal anomaly in AML, targets eight twenty one [ETO, also known as myeloid transforming gene on chromosome 8 (MTG8) or RUNX1T1] on chromosome 8 and acute myeloid leukemia 1 [AML1, also known as Runt-related transcription factor 1

(RUNX1), core binding factor (CBF) $\alpha 2$, and Phosphatidylethanolamine-binding protein (PEBP) 2 α B] on chromosome 21, yielding two fusion transcripts, the AML1-ETO (AE) (9) and AML1-ETO9a (AE9a), lacking the neutralized homology repeat (NHR)3–4 domains at the C terminus of ETO moiety (10). It has been established that AE9a bears a much stronger leukemogenic activity than AE in murine system (10), and a similar situation might exist in human setting (11). Studies showed that t(8;21) AML follows a stepwise leukemogenesis (i.e., AE/AE9a represent the first, fundamental genetic hit to initiate the disease), whereas activation of the C-KIT pathway may be a second but also crucial hit for the development of a full-blown leukemia (8, 12). Although AE impairs hematopoietic differentiation, aberrant C-KIT increases the stem cell capacity of normal hematopoietic stem cells and enhances the leukemogenic potential of and confers proliferative/survival advantages to AE-positive stem cells (13, 14). However, the mechanism of C-KIT in rendering apoptosis-evading potential to leukemic cells remains elusive. Moreover, novel therapeutic strategy remains a practical need for t(8;21) AML, because the clinical outcome of this subtype of AML remains unsatisfied (in particular, the non-Caucasian patients) (8, 15); additionally, patients receiving chemotherapy (16) or hematopoietic stem cell transplantation (17) had shorter overall survival than those patients with inv(16).

The ubiquitin-proteasome pathway plays a central role in the targeted destruction of endogenous proteins in eukaryotic cells, and its inhibition may result in apoptosis through the accumulation of proapoptotic molecules (18). However, recent studies showed that some proteasome inhibitors may exert effects on cancer through much more complex mechanisms than initially expected. For example, it was reported that (19) bortezomib (B α R), a clinically proven proteasome inhibitor, induces canonical NF- κ B activation in multiple myeloma cells; others showed that apoptosis induced by proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) can be blocked by caspase inhibitors, whereas caspase activation during apoptosis inhibits proteasome function by cleavage of some key subunits of the 19S regulatory complex (20). Therefore, scrutinized investigation of the complicated relationship between the protein degradation by

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proteasome-dependent and -independent pathways and cancer cell apoptosis may allow mechanisms of action of classic proteasome inhibitors to be discovered.

Using molecules, including medicinal compounds, as probes, chemical biology can not only reveal key factors/pathways involved in physiology and human diseases such as cancer but also provide drug leads or use of existing drugs. Recently, when conducting chemical biology study in a number of leukemia and solid tumor cell models, we were attracted by unexpected discoveries that, in *t*(8;21) leukemia and GIST cells with constitutively activated C-KIT, BOR triggered a clathrin-mediated endocytosis and lysosomal degradation of C-KIT, and the dynamin inhibitor dynasore (DY) (21) suppressed BOR- but not tyrosine kinase inhibitor imatinib (IM)-induced apoptosis of these cells. These results suggested that C-KIT may interact with an apoptosis initiator, whereas BOR-triggered degradation but not IM-caused kinase inhibition releases this factor and activates caspases as well as other key downstream molecular cascade. We addressed the hypothesis in this work.

Results

BOR-Induced a Caspase-Dependent Apoptosis of C-KIT-Driven Cells. We found that BOR significantly inhibited proliferation of *t*(8;21) AML lines Kasumi-1 and SKNO-1 and GIST line GIST882, with IC₅₀ values of 12.3, 21.9, and 80.5 nM, respectively. BOR inhibited cell growth (Fig. S1A) and induced apoptosis (Fig. S1B and C) of *t*(8;21)-positive lines and CD34+ primary leukemia cells isolated from bone marrow from three patients in 24–48 h of treatment time course. BOR inhibited chymotrypsin-like activity (Fig. S1D), down-regulated β 5/ β 5i-component (Fig. S1E), and caused cleavage of the Rpt5 subunit (Fig. S1F and G) of the proteasome. Interestingly, pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone (z-VAD.fmk) suppressed apoptosis of Kasumi-1, chronic myeloid leukemia K562, and myeloma U266 cells induced by treatment with BOR or another proteasome inhibitor Z-Ile-Glu(OtBu)-Ala-Leucinal or PSI (P, 1 μ M) for 24 h (Fig. S1C) and reversed BOR-caused Rpt5 cleavage (Fig. S1G). However, z-VAD could not repress BOR-induced inhibition of chymotrypsin-like activity (Fig. S1D) and down-regulation of β 5/ β 5i-component (Fig. S1E) of the proteasome. These results indicate that BOR is a caspase activator with detailed mechanisms in inducing apoptosis that warrant careful dissection.

BOR Induces Internalization and Lysosomal Degradation of C-KIT. As a cell surface molecule, C-KIT plays a crucial role in leukemogenesis of *t*(8;21) AML (8, 12), suggesting that it could be targeted by effective therapeutics. We, therefore, tested the effects

of BOR on C-KIT and found that treatment with BOR at 10 nM in Kasumi-1 cells resulted in down-regulation of C-KIT expression at the mRNA level (Fig. S2A). Importantly, C-KIT protein was down-regulated at 6 h and became very low at 12 h in cells on BOR (Fig. 1A). Other proteasome inhibitors PSI and MG-132 also caused C-KIT catabolism (Fig. S2B). In CD34+ primary leukemia cells with wild-type (WT) C-KIT, treatment with BOR for 12 h decreased the expression of C-KIT, which was revealed by Western blotting (Fig. S2C) and immunofluorescence assay, which also suggested a C-KIT internalization (Fig. S2D). In GIST882 cells with an activating C-KIT mutation (K642E) (22), treatment with BOR at 100 nM for 12 h markedly down-regulated C-KIT (Fig. 1B). We showed that ectopic expression of a degradable C-KIT with D816V mutation reduced BOR-induced inhibition rate of Kasumi-1 cells, but this reduction is not statistically significant (Fig. S2E). BOR could drastically potentiate the effect of the protein synthesis inhibitor cycloheximide (CHX) in suppressing C-KIT in Kasumi-1 cells (Fig. 1C). We found that, although z-VAD could not prevent BOR-triggered C-KIT turnover (Fig. S2F), lysosome inhibitor chloroquine (Chl; 40 μ M) substantially reduced C-KIT catabolism (Fig. 1D).

Immunofluorescence analyses showed that BOR caused a dynamic change of C-KIT in that, in an early stage (2 h), C-KIT molecules were slightly up-regulated, possibly because of inhibition of proteasomal degradation; however, in a middle stage (6–8 h), they colocalized with lysosomes in cytoplasm and down-regulated. In a relatively late stage (12 h), they became markedly reduced, and the cells underwent apoptosis reflected by nuclear fragmentation with intact cell membrane (Fig. 1E). In the presence of Chl (40 μ M), C-KIT was colocalized with lysosomes but not down-regulated (Fig. 1F and Fig. S2G). Nevertheless, z-VAD was unable to perturb C-KIT expression or cellular localization in Kasumi-1 cells on BOR (Fig. S2G).

C-KIT Internalization/Degradation Is Required for BOR-Caused Cell Apoptosis. C-KIT internalization is mediated by clathrin (3, 4, 23). We evaluated whether clathrin plays a role in BOR-induced C-KIT internalization using DY, a potent inhibitor of dynamin GTPase that is essential for clathrin-dependent coated vesicle formation (21). We found that, although treatment with BOR for 6 h caused C-KIT internalization in Kasumi-1 cells, coinubation with BOR and DY (80 μ M) or pretreatment with BOR for 1 h followed by treatment with DY for 5 h rendered C-KIT localization mainly on the cell surface (Fig. 2A), a sign of blockage of internalization. Interestingly, DY not only drastically attenuated BOR-caused inhibition of Kasumi-1 cell growth (Fig. 2B) but also significantly inhibited apoptosis of Kasumi-1, SKNO-1, and

Fig. 1. BOR triggers a lysosomal degradation of C-KIT. (A) Kasumi-1 cells were treated with BOR at 10 nM for indicated time points and lysed, and Western blotting was performed using the indicated antibodies. (B) Western blot analysis using lysates of GIST882 cells treated with BOR at 100 nM for 12 h. (C) Kasumi-1 cells were treated with CHX and/or BOR and lysed, and Western blotting was performed. (D) Western blot analyses using lysates of Kasumi-1 cells treated with BOR and/or Chl. (E) Kasumi-1 cells were treated with BOR at 10 nM for indicated time points, and immunofluorescence analyses were performed using the antibodies indicated. (F) Immunofluorescence analyses of Kasumi-1 cells treated with BOR and/or Chl using the antibodies indicated.

Fig. 2. DY inhibits C-KIT internalization and apoptosis induced by BOR. (A) Kasumi-1 cells were treated with BOR and/or DY (80 μ M) for 6 h, or they were pretreated with BOR for 1 h and then incubated with DY for 5 h followed by immunofluorescence assays using indicated antibodies. (B) Cells were treated with BOR and/or DY for indicated time points, and they were assessed by trypan blue exclusion analysis. (C and D) Cells driven by C-KIT (C) or not (D) were treated with BOR (10 nM) in the absence or presence of DY for 12 h, and they were assayed by Annexin V/7-AAD and flow cytometry. (E) Cells were treated with IM (1 μ M) and/or DY for 12 h, and apoptotic cells were determined. The mean $\pm$ SD of three independent experiments is shown.

GIST882 cells induced by BOR (Fig. 2C). However, DY could not inhibit BOR-caused apoptosis of U266 cells (Fig. 2D) or apoptosis of Kasumi-1 or SKNO-1 cells triggered by IM (Fig. 2E).

At the molecular level, DY attenuated BOR-induced C-KIT degradation and reversed BOR-caused suppression of phosphorylated AKT (pAKT), pSTAT3, and pERK, which are C-KIT targets (Fig. S3A). Although BOR up-regulated phospho-Stress-Activated Protein Kinase (pSAPK)/JNK, which is not a C-KIT target, DY could not reverse this effect (Fig. S3A). Furthermore, DY inhibited BOR-caused activation of caspase (Casp)-9 and -3 (reflected by down-regulation of pro-Casp-9 and -3) as well as cleavage of Casp-3 substrate Poly(ADP)-Ribose Polymerase (PARP) (Fig. S3A). However, DY could not reverse IM-induced inhibition of C-KIT signal pathway or cleavage of PARP (Fig. S3B), consistent with the observation fact that DY could not inhibit IM-induced apoptosis (Fig. 2E). Although capable of triggering degradation of C-KIT, SCF did not decrease pAKT or pERK (Fig. S3C) and could not induce apoptosis of Kasumi-1 cells (Fig. S3D). In this context, DY could not inhibit SCF-

caused C-KIT catabolism (Fig. S3E). These results indicate that C-KIT internalization and subsequent degradation are required for BOR-induced apoptosis of *t*(8;21) leukemia and GIST cells, and suggest that C-KIT might directly or indirectly sequester a factor that could activate Casp-9/-3, whereas BOR, but not IM, could release this factor and induce programmed cell death.

C-KIT Binds and Phosphorylates Heat Shock Protein 90 β . To identify the putative C-KIT binding factor, Kasumi-1 cells (with N822K mutant C-KIT) were treated with or without BOR and lysed, and the supernatant was immunoprecipitated with a monoclonal anti-C-KIT antibody. The bands of silver-stained gel of eluates were analyzed by tandem mass spectrometric peptide sequencing. Interestingly, heat shock protein 90 (Hsp90) was identified (Fig. S4A). We further confirmed that Hsp90 β , but not Hsp90 α (Fig. S4B), was the C-KIT binding protein.

Studies showed that phosphorylation modification modulates the function of Hsp90 β (24). We, therefore, tested whether C-

Fig. 3. C-KIT binds and phosphorylates Hsp90 β . (A) 293T cells were transfected with the plasmids indicated and lysed, and coimmunoprecipitation and Western blot assays were performed. (B and C) 293T cells were transfected with the plasmids indicated and lysed, and the proteins were purified. The extracts were supplemented with ATP followed by coimmunoprecipitation and Western blot analyses. (D) In vitro phosphorylation assay using kinase domain (KD) of C-KIT, purified Hsp90 β , and ATP. Western blot analysis was performed to evaluate pHsp90 β . (E) Hsp90 β protein was purified from lysates of CD34+ cells isolated from four patients with *t*(8;21) AML, and pHsp90 β was detected using an anti-pTyr antibody. (F) CD117+ and CD117- cells were isolated by fluorescence activated cell sorting from bone marrow mononuclear cells of a *t*(8;21) AML patient with WT C-KIT and lysed, and coimmunoprecipitation and Western blotting were performed.

KIT could phosphorylate Hsp90 β or not. To do this testing, 293T cells were transfected with Flag-Hsp90 β and/or Flag-C-KIT with or without D816V mutation and lysed 48 h later, and coimmunoprecipitation assays were performed. We found that, in the presence of mutant or WT C-KIT, the phosphorylated Hsp90 β (pHsp90 β) was up-regulated (Fig. 3A). C-KIT with N822K mutation was also able to induce phosphorylation of Hsp90 β (Fig. 3B). The residue Y301 was shown to be the phosphorylation site of Hsp90 β in Src-mediated phosphorylation of Hsp90 β in response to VEGF (25). Plasmids containing Flag-Hsp90 β , Flag-Hsp90 β with Y301F mutation (Flag-mHsp90 β), or Flag-C-KIT were transfected into 293T cells. Although C-KIT increased the expression of pHsp90 β , Y301F substitution could attenuate this effect (Fig. 3B and C), suggesting that Y301 is a phosphorylation site. In an in vitro phosphorylation assay, both WT and D816V C-KIT induced phosphorylation of Hsp90 β (Fig. 3D). We investigated the expression of pHsp90 β in CD34+ cells from *t*(8;21) AML patients with N822K or WT C-KIT, and we found that pHsp90 β was the main type of Hsp90 β in these cells (Fig. 3E). Moreover, the expression of pHsp90 β was much higher in CD117/C-KIT+ than CD117- cells from bone marrow mononuclear cells of a *t*(8;21) AML patient with WT C-KIT (Fig. 3F).

C-KIT Indirectly Sequesters Apoptotic Protease Activating Factor 1, Whereas BOR Releases It. Studies (24, 26) showed that Hsp90 β can bind strongly to apoptotic protease activating factor 1 (Apaf-1), a caspase recruitment domain-containing protein that forms an oligomeric apoptosome on binding cytochrome *c* and dATP (27). To investigate the possible interaction between C-KIT, Hsp90 β , and Apaf-1, plasmids containing Flag-Hsp90 β and His-Apaf-1 were transfected into 293T cells, and the proteins were purified and incubated with C-KIT isolated from Kasumi-1 cells. By reciprocal coimmunoprecipitation and Western blot analyses, we found that C-KIT not only induced phosphorylation of Hsp90 β but also markedly enhanced the binding affinity between Hsp90 β and Apaf-1 (Fig. 4A), suggesting that C-KIT could sequester Apaf-1 by phosphorylation of Hsp90 β . Y301F substitution in Hsp90 β reduced Apaf-1 binding activity (Fig. 4A). In Kasumi-1 cells, pHsp90 β bound Apaf-1, whereas BOR decreased phosphorylation of Hsp90 β and released Apaf-1 (Fig. 4B). In CD34+ leukemia cells from patients with *t*(8;21) AML and GIST882 cells, BOR drastically down-regulated pHsp90 β and released Apaf-1 from Hsp90 β (Fig. 4C).

To define the interaction between C-KIT and Hsp90 β , different intracellular domains of C-KIT (Fig. 4D) were subcloned into pEGFP-C1 plasmids and transfected into 293T cells. We

found that the tyrosine kinase domains 1 and 2 and the kinase insertion domain could bind Hsp90 β , whereas the juxtamembrane domain and the C-terminal region could not (Fig. 4E).

Released Apaf-1 Activates Casp-3, Which Is Not Sufficient to Cause Marked Apoptosis. We found that, in Kasumi-1 cells treated with BOR for 6 h, the binding affinity between Hsp90 β and Apaf-1 was markedly decreased, and cytochrome *c* was recruited to Apaf-1 (Fig. S4C), which was confirmed by a reciprocal coimmunoprecipitation assay (Fig. S4D). Chronologically, this event was followed by the activation of Casp-9 and -3 with BOR treatment for 6–8 h (Fig. S4E). However, at these time points, BOR failed to induce obvious apoptosis in Kasumi-1 cells (Fig. S4F). Compared with vehicle control, after treatment with BOR for 12 h, only 8% of the cells were committed to apoptosis (Fig. S4F), whereas no significant cell growth inhibition was visible (Fig. S14). Nevertheless, marked apoptotic effect was seen 24–48 h after incubation with BOR (Figs. S1B and S4F). In agreement with these observations, apoptosis was seen in CD34+ primary leukemia cells treated with BOR for 24–48 h (Fig. S1B). While Casp-3 is shown to be a major growth-stimulating signal to stimulate the repopulation of tumors undergoing radiotherapy (28), our results indicate that an early activation of Casp-3 is unable to initiate suicide program in *t*(8;21) cells, and other signals are required to amplify the apoptotic cascade.

We found that DY was able to inhibit BOR-caused down-regulation of pHsp90 β and release of Apaf-1 (Fig. S4D), consistent with the fact that DY could attenuate BOR-induced degradation of C-KIT (Fig. S3A). Moreover, we showed that Hsp90 β inhibitor 17-allylamino-demethoxy geldanamycin (17-AAG) reduced Kasumi-1 cell proliferation (Fig. S4G), and combined use of BOR (2.5 nM) and 17-AAG (0.1 μ M) caused a potentiated suppression of cell proliferation, whereas no such enhanced effect was seen in BOR/IM and 17-AAG/IM combinations (Fig. S4H).

BOR-Triggered Degradation of AE/AE9a and Generation of Cleavage Fragments. Interestingly, treatment of Kasumi-1 cells with BOR for 12 h resulted in degradation of the AE oncoprotein with generation of a 70-kDa cleavage fragment (CF), Δ AE (Fig. S5A), reminiscent of *t*(8;21) AML cells treated with oridonin (29) and triptolide (30). These phenomena were also seen in BOR-treated CD34+ cells derived from a *t*(8;21) patient (Fig. S5B). Moreover, when murine AML with expression of AE9a (10) was used as a model, in vitro and in vivo treatment with BOR caused AE9a down-regulation (Fig. S5C). Indeed, in HeLa cells transfected with a construct of AE9a coding region fused in

Fig. 4. Protein–protein interactions among C-KIT, Hsp90 β , and Apaf-1. (A) 293T cells were transfected with the plasmids indicated and lysed, and the proteins were purified by immunoprecipitation followed by incubation with C-KIT isolated from Kasumi-1 cells. Reciprocal coimmunoprecipitation and Western blot analyses were performed. (B and C) The cells were treated without or with BOR and lysed, and reciprocal coimmunoprecipitation and Western blotting were performed. (D) Diagram of constructs of C-KIT. CY, cytoplasmic domain. (E) 293T cells were transfected with pEGFP-C1 plasmids containing different domains of C-KIT and lysed, and coimmunoprecipitation and Western blot assays were conducted.

C-KIT tyrosine kinase activity is not responsible to apoptosis induced by BOR, and instead, C-KIT degradation might release an apoptosis initiator. These data also suggest that C-KIT may have an unrecognized role in programmed cell death. Indeed, we identify Hsp90 β as both a binding factor and a substrate of C-KIT. We find that, in the presence of C-KIT, Hsp90 β -Apaf-1 binding affinity (24, 26) is markedly enhanced; however, on BOR, Apaf-1 is released and then recruits cytochrome *c* to activate caspases. Therefore, our data not only uncover the critical role in apoptosis for C-KIT by indirect sequestration of Apaf-1 through phosphorylation of Hsp90 β , but also unveil mechanisms of action of BOR in cancer.

Ligand-induced down-regulation is an important aspect of the normal physiology of the cell surface receptors. While binding to its receptor, SCF accelerates the turnover of C-KIT by inducing internalization of the receptor ligand complexes followed by polyubiquitination and degradation (32). However, unlike BOR-induced C-KIT degradation, which leads to inactivation of pAKT/pSTAT3/pERK (Fig. S3A), SCF does not inhibit pAKT/pSTAT3 (Fig. S3C) and does not induce cell apoptosis (Fig. S3D). Because AKT is critical for C-KIT-mediated growth and survival of neoplastic cells (33) and AKT inhibitors can induce apoptosis of malignant cells (34), our results may at least partially explain the difference between the effects of BOR and SCF on C-KIT-driven cells. However, why BOR but not SCF inactivates AKT remains elusive, whereas their effects on protein-protein interaction may be critical. This possibility warrants additional exploration.

AE/AE9a-targeting strategies have been emerging in the recent years to further improve clinical outcome of *t*(8;21) AML (29, 30, 35). We show that AE/AE9a CFs can perturb AE/AE9a oligomerization, leading to inhibition of parental oncoproteins

and amplification of the Casp-3 signal to efficiently trigger apoptosis (Fig. S9). In *t*(8;21) AML, AE and AE9a are associated with C-KIT mutation/overexpression (8, 11), and AE is able to up-regulate C-KIT (8). Therefore, BOR represents a C-KIT, AE/AE9a double targeting agent that triggers a positive feedback signal network to induce apoptosis, and its efficacy on the murine *t*(8;21) AML model suggests its potential of clinical application in *t*(8;21) AML as well as other C-KIT-driven cancers.

Methods

Methods and associated references are in *SI Methods*. The CD34+ primary cells were isolated from patients with *t*(8;21) AML. The protein extracts of BOR-treated cells were analyzed by immunoprecipitation/Western blot assays. Immunofluorescence microscopy, clonogenic, in vitro phosphorylation, EMSA and luciferase assays were performed. The interested genes were transfected into cells or *Xenopus* embryos and their biological functions were investigated. AE9a+ cells were injected into irradiated mice, which were then treated with BOR.

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