Histone demethylase RBP2 mediates the blast crisis of chronic myeloid leukemia through an RBP2/PTEN/BCR-ABL cascade

Xiaolin Yin, Minran Zhou, Yue Fu, Lin Yang, Man Xu, Ting Sun, Xiaoming Wang, Tao Huang, Chunyan Chen*

Department of Hematology, Qilu Hospital, Shandong University, No. 107,Wenhua Xi Road, Jinan 250012, Shandong, PR China

ABSTRACT

Epigenetic disorders play a key role in tumorigenesis and development, among which histone methylation abnormalities are common. While patients living with chronic myeloid leukemia in the chronic phase (CML-CP) have a good response to TKI, blastic phase (CML-BP) patients demonstrate poor efficacy and high fatality rates. However, while the mechanism of blast crisis of chronic myeloid leukemia remains unclear, high expression and activation of BCR-ABL are usually related to CML blast crisis transition. We found that histone H3 lysine 4 (H3K4) demethylase RBP2 expression is negatively correlated with BCR-ABL expression, which suggests a regulatory link between these two genes. We also discovered that RBP2 mediates the dephosphorylation of BCR-ABL by directly downregulating PTEN expression, depending on histone demethylase activity, while PTEN targets protein phosphatase activity of BCR-ABL, a phosphatase which directly dephosphorylates BCR-ABL. In clinical specimens, the mRNA expression of RBP2 was found to be positively correlated with that of PTEN. These data suggest that the under-expression of RBP2 promotes blast crisis transition by activating an RBP2/PTEN/BCR-ABL cascade.

1. Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the BCR-ABL fusion gene, which forms a chimeric protein with deregulated tyrosine kinase activity [1,2]. The disease starts with an initial chronic phase (CP), before spontaneous progression to an accelerated phase (AP) and then finally a blastic phase (BP) [3,4].The vast majority of CML-CP patients are sensitive to tyrosine kinase inhibitors (TKI), which demonstrate a good curative effect. However, approximately 20% of CML-CP patients , and so spontaneously progress into the blastic phase are insensitive to TKI, including both Imatinib and second-generation TKI. Once the disease moves into the blastic phase, existing therapy does not have positive responses; fatality rates are extremely high. The median survival for CML-BP patients is just six months [5].

While the mechanism of blast crisis transition is both complex and highly heterogeneous, it tends to be accompanied by a high expression and activation of BCR-ABL. Uncontrolled activation of BCR-ABL is considered the driving force in promoting CML [5–7]. It may be speculated that CML-BP is a multi-step, time-dependent process initiated by mechanisms which are both dependent and independent of

BCR-ABL1 and which cause DNA damage associated with inefficient, unfaithful DNA repair in CML-CP, facilitating accumulation of additional genetic changes that lead to selection of CML-BP clones [8]. At present, BCR-ABL is mainly regulated by both transcription and post-transcriptional regulation. BCR-ABL dephosphorylation (inactivation) which is a post-transcriptional regulation, can be regulated with PP2A, SET, CIP2A, SHP1 and PTP1B [9–14]. There are many binding sites of SP1, Sry, E47 and other transcription factors in the BCR promoter [15]. BCR-ABL is also regulated by epigenetics at the transcriptional level. This means that β-arrestin1 in the nucleus, binding to EZH2, promotes CML progression by regulating BCR-ABL H4 acetylation [16]. However, whether or not BCR-ABL could be regulated by histone demethylase has not been well explored.

Epigenetics lead to in heritable changes in gene expression but no alteration in DNA sequence. Epigenetic modification, including DNA methylation, histone modification and so on, play a key role in carcinogenesis [17–19]. The retinoblastoma binding protein 2 (RBP2) belongs to the KDM5 family, and is also known as JARID1A or KDM5A. RBP2 can specifically target both tri- and di-methylated lysine 4 of histone H3 (H3-K4) for demethylation [20]. RBP2, which can regulate transcription and differentiation, contains a JmjC domain, which is a

---

histone demethylase signature motif [21]. The role of histone demethylase RBP2 as an oncogene or tumor suppressor in cancer remains controversial. It may have different functions in different types of cancer cells. Recently, RBP2 has been reported to participate in the initiation of cancers including gastric [22,23], lung [24], breast [25], malignant gliomas [26], renal cell carcinoma [27], ovarian [28] and hepatocellular carcinoma [29]. In our previous research, we found that RBP2 is under-expressed in CML-BP. RBP2 mediated CML blast crisis transition by regulating miR-21 in the BCR-ABL independent pathway [30]. However, whether RBP2 could mediate CML progression through the BCR-ABL dependent pathway remains unknown.

Protein tyrosine phosphorylation plays a key role in regulating the activity and stability of proteins. The state of tyrosine phosphorylation will depend on the balance between protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs) [31]. The phosphatases and tension homolog (PTEN) is the first tumor suppressor gene found to have a double specific phosphatase activity; that is, dephosphorylate protein and peptide substrates phosphorylated on serine, threonine and tyrosine residues [32,33]. PTEN can lead to PTK6 dephosphorylation of PY342 as well as PTK6 inhibition in prostate cancer [34]. The crystal structure analysis of PTEN demonstrated that its N-terminal domain contained a specific sequence of protein tyrosine phosphatase (PTP), which has a structure similar to that of double specific phosphatase VHR [35,36]. In contrast, PTP1B can mediate the dephosphorylation of BCR-ABL and inhibit transcriptional activation induced by BCR-ABL [12,13]. Peng and colleagues have shown that PTEN over-expression has a synergistic effect with imatinib. This synergy can significantly prolong survival time of mice [37]. PTEN can be inactivated by BCR-ABL in chronic myeloid leukemia [38–40]. Therefore, we speculate that PTEN may inhibit activation of BCR-ABL by dephosphorylating BCR-ABL.

We hypothesize that histone demethylase RBP2 mediates CML progression in the BCR-ABL dependent pathway. That is, under-expression of RBP2 leads to low PTEN expression depending on histone demethylase activity, which could upregulate BCR-ABL phosphorylation and activate the BCR-ABL signaling pathway as well as promoting CML blast crisis transition.

2. Materials and methods

2.1. Cell lines and cell culture

K562, MEG01 and HEK293 cell lines were obtained from and authenticated by the Typical Culture Preservation Commission Cell Bank, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI 1640 which contained 10% fetal bovine serum without antibiotics (FBS; Gibco, Carlsbad, CA, USA).

2.2. Transfection

Cells were transfected using RBP2, PTEN, BCR-ABL(P210) expression plasmid (purchased from addgene) or with stealth interference RNAs (siRNA) of PTEN, BCR-ABL [41] with Lipofactamine2000 (Invitrogen, Carlsbad, CA, USA), according to the protocol. Sequences of these siRNAs are listed in Table 1.

2.3. Patients and sample preparation

Bone-marrow samples were obtained from patients with newly diagnosed CML-CP (n = 26) and CML-BP (n = 16) who were treated at the Department of Hematology, Qilu Hospital of Shandong University in Jinan, China. Clinical characteristics of CML patients can be seen in Table 2. Mononuclear cells were isolated from samples and stored at −80 ˚C. This study was approved by the Ethics Committee of Shandong University School of Medicine.

Table 1
Sequences of siRNAs.

siRNAs		Sequences
BCR-ABL NC	Sense	5′-CAGUGUUCAUAAGCCGUUCAG-3′
	Antisense	3′-UCGUCACAAGUAUUCGGCAAGUC-5′
BCR-ABL siRNA	Sense	5′-CAGAGUUCAAAAGCCCUUCAG-3′
	Antisense	3′-UCGUCUCAAGUUUUCGGGAAGUC-5′
PTEN NC	Sense	5′-UUCUCCGAACGUGUCACGUTT-3′
	Antisense	5′-ACGUGACGUUCGGAGAATT-3′
PTEN siRNA	Sense	5′-CUAUUCAUGGAAGGAUUUAdTdT-3′
	Antisense	5′-UAAAUCCUUCCAUAG-dTdT-3′

Table 2
Clinical characteristics of CML patients.

Characteristic		Patients (n = 42)
Progression	CP	26
	BP	16
Gender	Male	25
	Female	17
Age(years)	Range	26–70
	Medium	44
WBC, ×10^9/L	Range	3.65–397.06
	Medium	55

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from human bone marrow samples and cells was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNA used a RevertAid First Strand DNA Synthesis (RT) kit (Fermentas Life Science, Canada) in order to reverse-transcriptase. Expressions of RBP2 and BCR-ABL mRNA were verified by PCR using TaqMan gene expression assay kit (Life Technologies, USA). The expression of other genes' mRNA were measured using SYBR Premix Ex Taq kit (Takara, Japan). Probes for RBP2 (Applied Biosystems) were Hs00231908_m1. TaqMan probes were placed to cover the fusion region of the b2a2 and b3a2 variants respectively. The probes b2a2: tgaccatcaataaggaagaagcccttcagc and b3a2: cagagttcaaaagcccttcagcggc were purchased labeled with 6-carboxy-fluorescein (FAM) (Applied Biosystems, Germany) [42]. Gene expression was normalized to that of β-actin. Expressions were calculated using the $2^{-\Delta\Delta CT}$method. The sequences of the primers used were shown in Table 3.

Table 3
Sequences of the primers used.

Primers		Sequences
ACTIN	Forward	AGTTGCGTTACACCCTTTCTTG
	Reverse	CACCTTCACCGTTCCAGTTTT
PTEN	Forward	TGGATTCGACTTAGACTTGAC-CT
	Reverse	GGTGGGTTATGGTCTTCAAAAGG
b2a2	Forward	TGTGAAACTCCAGACTGTCCACA
	Reverse	AAAGTCAGATGCTACTGGCCG
b3a2	Forward	TCCACTCAGCCACTGGATTTAA
	Reverse	CAGAGTTCAAAAGCCCTTCAGCGGC
PTP1B	Forward	GCAGATCGACAAGTCCGGG
	Reverse	GCCACTCTACATGGGAAGTCAC
SHP1	Forward	GGTGTCCACGGTAGCTTCC
	Reverse	ACAGGTCATAGAAATCCCCTGAG
SET	Forward	AGCAAGAAGCGATTGAACACA
	Reverse	TGGTTGGCGGAGTTTGTTATATT
PP2A	Forward	TCTCAGGCATACGCTGACTAC
	Reverse	GGAGACTCTGTACTCGAAGGT

Table 4
The detail information of primary antibodies used.

Antibody	Company	Product
RBP2	Abcam	ab70892
H3K4me2	Abcam	ab32356
H3K4me3	Abcam	ab8580
ACTIN	Sigma	A5441
P-STAT5	Cell Signaling Technology	4322
STAT5	Cell Signaling Technology	9363
P-ERK	Cell Signaling Technology	4376
ERK	Cell Signaling Technology	4695
PTEN	Cell Signaling Technology	9552
c-ABL	Cell Signaling Technology	2862
4G10	Millipore	2,654,211
GAPDH	Santa Cruz	sc-47,724

2.5. Western blotting

Cells were collected, washed twice in PBS and then lysed for 30 min on ice in DTT-buffer, supplemented with 1 mM PMSF. Total cellular proteins were separated using SDS-PAGE and transferred to PVDF membranes. Primary antibodies against RBP2 (1:1000, Abcam), PTEN (1:500, Cell Signaling Technology), c-ABL (1:1000, Cell Signaling Technology), and β-actin (1:10000, Sigma) were incubated overnight at 4 °C. The detail information of these primary antibodies can be seen in Table 4. Horseradish peroxidase-conjugated anti-rabbit as well as anti-mouse secondary antibodies (Jackson) were diluted 1:6000 and incubated at room temperature for 50 min. Antigens were revealed with Enhanced Chemiluminescence Reaction (ECL + , Millipore, USA).

2.6. Co-immunoprecipitation(Co-IP) and immunoblot analysis

Co-IP analysis was performed using a Pierce TM Co-Immunoprecipitation Kit (Thermo-Fisher, Waltham, MA, USA), following the manufacturer's instructions. Protein extracts were then incubated with 5 μg antibodies.

2.7. Chromatin immunoprecipitation (ChIP)

A Cell Signaling Technology ChIP Assay Kit was utilized to treat prepared K562 cells according to kit protocol. K562 cells were cross-linked by incubation in 37% formaldehyde solution for 10 min at 37 °C and then sonicated to develop soluble chromatin with DNA fragments, ranging in size from 200 to 800 bps. DNA was purified from the chromatin fragments which had been immunoprecipitated with antibodies against RBP2, H3K4me3, H3K4me2 (Abcam) and then used for PCR amplification. PCR primers for the PTEN promoter were as follows: forward primer: 5′-GTCGGAGTCAAGCTCGGT-3′, reverse primer: 5′-TCCTACCGTTCCGTACTTTC-C-3′.

2.8. Cell proliferation

We used 5-ethynyl-2′-deoxyuridine (EDU) assay to detect proliferative rates of K562 and MEG01 cells. Transfected cells were incubated with EDU for 2 h before fluorescent detection. Cells which had been treated were used to prepare cell smear with glass slides, fixed with 4% paraformaldehyde for 30 min and then stained using a Cell-Light™ EDU Apollo®488 In Vitro Imaging Kit (RioBio, China), following the manufacturer's instructions. Slides were examined by confocal laser scanning microscopy.

2.9. Soft agar assay

One ml of 1% agar in complete 2xDMEM, containing 20% fetal bovine serum (FBS), was plated as the basal layer in 6-well plates. Cells in complete medium containing 0.4% agar were seeded on the basal

layer. Plates were then incubated at 37 °C in a CO_2 incubator for a total of 21 days. Dense colonies were microscopically examined and counted on the final day.

2.10. Luciferase reporter assay

We transfected HEK293 cells with RBP2 plasmid on day one and the wild-type or mutant PTEN promoter reporter plasmid the following day. A thymidine kinase promoter was co-transfected to monitor transfection efficiency. After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity of the PTEN promoter reporter was found to be normalized to thymidine kinase renilla activity.

2.11. Statistical analysis

Data obtained from biological replicates are presented as means (± SD or SEM). Student's *t*-test and Pearson correlation efficiency were used to analyze differences between the groups, using GraphPad Prism for Windows version 5.00 (GraphPad Software, La Jolla, CA, USA). $P < .05$ was considered to signify a statistically significant difference.

3. Results

3.1. RBP2 over-expression inhibited BCR-ABL phosphorylation

Our previous study has shown that RBP2 is under-expressed in blast crisis of chronic myeloid leukemia (CML). RBP2 over-expression activated leukemia cell differentiation while inhibiting cell proliferation through BCR-ABL independent pathway [30]. Whether RBP2 plays a role in CML progression in the BCR-ABL dependent pathway remains unknown. Compared to the RBP2 expression in BCR-ABL positive and negative cell lines, we discovered that RBP2 protein level is lower in BCR-ABL positive cell lines than BCR-ABL negative cell lines (Fig. 1A–B). When transfecting RBP2 expression plasmid into MEG01 and K562 cell lines, levels of P-BCR-ABL protein decrease (Fig. 1C–D). However, no significant change of BCR-ABL protein (Fig. 1C) and mRNA (Fig. 1E) was found. Mononuclear cells were isolated from the bone marrow of one patient with newly diagnosed CML-CP, and RBP2 expression plasmid was transfected into these cells. When the RBP2 mRNA level increased, the level of BCR-ABL mRNA displayed no obvious change (Fig. 1G). These results suggest that RBP2 cannot regulate BCR-ABL expression transcriptionally, but is able to regulate BCR-ABL phosphorylation.

3.2. PTEN is a potential target for RBP2

Thus far, it remains unknown whether RBP2 has phosphorylation function. We therefore hypothesized that RBP2 inhibited BCR-ABL phosphorylation indirectly. We searched core regulatory factors related to the phosphorylation of BCR-ABL, such as PP2A, SET, SHP1, PTP1B and PTEN. Only the expression of phosphatase PTEN mRNA could be regulated by RBP2 (Fig. 2A–B). We found that the expression of RBP2 is positively correlated with the expression of PTEN. We investigated whether there was a correlation between RBP2 and PTEN and found that the expression of RBP2 is positively correlated with that of PTEN in samples of newly diagnosed CML-CP and CML-BP patients collected from Qilu hospital ($N = 42$, $R = 0.5621$, $P < .0001$) (Fig. 2C). Demographic details relating to these patients can be seen in Table 4. Following transfection of RBP2 expression plasmid into MEG01 and K562 cell lines, PTEN protein (Fig. 2D–F) and mRNA (Fig. 2A–B) levels showed obvious increases. In addition, in primary cells from the bone marrow of a CML-CP patient with RBP2 overexpression, PTEN expression was significantly increased (Fig. 2G). Furthermore, when compared to wild mice, accompanied with RBP2 under-expression, PTEN mRNA level was lower in RBP2 hybrid knock-out mice (Fig. 2H).
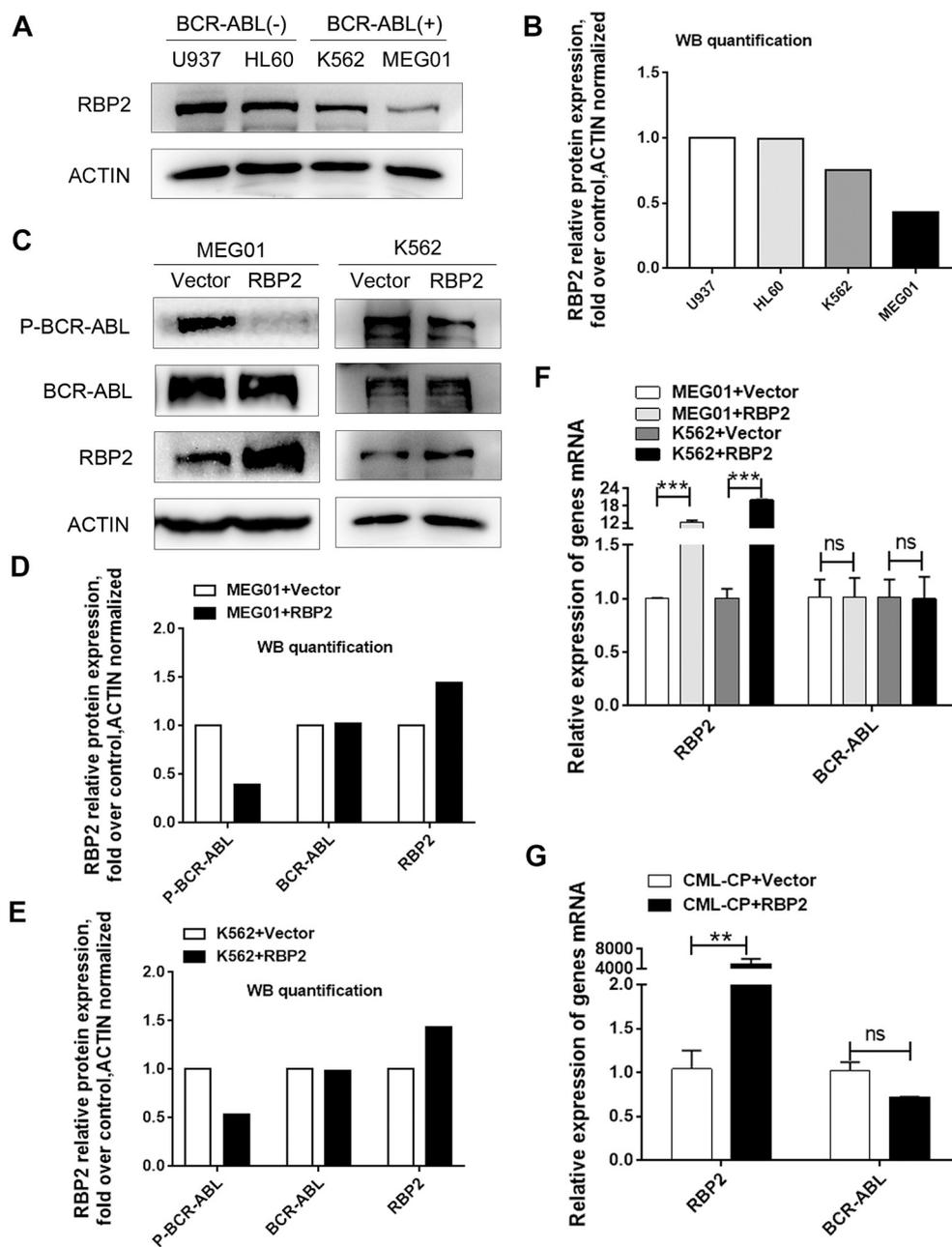
Fig. 1. RBP2 overexpression inhibited BCR-ABL phosphorylation. (A) Western Blot analysis of RBP2 protein level in leukemia cell lines, actin was a loading control. (B) Relative protein levels were quantified using the ImageJ program, with actin for normalization. (C)Western Blot analysis of RBP2, BCR-ABL and P-BCR-ABL protein levels in MEG01 and K562 cells following transfection with RBP2 expression plasmid, actin was a loading control. (D, E) Relative protein levels were quantified using the ImageJ program, with actin for normalization. (F) qRT-PCR analysis of RBP2 and BCR-ABL mRNA level following transfection with RBP2 expression plasmid in MEG01 and K562 for 48 h. (G)Primary cells were from the bone marrow of a CML-CP patient, transfected with RBP2 expression plasmid for 48 h. **$P < .01$, ***$P < .001$.

Therefore, PTEN is upregulated by RBP2 and may be a target gene of RBP2.

3.3. RBP2 upregulates the expression of PTEN by binding to its promoter depending on histone demethylase activity

In order to determine the mechanism by which RBP2 upregulates PTEN, RBP2 wild-type and RBP2-mutant defective in demethylase activity (H483A) expression plasmids were selected. Two plasmids were separately transfected into MEG01 and K562 cell lines. With RBP2 over-expression, PTEN protein (Fig. 3A–C) and mRNA (Fig. 3D) levels rose significantly. RBP2-H483A plasmid, over-expressing RBP2 but with no histone demethylase activity, could not regulate expression of PTEN protein (Fig. 3A–C) or mRNA (Fig. 3D). This suggests that RBP2 regulates PTEN expression depending on enzyme activity. To further explore whether PTEN is a direct target of RBP2, we found a binding motif (CCGCCC) in PTEN promoter region (Fig. 3E). To verify whether RBP2 directly binds to PTEN promoter, we performed a ChIP assay in K562

cells and found that RBP2 is bound to the region (Fig. 3F). Therefore, it appears that RBP2 upregulates the expression of PTEN by binding to its promoter, depending on histone demethylase activity (Fig. 3G). In order to determine the effect of RBP2 on PTEN promoter activity, we transfected both RBP2 plasmid and PTEN promoter reporter plasmid into the HEK293 cell line. With RBP2 over-expression, PTEN promoter activity was significantly increased, while there was no change in PTEN promoter activity on mutation of the binding site (Fig. 3H). There was no significant change in PTEN promoter activity with RBP2-H483A over-expression (Fig. 3H). These results suggest that RBP2 can regulate PTEN expression transcriptionally depending on enzyme activity.

3.4. PTEN regulates the downstream signaling pathway of BCR-ABL by regulating the phosphorylation of BCR-ABL

PTEN has the same domain PTP as PTP1B, which mediates the dephosphorylation of BCR-ABL as well as inhibiting the transcriptional activation induced by BCR-ABL. Therefore, we speculated that PTEN
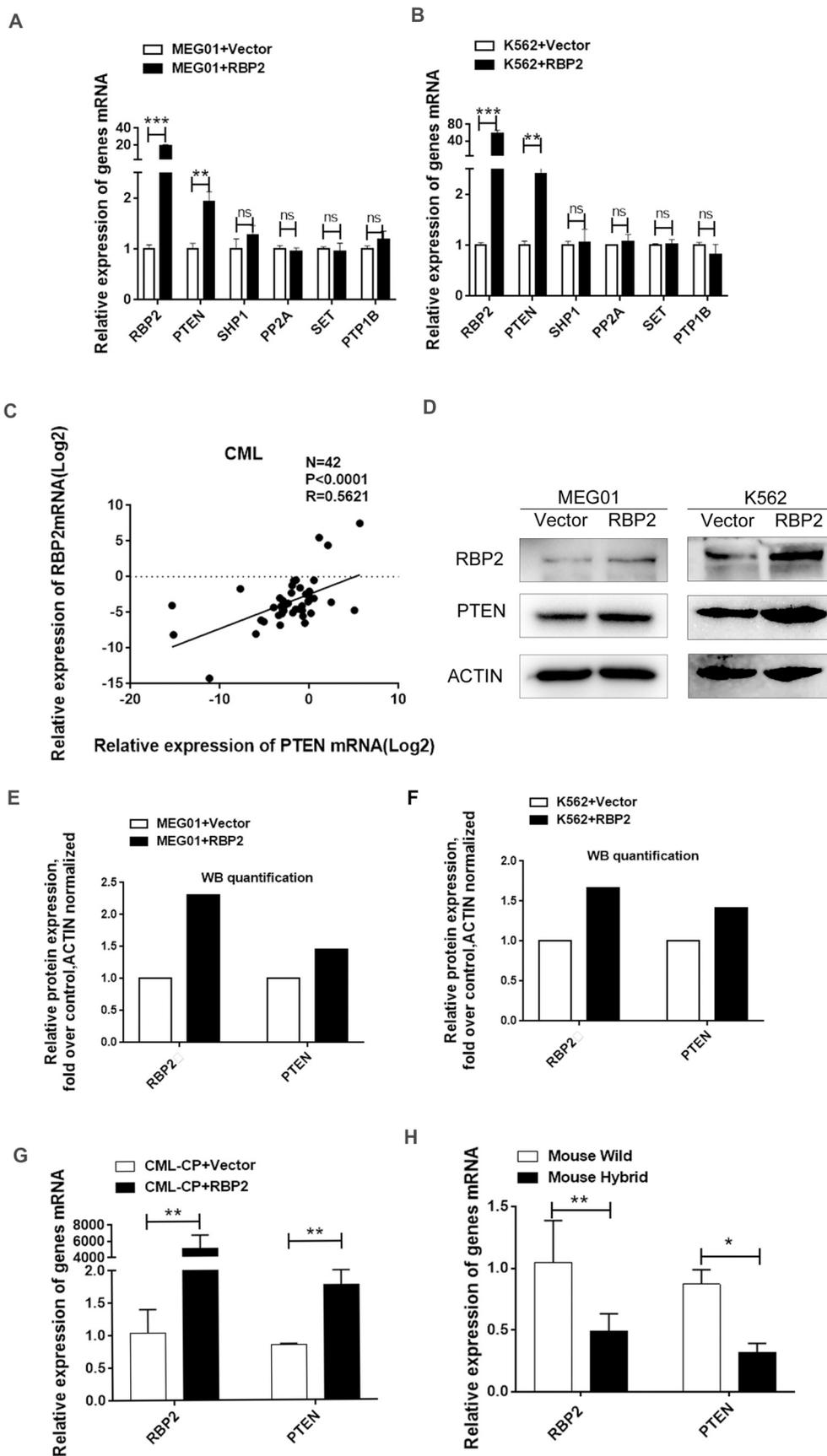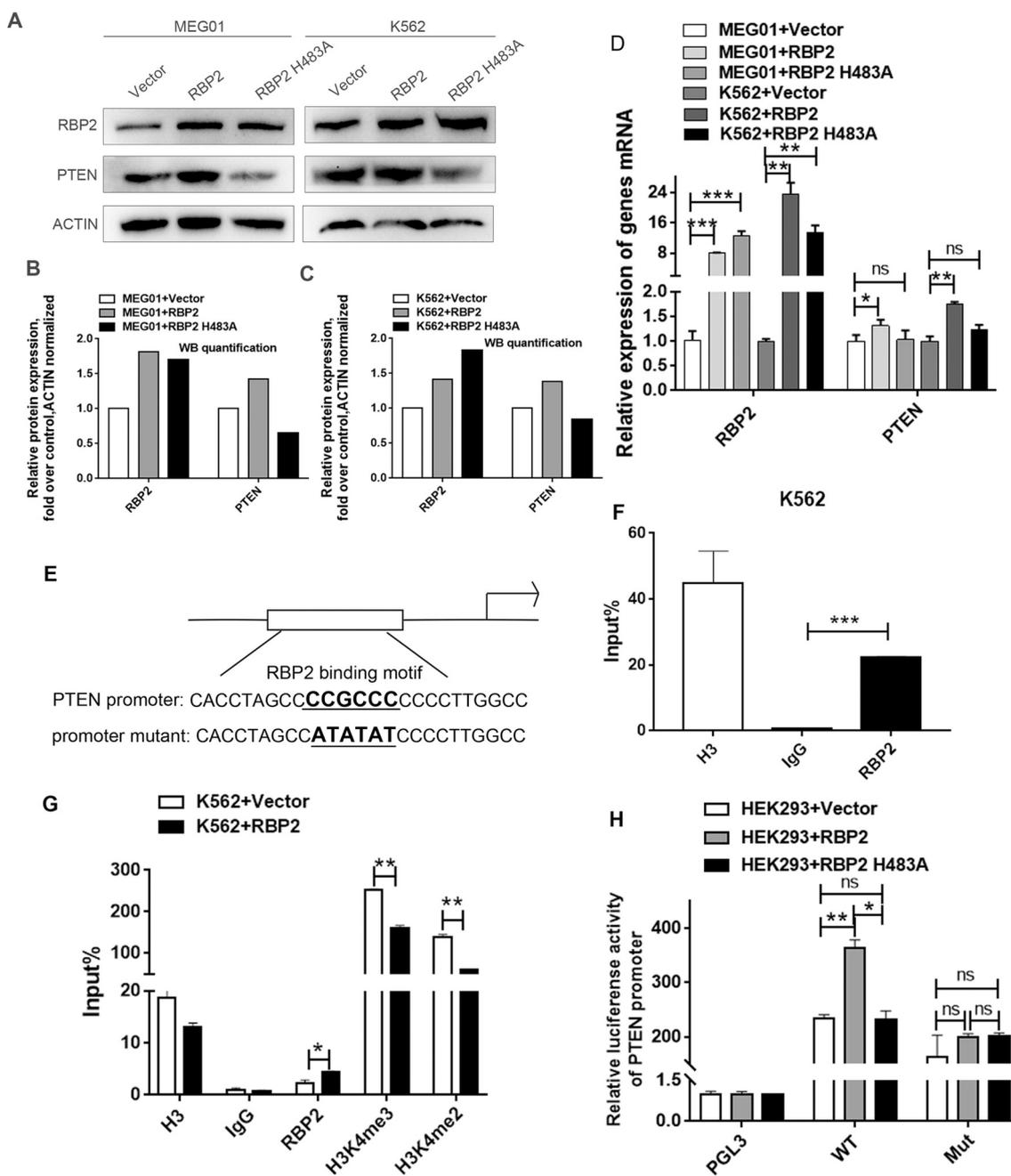
Fig. 2. PTEN is a potential target for RBP2. (A, B) qRT-PCR analysis of RBP2, SHP1, PP2A, SET, PTP1B and PTEN mRNA levels following transfection with RBP2 expression plasmid in MEG01 and K562 for 48 h. (C) Expressions of RBP2 and PTEN were analyzed by linear correlation in samples obtained from Qilu hospital. (D) Western Blot analysis of RBP2, PTEN protein levels in MEG01 and K562 cells following transfection with RBP2 expression plasmid. β-actin was a loading control. (E, F) Relative protein levels were quantified using the ImageJ program, with actin for normalization. (G) qRT-PCR analysis of RBP2 and PTEN mRNA levels following transfection with RBP2 expression plasmid in CML-CP primary cells for 48 h. (H) qRT-PCR analysis of RBP2 and PTEN mRNA levels in RBP2 hybrid knockout mice compared to wild mice. *P < .05, **P < .01, ***P < .001.

Fig. 3. RBP2 upregulates the expression of PTEN by binding to its promoter, depending on histone demethylase activity. (A) Western Blot analysis of RBP2 and PTEN protein levels in leukemia cell lines transfected with either RBP2 expression plasmid or RBP2-H483A plasmid. β-actin was a loading control. (B, C) Relative protein levels were quantified using the ImageJ program, with actin for normalization. (D) qRT-PCR analysis of RBP2 and PTEN mRNA level following transfection with RBP2 expression plasmid or RBP2-H483A plasmid in MEG01 and K562 cell lines. Data are mean ± SEM of three independent experiments. (E) RBP2 binding motif in PTEN promoter. (F) ChIP assay for binding RBP2 to PTEN promoter in K562 cells. (G) ChIP assay for binding RBP2, H3K4me3 and H3K4me2 to PTEN promoter in K562 cells. (H) PTEN promoter and mutated activity with RBP2 expression plasmid or RBP2-H483A expression plasmid transfection for HEK293 cells. Luciferase activities were determined at 48 h and normalized using Renilla luciferase activity. Results are from three independent experiments, only exemplary blot pictures are shown. $*P < .05$, $**P < .01$, $***P < .001$.

may inhibit BCR-ABL activation via the dephosphorylation of BCR-ABL. In order to determine whether PTEN regulates phosphorylation level of BCR-ABL, K562 cells were transfected with control siRNA or PTEN siRNA (Fig. 4A–C). PTEN was depleted, BCR-ABL phosphorylation level and its downstream targets p-STAT5 and p-ERK increased (Fig. 4A–C). MEG01 cells were transfected with PTEN expression plasmid; with PTEN overexpression, both BCR-ABL phosphorylation levels and BCR-ABL signaling pathway downstream targets p-STAT5 and p-ERK decreased (Fig. 4A–C). Following PTEN depletion in K562 cells or PTEN overexpression in MEG01 cells, BCR-ABL mRNA levels demonstrated no

significant change (Fig. 4D).

To explore the ways in which PTEN regulates BCR-ABL phosphorylation, we conducted both exogenous and endogenous co-immunoprecipitation experiments. PTEN and c-ABL antibodies were used for IP assay in K562 cells, which confirmed that PTEN and c-ABL can endogenously bind to each other (Fig. 4E). When PTEN and BCR-ABL expression plasmid were transfected into HEK293 cells at the same time, PTEN and c-ABL antibodies were used for IP assay after 48 h, which confirmed that ectopic PTEN and BCR-ABL can bind to each other (Fig. 4E). Therefore, overexpression of PTEN inhibits activation of
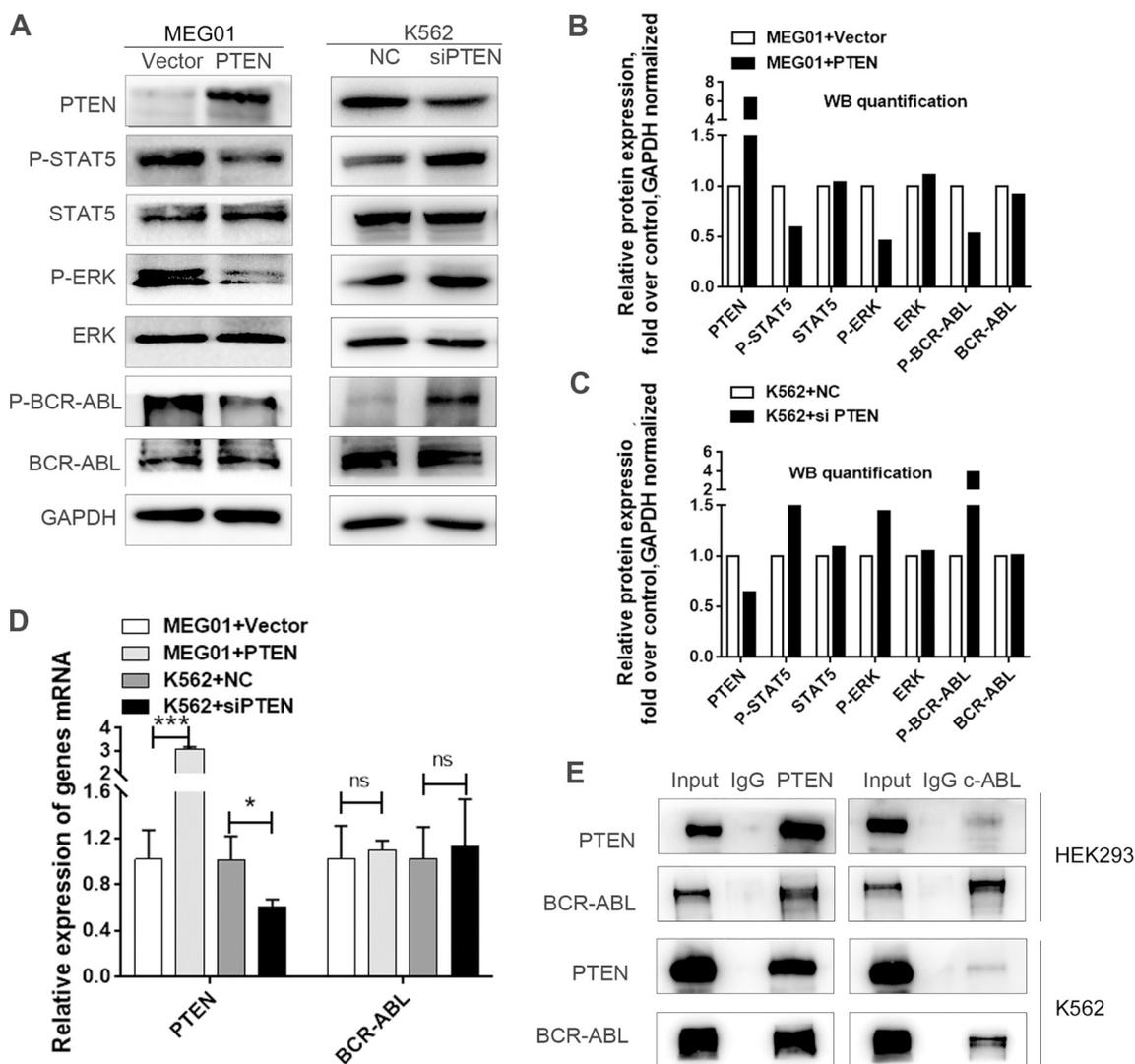
Fig. 4. PTEN regulates the downstream signaling pathway of BCR-ABL by regulating the phosphorylation of BCR-ABL. (A) Western Blot analysis of PTEN, BCR-ABL/ P- BCR-ABL, ERK/P-ERK and STAT5/P-STAT5 protein levels in leukemia cell lines transfected with PTEN expression plasmid or siRNA in MEG01 and K562 cells. GAPDH was a loading control. (B, C) Relative protein levels were quantified using the ImageJ program, with GAPDH for normalization. (D) qRT-PCR analysis of PTEN and BCR-ABL mRNA levels following transfection with PTEN expression plasmid or siRNA in MEG01, K562 for 48 or 72 h. Data are mean ± SEM of three independent experiments. (E) IP assay for binding PTEN to BCR-ABL in K562 and HEK293 cells. *$P < .05$.

BCR-ABL and its downstream signaling pathway by downregulating BCR-ABL phosphorylation through protein-protein interaction with BCR-ABL.

3.5. BCR-ABL inhibits RBP2 expression reversely forming a feedback loop

It is worth mentioning that we found RBP2 to be reversely regulated by BCR-ABL. K562 cells were incubated with imatinib (IM) at various concentrations (0, 0.5, 1.0 and 2.0 μM) for 24 h. The mRNA and protein expression of RBP2 were upregulated dose-dependently (Fig. 5A–C). Furthermore, K562 cells were incubated with IM at differing times (0, 12, 24 and 36 h), while RBP2 mRNA and protein expression were upregulated time-dependently (Fig. 5D–F). These results indicate that IM, a small molecule that inhibits the BCR-ABL kinase activity, is able to upregulate RBP2 expression in both concentration and a time-dependent manner. Moreover, BCR-ABL expression plasmid which has been transfected into K562 cells, RBP2 mRNA and protein expression was clearly downregulated (Fig. 5G–I). In contrast, when transfecting K562 cells with BCR-ABL siRNA, the mRNA and protein expression of RBP2 were upregulated (Fig. 5G–I). This means that BCR-ABL inhibits RBP2 expression, reversely forming a feedback loop.

3.6. Ectopic expression of RBP2 inhibits leukemia cell proliferation depending at least partially on BCR-ABL inhibition by RBP2

We investigated the potential role of RBP2 in leukemia cell proliferation by transfecting RBP2 expression plasmid into MEG01 and K562 cell lines. Following this, cells with RBP2 overexpression proliferated at a slower rate than the control group (Fig. 6A, C), while their ability to form colonies was also impaired (Fig. 6B, D). This means that RBP2 inhibited the proliferation of leukemia cells. Following transfection of RBP2 and BCR-ABL expression plasmid into MEG01 and K562 cell lines, BCR-ABL restored the inhibition of proliferation by RBP2 (Figs. 7A, 8B), while their ability to form colonies increased (Fig. 8A, C). The key finding was that RBP2 partially regulates CML cell proliferation through the BCR-ABL pathway.

4. Discussion

The blast crisis phase is the fatal stage of CML. While its mechanism is complex and highly heterogeneous, it tends to be accompanied by high expression and activation of BCR-ABL. Uncontrolled activation of BCR-ABL
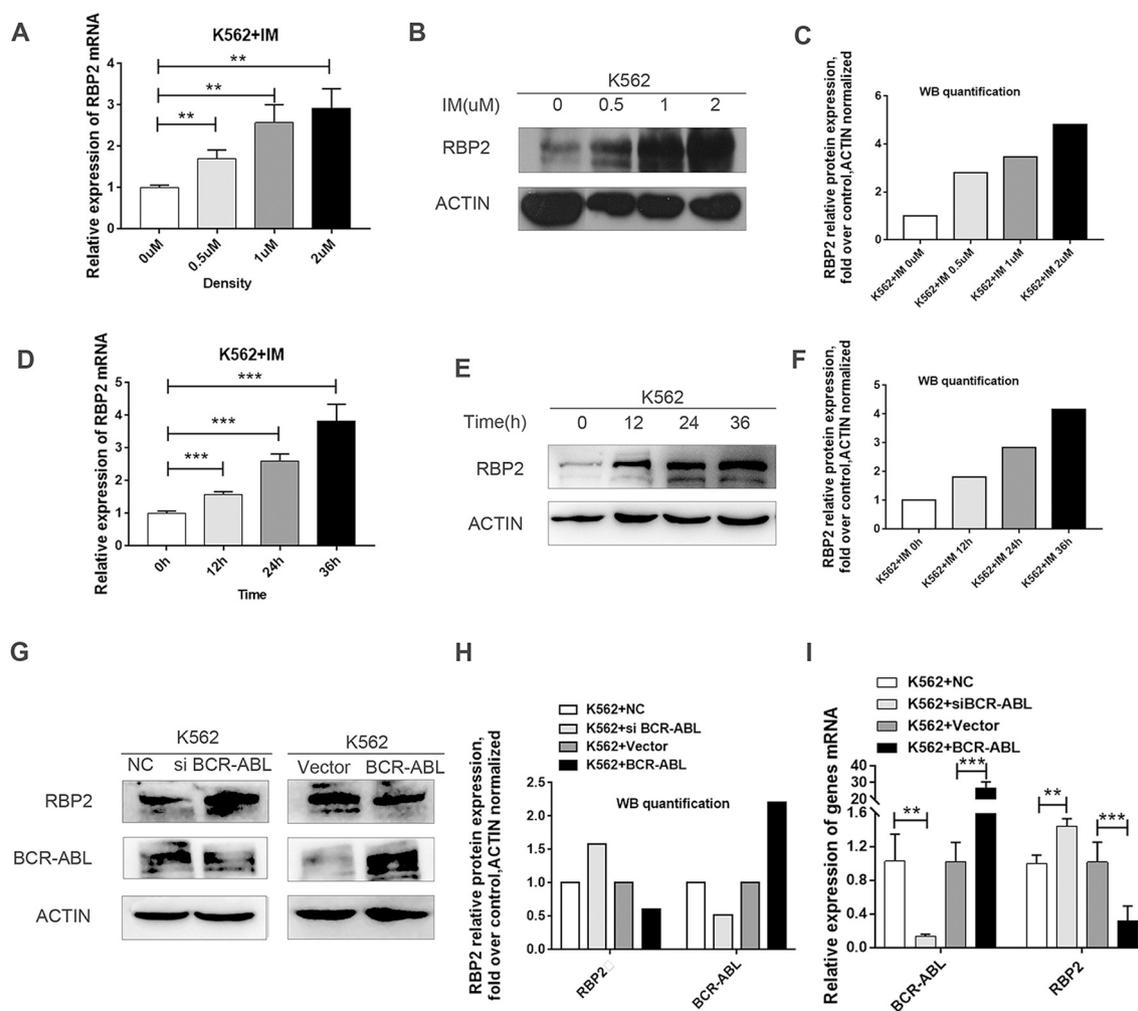
Fig. 5. BCR-ABL inhibits RBP2 expression, reversely forming a feedback loop. (A, D) qRT-PCR analysis of RBP2 mRNA levels following treatment with IM in K562 cells for different concentrations or times. Data are mean ± SEM of three independent experiments. (B, E) Western Blot analysis of RBP2 protein levels following treatment with IM in K562 cells for different concentration or time. β-actin was a loading control. (C, F) Relative protein levels were quantified using the ImageJ program, with actin for normalization. (G, I) Western Blot and qRT-PCR analysis of RBP2 and BCR-ABL protein and mRNA levels in leukemia cell lines transfected with BCR-ABL expression plasmid or siRNA. (H)Relative protein levels were quantified using the ImageJ program, with actin for normalization. **P < .01, ***P < .001.

may be considered the driving force for promoting CML [5,43,44]. A growing number of studies have suggested that epigenetic regulation is involved in the progression of CML from CP to BP [45,46]. We posit that there is a relationship between BCR-ABL and epigenetic regulation. Our focus is on histone demethylase. Until now, regulation of BCR-ABL by histone demethylase in this type of disease progression has not been described.

It has been reported that histone demethylase RBP2 participates in carcinogenesis [22–29]. Our previous study demonstrated that histone demethylase RBP2 is downregulated in CML-BP compared with CML-CP. RBP2 is involved in the progression of CML from CP to BP by downregulating miR-21 expression, independent of the BCR-ABL pathway [30]. In this study, we found that RBP2 is involved in CML progression in the BCR-ABL dependent pathway. By examining RBP2 and PTEN expression in samples from Qilu hospital, we found that expression of RBP2 correlates positively with PTEN expression. RBP2 overexpression significantly upregulated PTEN expression in K562, HL60 and CML primary cells, while RBP2 binds to the PTEN promoter and upregulates its transcription. However, when the binding site was mutated, activation disappeared. In addition, a ChIP assay demonstrated that RBP2 directly bound to the promoter of PTEN.

PTEN is often lost or inactivated in multiple solid tumor types, but is a critical regulator of the PI3K/Akt signaling pathway [47]. PTEN

results in PTK6 dephosphorylation of PY342 and PTK6 inhibition in prostate cancer [34]. PTEN has the same function as PTP1B, which can regulate BCR-ABL dephosphorylation [13]. However, whether PTEN can mediate BCR-ABL dephosphorylation remains unknown. We found that under-expression of PTEN significantly upregulated phosphorylation level of BCR-ABL. In order to verify the mechanisms, co-IP assays were applied, demonstrating the ways in which PTEN and BCR-ABL interact with each other. Further investigation is needed to confirm at which tyrosine residue PTEN dephosphorylates BCR-ABL.

Furthermore, BCR-ABL is able to regulate RBP2 expression in return. However, the exact mechanism for how BCR-ABL regulates RBP2 needs further investigation. The major finding here is a new epigenetic mechanism for regulating BCR-ABL in CML-BP pathogenesis (Fig. 9). In CML, a low expression of RBP2 leads to low PTEN expression, which results in a high level of BCR-ABL phosphorylation and promotes the transition of CML from CP to BP.

5. Conclusion

Downregulation of RBP2 promoted BCR-ABL phosphorylation by directly downregulating PTEN expression, while the under-expression of RBP2 promoted blast crisis transition via an RBP2/PTEN/BCR-ABL
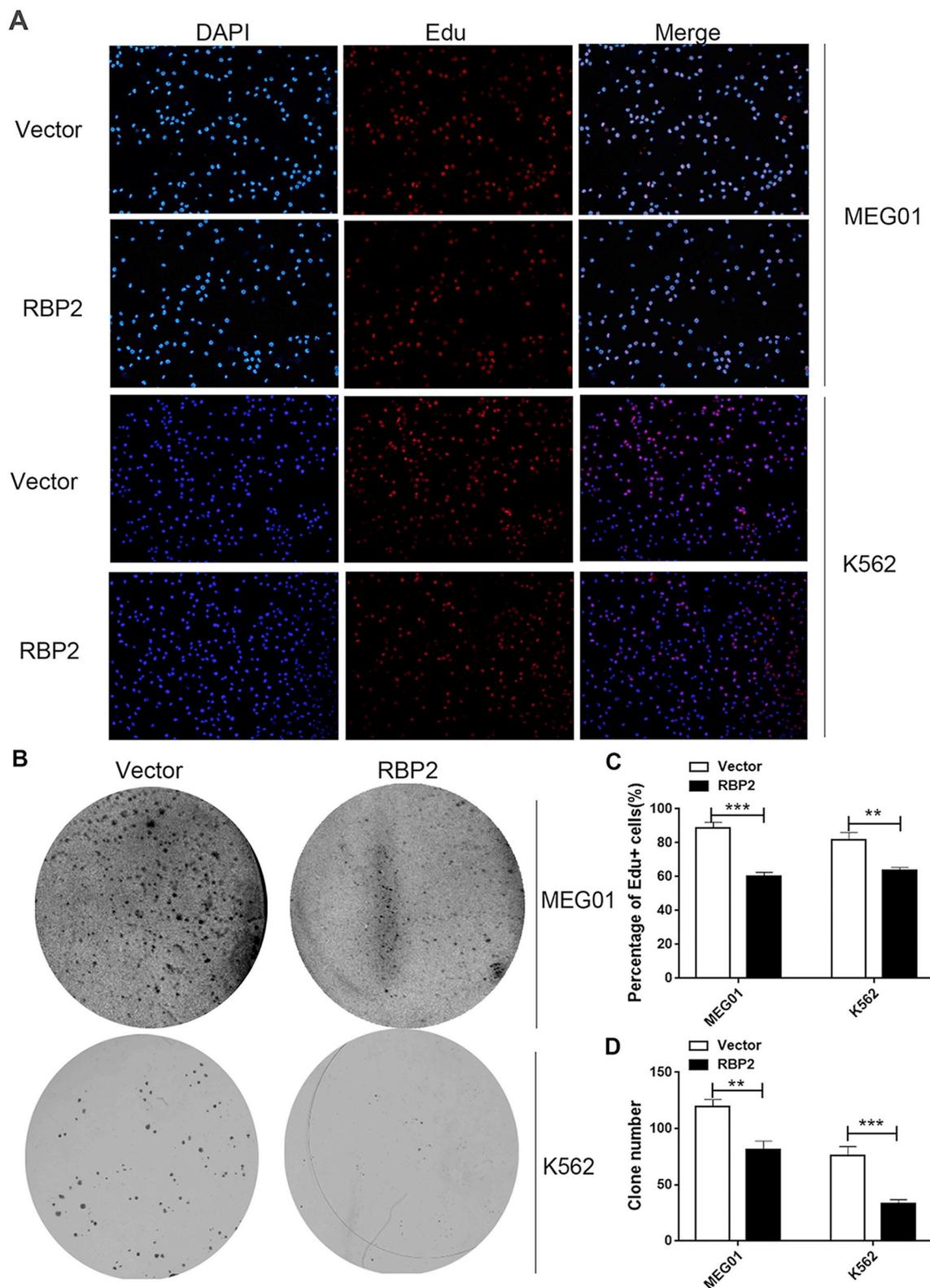
Fig. 6. Ectopic expression of RBP2 inhibits leukemia cell proliferation. (A, C) EDU of MEG01 and K562 cells following transfection with RBP2 expression plasmid. (B, D) Foci formation of MEG01 and K562 cells following transfection with RBP2 expression plasmid. *P < .05, **P < .01, ***P < .001.

cascade. These findings may provide new insights into the mechanisms of CML blast crisis transition.

Availability of data and materials

All data generated or analyzed during this study were included in this published article and its additional file.

Consent for publication

Each person provided signed informed consent for publication of the results of the study.

Fig. 7. Ectopic expression of RBP2 inhibits leukemia cell proliferation depending at least partially on BCR-ABL inhibition by RBP2. (A) EDU of MEG01 and K562 cells following transfection with RBP2 expression plasmid or RBP2 + BCR-ABL expression plasmid.

Declaration of Competing Interest
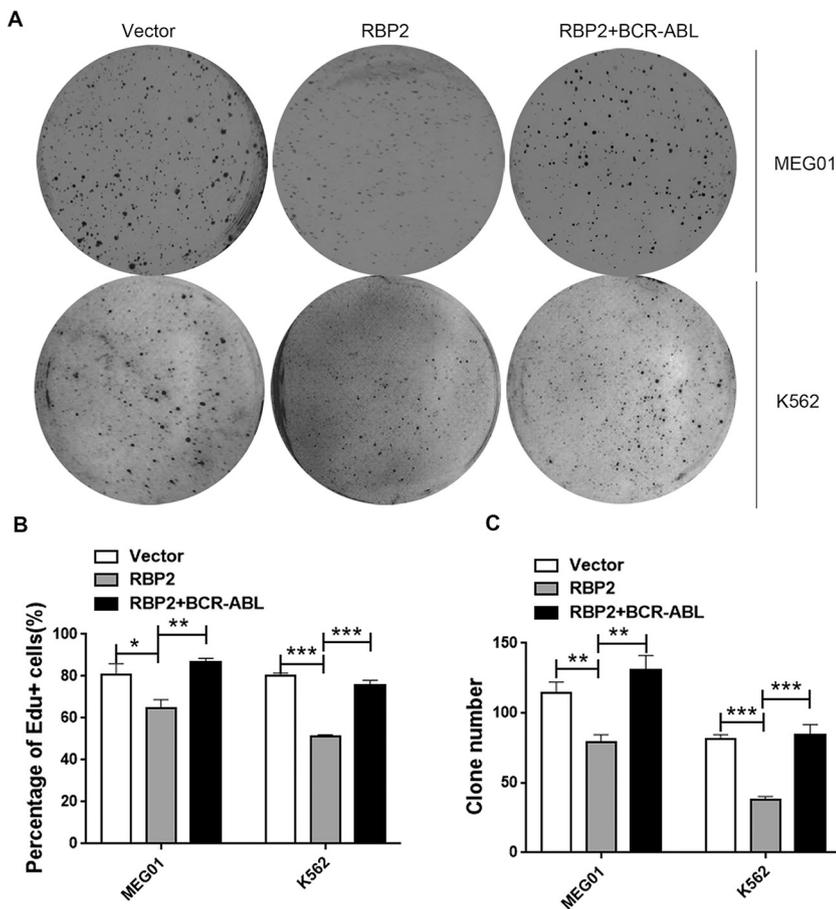
None.

A

B

C

Fig. 8. Ectopic expression of RBP2 inhibits leukemia cell proliferation depending at least partially on BCR-ABL inhibition by RBP2. (A, C) Foci formation of MEG01 and K562 cells following transfection with RBP2 expression plasmid or RBP2 + BCR-ABL expression plasmid. (B) EDU of MEG01 and K562 cells following transfection with RBP2 expression plasmid or RBP2 + BCR-ABL expression plasmid. *$P < .05$, **$P < .01$, ***$P < .001$.
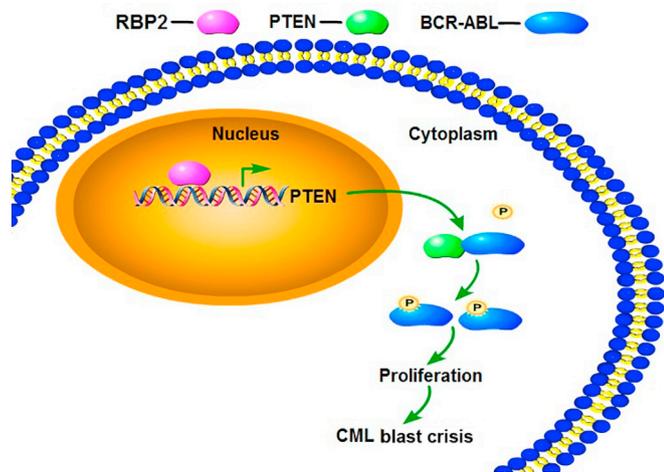

Fig. 9. A model of RBP2/PTEN/BCR-ABL cascade. A new mechanism involved in CML blast crisis transition; RBP2 is under-expressed during CML progression. RBP2 can regulate expression of PTEN by directly binding to the PTEN promoter locus. PTEN, which is down-regulated by low-expressed RBP2, activates the BCR-ABL signal pathway. In CML progression, low levels of RBP2 can repress PTEN, which increases p-BCR-ABL expression to stimulate cell proliferation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.109360.

References

[1] J.D. Rowley, Letter: a new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining, Nature 243 (1973) 290–293.

[2] N.C. Cross, G.Q. Daley, A.R. Green, T.P. Hughes, C. Jamieson, P. Manley, T. Mughal, D. Perrotti, J. Radich, R. Skoda, S. Soverini, W. Vainchenker, S. Verstovsek, J.L. Villeval, J.M. Goldman, BCR-ABL1-positive CML and BCR-ABL1-negative chronic myeloproliferative disorders: some common and contrasting features, Leukemia 22 (2008) 1975–1989.

[3] S. Prost, F. Relouzat, M. Spentchian, Y. Ouzegdouh, J. Saliba, G. Massonnet, J.P. Beressi, E. Verhoeyen, V. Raggueneau, B. Maneglier, S. Castaigne, C. Chomienne, S. Chretien, P. Rousselot, P. Leboulch, Erosion of the chronic myeloid leukaemia stem cell pool by PPAR gamma agonists, Nature 525 (2015) 380.

[4] M. Albajar, M.T. Gomez-Casares, J. Llorca, I. Mauleon, J.P. Vaque, J.C. Acosta, A. Bermudez, N. Donato, M.D. Delgado, J. Leon, MYC in chronic myeloid leukemia: induction of aberrant DNA synthesis and association with poor response to imatinib, Mol. Cancer Res. 9 (2011) 564–576.

[5] D. Perrotti, C. Jamieson, J. Goldman, T. Skorski, Chronic myeloid leukemia: mechanisms of blastic transformation, J. Clin. Invest. 120 (2010) 2254–2264.

[6] A.H. Elmaagacli, D.W. Beelen, B. Opalka, S. Seeber, U.W. Schaefer, The amount of BCR-ABL fusion transcripts detected by the real-time quantitative polymerase chain reaction method in patients with Philadelphia chromosome positive chronic myeloid leukemia correlates with the disease stage, Ann. Hematol. 79 (2000) 424–431.

[7] D.J. Barnes, B. Schultheis, S. Adedeji, J.V. Melo, Dose-dependent effects of Bcr-Abl in cell line models of different stages of chronic myeloid leukemia, Oncogene 24 (2005) 6432–6440.

[8] T. Skorski, Genetic mechanisms of chronic myeloid leukemia blastic transformation, Curr. Hematol. Malig. Rep. 7 (2012) 87–93.

[9] P. Neviani, R. Santhanam, R. Trotta, M. Notari, B.W. Blaser, S. Liu, H. Mao, J.S. Chang, A. Galietta, A. Uttam, D.C. Roy, M. Valtieri, R. Bruner-Klisovic, M.A. Caligiuri, C.D. Bloomfield, G. Marcucci, D. Perrotti, The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein, Cancer Cell 8 (2005) 355–368.

[10] P. Neviani, R. Santhanam, J.J. Oaks, A.M. Eiring, M. Notari, B.W. Blaser, S. Liu, R. Trotta, N. Muthusamy, C. Gambacorti-Passerini, B.J. Druker, J. Cortes, G. Marcucci, C.S. Chen, N.M. Verrills, D.C. Roy, M.A. Caligiuri, C.D. Bloomfield, J.C. Byrd, D. Perrotti, FTY720, a new alternative for treating blast crisis chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphocytic leukemia, J. Clin. Invest. 117 (2007) 2408–2421.

[11] P. Chen, M. Levis, P. Brown, K.T. Kim, J. Allebach, D. Small, FLT3/ITD mutation

signaling includes suppression of SHP-1, J. Biol. Chem. 280 (2005) 5361–5369.

[12] N. Koyama, S. Koschmieder, S. Tyagi, I. Portero-Robles, J. Chromic, S. Myloch, H. Nurnberger, T. Rossmanith, W.K. Hofmann, D. Hoelzer, O.G. Ottmann, Inhibition of phosphotyrosine phosphatase 1B causes resistance in BCR-ABL-positive leukemia cells to the ABL kinase inhibitor STI571, Clin. Cancer Res. 12 (2006) 2025–2031.

[13] D. Alvira, R. Naughton, L. Bhatt, S. Tedesco, W.D. Landry, T.G. Cotter, Inhibition of protein-tyrosine phosphatase 1B (PTP1B) mediates ubiquitination and degradation of Bcr-Abl protein, J. Biol. Chem. 286 (2011) 32313–32323.

[14] C.M. Lucas, R.J. Harris, A. Giannoudis, M. Copland, J.R. Slupsky, R.E. Clark, Cancerous inhibitor of PP2A (CIP2A) at diagnosis of chronic myeloid leukemia is a critical determinant of disease progression, Blood 117 (2011) 6660–6668.

[15] M. Marega, R.G. Piazza, A. Pirola, S. Redaelli, A. Mogavero, I. Iacobucci, I. Meneghetti, M. Parma, E.M. Pogliani, C. Gambacorti-Passerini, BCR and BCR-ABL regulation during myeloid differentiation in healthy donors and in chronic phase/ blast crisis CML patients, Leukemia 24 (2010) 1445–1449.

[16] R. Qin, K. Li, X. Qi, X. Zhou, L. Wang, P. Zhang, L. Zou, beta-Arrestin1 promotes the progression of chronic myeloid leukaemia by regulating BCR/ABL H4 acetylation, Br. J. Cancer 111 (2014) 568–576.

[17] P. Sroczynska, V.A. Cruickshank, J.P. Bukowski, S. Miyagi, F.O. Bagger, J. Walfridsson, M.B. Schuster, B. Porse, K. Helin, shRNA screening identifies JMJD1C as being required for leukemia maintenance, Blood 123 (2014) 1870–1882.

[18] M. Chen, N. Zhu, X.C. Liu, B. Laurent, Z.Y. Tang, R. Eng, Y. Shi, S.A. Armstrong, R.G. Roeder, JMJD1C is required for the survival of acute myeloid leukemia by functioning as a coactivator for key transcription factors, Genes Dev. 29 (2015) 2123–2139.

[19] A. Duckworth, M. Glenn, J.R. Slupsky, G. Packham, N. Kalakonda, Variable induction of PRDM1 and differentiation in chronic lymphocytic leukemia is associated with anergy, Blood 123 (2014) 3277–3285.

[20] J. Christensen, K. Agger, P.A.C. Cloos, D. Pasini, S. Rose, L. Sennels, J. Rappsilber, K.H. Hansen, A.E. Salcini, K. Helin, RBP2 belongs to a family of demethylases, specific for tri- and dimethylated lysine 4 on histone 3, Cell 128 (2007) 1063–1076.

[21] R.J. Klose, Q. Yan, Z. Tothova, K. Yamane, H. Erdjument-Bromage, P. Tempst, D.G. Gilliland, Y. Zhang, W.G. Kaelin Jr., The retinoblastoma binding protein RBP2 is an H3K4 demethylase, Cell 128 (2007) 889–900.

[22] L. Li, L. Wang, P. Song, X. Geng, X. Liang, M. Zhou, Y. Wang, C. Chen, J. Jia, J. Zeng, Critical role of histone demethylase RBP2 in human gastric cancer angiogenesis, Mol. Cancer 13 (2014) 81.

[23] J. Zeng, Z. Ge, L. Wang, Q. Li, N. Wang, M. Bjorkholm, J. Jia, D. Xu, The histone demethylase RBP2 is overexpressed in gastric cancer and its inhibition triggers senescence of cancer cells, Gastroenterology 138 (2010) 981–992.

[24] Y.C. Teng, C.F. Lee, Y.S. Li, Y.R. Chen, P.W. Hsiao, M.Y. Chan, F.M. Lin, H.D. Huang, Y.T. Chen, Y.M. Jeng, C.H. Hsu, Q. Yan, M.D. Tsai, L.J. Juan, Histone demethylase RBP2 promotes lung tumorigenesis and cancer metastasis, Cancer Res. 73 (2013) 4711–4721.

[25] J. Cao, Z. Liu, W.K. Cheung, M. Zhao, S.Y. Chen, S.W. Chan, C.J. Booth, D.X. Nguyen, Q. Yan, Histone demethylase RBP2 is critical for breast cancer progression and metastasis, Cell Rep. 6 (2014) 868–877.

[26] B. Dai, H. Huang, F. Guan, G. Zhu, Z. Xiao, B. Mao, H. Su, Z. Hu, Histone demethylase KDM5A inhibits glioma cells migration and invasion by down regulating ZEB1, Biomed. Pharmacother. 99 (2018) 72–80.

[27] D. Zhou, V. Kannappan, X. Chen, J. Li, X. Leng, J. Zhang, S. Xuan, RBP2 induces stem-like cancer cells by promoting EMT and is a prognostic marker for renal cell carcinoma, Exp. Mol. Med. 48 (2016) e238.

[28] T. Feng, Y. Wang, Y. Lang, Y. Zhang, KDM5A promotes proliferation and EMT in ovarian cancer and closely correlates with PTX resistance, Mol. Med. Rep. 16 (2017) 3573–3580.

[29] Z.Y. Wang, J. Yang, C.K. Liu, S.Q. Shen, High expression of retinoblastoma-binding protein 2 (RBP2) in patients with hepatocellular carcinoma and its prognostic significance, Med. Sci. Monit. 23 (2017) 2736–2744.

[30] M. Zhou, J. Zeng, X. Wang, X. Wang, T. Huang, Y. Fu, T. Sun, J. Jia, C. Chen, Histone demethylase RBP2 decreases miR-21 in blast crisis of chronic myeloid leukemia, Oncotarget 6 (2015) 1249–1261.

[31] H.P. Monteiro, R.J. Arai, L.R. Travassos, Protein tyrosine phosphorylation and protein tyrosine nitration in redox signaling, Antioxid. Redox Signal. 10 (2008) 843–889.

[32] M.P. Myers, J.P. Stolarov, C. Eng, J. Li, S.I. Wang, M.H. Wigler, R. Parsons, N.K. Tonks, P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 9052–9057.

[33] M. Cui, A. Augert, M. Rongione, K. Conkrite, S. Parazzoli, A.Y. Nikitin, N. Ingolia, D. MacPherson, PTEN is a potent suppressor of small cell lung cancer, Mol. Cancer Res. 12 (2014) 654–659.

[34] D.J. Wozniak, A. Kajdacsy-Balla, V. Macias, S. Ball-Kell, M.L. Zenner, W. Bie, A.L. Tyner, PTEN is a protein phosphatase that targets active PTK6 and inhibits PTK6 oncogenic signaling in prostate cancer, Nat. Commun. 8 (2017) 1508.

[35] M.S. Song, L. Salmena, P.P. Pandolfi, The functions and regulation of the PTEN tumour suppressor, Nat. Rev. Mol. Cell Biol. 13 (2012) 283–296.

[36] J.O. Lee, H. Yang, M.M. Georgescu, A. Di Cristofano, T. Maehama, Y. Shi, J.E. Dixon, P. Pandolfi, N.P. Pavletich, Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association, Cell 99 (1999) 323–334.

[37] C. Peng, Y. Chen, Z. Yang, H. Zhang, L. Osterby, A.G. Rosmarin, S. Li, PTEN is a tumor suppressor in CML stem cells and BCR-ABL-induced leukemias in mice, Blood 115 (2010) 626–635.

[38] A. Morotti, D. Torti, G. Carra, C. Panuzzo, S. Crivellaro, R. Taulli, C. Fava, A. Guerrasio, G. Saglio, Unleashing the guardian: the targetable BCR-ABL/HAUSP/ PML/PTEN network in chronic myeloid leukemia, Curr. Drug Targets 18 (2017) 389–395.

[39] A. Morotti, C. Panuzzo, S. Crivellaro, G. Carra, C. Fava, A. Guerrasio, P.P. Pandolfi, G. Saglio, BCR-ABL inactivates cytosolic PTEN through Casein Kinase II mediated tail phosphorylation, Cell Cycle 14 (2015) 973–979.

[40] C. Panuzzo, S. Crivellaro, G. Carra, A. Guerrasio, G. Saglio, A. Morotti, BCR-ABL promotes PTEN downregulation in chronic myeloid leukemia, PLoS One 9 (2014) e110682.

[41] L. Wohlbold, H. van der Kuip, C. Miething, H.P. Vornlocher, C. Knabbe, J. Duyster, W.E. Aulitzky, Inhibition of bcr-abl gene expression by small interfering RNA sensitizes for imatinib mesylate (STI571), Blood 102 (2003) 2236–2239.

[42] M. Eder, K. Battmer, S. Kafert, A. Stucki, A. Ganser, B. Hertenstein, Monitoring of BCR-ABL expression using real-time RT-PCR in CML after bone marrow or peripheral blood stem cell transplantation, Leukemia 13 (1999) 1383–1389.

[43] E. Bolton-Gillespie, M. Schemionek, H.U. Klein, S. Flis, G. Hoser, T. Lange, M. Nieborowska-Skorska, J. Maier, L. Kerstiens, M. Koptyra, M.C. Muller, H. Modi, T. Stoklosa, I. Seferynska, R. Bhatia, T.L. Holyoake, S. Koschmieder, T. Skorski, Genomic instability may originate from imatinib-refractory chronic myeloid leukemia stem cells, Blood 121 (2013) 4175–4183.

[44] A. Slupianek, R. Falinski, P. Znojek, T. Stoklosa, S. Flis, V. Doneddu, D. Pytel, E. Synowiec, J. Blasiak, A. Bellacosa, T. Skorski, BCR-ABL1 kinase inhibits uracil DNA glycosylase UNG2 to enhance oxidative DNA damage and stimulate genomic instability, Leukemia 27 (2013) 629–634.

[45] T. Dunwell, L. Hesson, T.A. Rauch, L. Wang, R.E. Clark, A. Dallol, D. Gentle, D. Catchpoole, E.R. Maher, G.P. Pfeifer, F. Latif, A genome-wide screen identifies frequently methylated genes in haematological and epithelial cancers, Mol. Cancer 9 (2010) 44.

[46] J. Roman-Gomez, A. Jimenez-Velasco, X. Agirre, J.A. Castillejo, G. Navarro, E.S. Jose-Eneriz, L. Garate, L. Cordeu, F. Cervantes, F. Prosper, A. Heiniger, A. Torres, Epigenetic regulation of PRAME gene in chronic myeloid leukemia, Leuk. Res. 31 (2007) 1521–1528.

[47] J.L. Zhang, Z.F. Xiang, P.A. Malaviarachchi, Y. Yan, N.J. Baltz, P.D. Emanuel, Y.L. Liu, PTEN is indispensable for cells to respond to MAPK inhibitors in myeloid leukemia, Cell. Signal. 50 (2018) 72–79.