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ORIGINAL ARTICLE

BCR–ABL-mediated upregulation of *PRAME* is responsible for knocking down *TRAIL* in CML patients

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Tumor necrosis factor-related apoptosis-inducing ligand—TNFSF10 (TRAIL), a member of the TNF-α family and a death receptor ligand, was shown to selectively kill tumor cells. Not surprisingly, TRAIL is downregulated in a variety of tumor cells, including BCR–ABL-positive leukemia. Although we know much about the molecular basis of TRAIL-mediated cell killing, the mechanism responsible for TRAIL inhibition in tumors remains elusive because (a) *TRAIL* can be regulated by retinoic acid (RA); (b) the tumor antigen preferentially expressed antigen of melanoma (PRAME) was shown to inhibit transcription of RA receptor target genes through the polycomb protein, enhancer of zeste homolog 2 (EZH2); and (c) we have found that *TRAIL* is inversely correlated with *BCR–ABL* in chronic myeloid leukemia (CML) patients. Thus, we decided to investigate the association of *PRAME*, *EZH2* and *TRAIL* in BCR–ABL-positive leukemia. Here, we demonstrate that *PRAME*, but not *EZH2*, is upregulated in BCR–ABL cells and is associated with the progression of disease in CML patients. There is a positive correlation between *PRAME* and *BCR–ABL* and an inverse correlation between *PRAME* and *TRAIL* in these patients. Importantly, knocking down *PRAME* or *EZH2* by RNA interference in a BCR–ABL-positive cell line restores *TRAIL* expression. Moreover, there is an enrichment of EZH2 binding on the promoter region of TRAIL in a CML cell line. This binding is lost after PRAME knockdown. Finally, knocking down PRAME or EZH2, and consequently induction of TRAIL expression, enhances Imatinib sensibility. Taken together, our data reveal a novel regulatory mechanism responsible for lowering *TRAIL* expression and provide the basis of alternative targets for combined therapeutic strategies for CML.

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Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder of transformed hematopoietic progenitor cells. It is characterized by the Philadelphia chromosome, which is generated by a t(9;22)(q34;q11) translocation (Kurzrock *et al*., 1988; Sawyers, 1999). This translocation results in the appearance of the constitutively active chimeric protein tyrosine kinase BCR–ABL, a hallmark of CML (Sawyers, 1997). Expression of BCR–ABL activates multiple signaling pathways leading to proliferation, reduced growth factor dependence, abnormal interaction with the bone marrow extracellular matrix and to a state of extreme resistance to apoptosis (Bedi *et al*., 1994; Amarante-Mendes *et al*., 1997, 1998; McGahon *et al*., 1997; Brumatti *et al*., 2003). Although the development of tyrosine-kinase inhibitors has revolutionized the treatment of BCR–ABL-positive leukemia, it has become increasingly clear that treatment with tyrosine-kinase inhibitors alone will not be curative in many cases, in particular in the advanced phase of the disease (Melo and Barnes, 2007). Thus, proper knowledge of molecular alterations triggered by the expression of BCR–ABL, especially during the disease progression, should provide rationale for the development of novel therapeutic strategies for CML.

Tumor necrosis factor-related apoptosis-inducing ligand—TNFSF10 (TRAIL) is a type II transmembrane protein belonging to the TNF-α family that is constitutively expressed in a wide range of tissues (Wiley *et al*., 1995). TRAIL is critically involved in anti-cancer surveillance by immune cells (Takeda *et al*., 2001, 2002, 2004; Schmaltz *et al*., 2002) and also has the ability to trigger apoptosis in a cell autonomous way, in a variety of tumor cell lines, but

not in most normal cells (Walczak *et al*., 1999; Wang and El-Deiry, 2003). Importantly, TRAIL$^{-/-}$ mice display an increased susceptibility to tumor initiation and metastasis (Cretney *et al*., 2002; Sedger *et al*., 2002), highlighting the importance of TRAIL in the defense against tumors. Therefore, it is not surprising that one of the key mechanisms of tumor escape is a general downregulation of *TRAIL*. Despite the enormous amount of knowledge gained in the last few years on TRAIL regulation and TRAIL-mediated cell death, the molecular basis of TRAIL inhibition in tumors is poorly understood.

Preferentially expressed antigen of melanoma (*PRAME*) is over-expressed in a variety of neoplasia, such as melanoma, breast carcinomas, renal cell carcinomas, non-small cell lung carcinomas, head and neck cancers, Hodgkin's lymphomas, medulloblastomas, sarcomas, Wilm's tumors, myelocytic and lymphocytic leukemia (Epping and Bernards, 2006), but its expression is low or absent in normal tissues (Ikeda *et al*., 1997). Although PRAME can trigger autologous cytotoxic T cell-mediated immune responses, its expression is preserved in several tumor cells, suggesting that *PRAME* expression can confer a selective advantage that outweighs its role in CTL-mediated tumor killing (Ikeda *et al*., 1997; Epping and Bernards, 2006). The manner in which *PRAME* over-expression contributes to the tumorigenic process still remains elusive.

We have recently observed that TRAIL is downregulated by BCR–ABL in cell lines as well as in peripheral blood mononuclear cells obtained from CML patients (JMGL and GPA-M, unpublished observations). Importantly, we found that decreased levels of TRAIL were associated with the progression of the disease. As it is known that *TRAIL* can respond to retinoic acid (RA) (Walczak *et al*., 1999; Altucci *et al*., 2001; Clarke *et al*., 2004) and that PRAME can act as a dominant repressor of RA/RA receptor signaling (Epping *et al*., 2005), we decided to investigate a possible association between upregulation of PRAME and decreased levels of TRAIL in CML.

We report here that BCR–ABL-mediated upregulation of PRAME is the molecular event responsible for knocking down TRAIL in Philadelphia chromosome-positive leukemia. The expression of polycomb protein enhancer of zeste homolog 2 (EZH2), which is necessary for PRAME-mediated transcriptional inhibition of *TRAIL*, is not altered in the presence of BCR–ABL. Finally, *in silico* analysis of gene-expression profiling studies suggest that the downregulation of TRAIL by PRAME may be a more general phenomenon characteristic of a variety of tumors. This implicates the transcription-repressive activity of the PRAME/EZH2 complex in the *TRAIL* promoter as a possible major target for novel anti-cancer therapies.

Results

PRAME *but not* EZH2 *is a BCR–ABL-induced gene*

We first surveyed human leukemia cell lines for expression of PRAME and EZH2 and verified that cell lines endogenously expressing BCR–ABL, such as KBM7, LAMA8.4 and K562, showed higher expression of PRAME than the *BCR–ABL*-negative cell lines, SKW6.4, THP1 and HL60 (Figure 1a). This result was confirmed by western blot (Supplementary Figure 1). The expression of EZH2 was not significantly different between BCR–ABL-positive and -negative cells (Figure 1b). To confirm that the relatively higher level of PRAME is a direct consequence of the expression of BCR–ABL, we compared the mRNA levels of PRAME in our HL-60.vector and HL-60.Bcr-Abl cell line and verified that the ectopic expression of BCR–ABL dramatically upregulated *PRAME* (Figure 1c). Interestingly, the treatment of BCR–ABL-positive cells, K562 and HL60.BCR–ABL, with 10 μM of Imatinib for 8 or 24 h does not decrease *PRAME* expression (Supplementary Figure 2). This result suggests that although *PRAME* expression can be induced in the presence of BCR–ABL, its tyrosine kinase activity is not necessary to maintain high *PRAME* expression.

Next, we quantitatively determined the mRNA of *PRAME* and *EZH2* in peripheral blood mononuclear cells from healthy individuals and CML patients in different phases of the disease. We found that *PRAME* (Figure 1d), but not EZH2 (Figure 1e), was upregulated in CML patients when compared with the healthy controls. In addition, there was significant positive correlation between levels of *PRAME* and *BCR–ABL* (Figure 1f; $P<0.0001$, $rs=0.6117$). Altogether, these data confirm that PRAME, but not EZH2, is positively regulated by BCR–ABL and that PRAME expression correlates with the progression of CML in our cohort of patients.

PRAME is responsible for BCR–ABL-mediated repression of TRAIL

In light that PRAME acts as a dominant repressor of RA/RA receptor signaling (Epping *et al*., 2005) and that TRAIL can be induced by RA treatment (Altucci *et al*., 2001; Clarke *et al*., 2004), we established a relationship analysis between the absolute gene expression of *PRAME* and *TRAIL* in our cohort of CML patients. We found out a moderate but significant inverse correlation ($P=0.0009$, $rs=-0.4971$) between the expression of these two genes (Figure 2a). In comparison, we found no correlation between *EZH2* and *TRAIL* (Figure 2b). It is important to note that the patients with higher TRAIL expression were in the chronic phase, while the patients with lower expression were in the advanced phases of the disease. This strengthens the hypothesis that decreased TRAIL expression may be important in the pathogenesis of CML.

To further confirm that the downregulation of *TRAIL* detected in BCR–ABL-positive cells is a direct consequence of BCR–ABL-mediated upregulation of PRAME, we generated a CML blastic crisis-derived cell line, namely K562, stably infected with short hairpin RNA against *PRAME* (K562.PRAME^{KD}). We specifically chose this cell line because of its high resistance to TRAIL-mediated cell death (Nimmanapalli *et al*., 2001). The sequence-specific short hairpin RNA vector

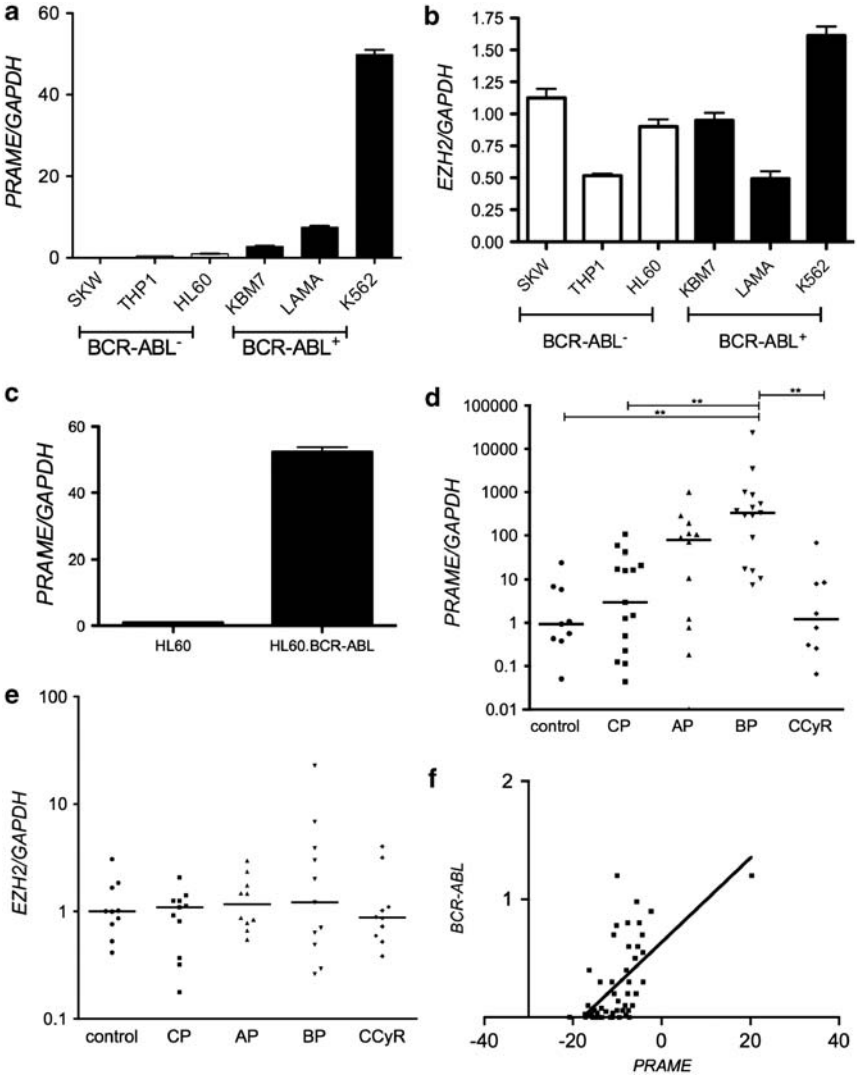

Figure 1 *PRAME*, but not *EZH2,* is a BCR–ABL-induced gene and is upregulated during the disease progression. (**a**) RT–qPCR analysis of *PRAME* mRNA in different leukemia cell lines. The BCR–ABL$^+$ cells show higher PRAME expression than the BCR–ABL$^-$ cells. (**b**) RT–qPCR analysis of *EZH2* mRNA in different leukemia cell lines. There is no difference in *EZH2* expression between the BCR–ABL-positive and -negative cells. (**c**) RT–qPCR analysis of *PRAME* expression in HL-60 empty vector (HL60) and HL60.BCR–ABL, which ectopically expresses *BCR–ABL*, cell lines. The graph shows a dramatic upregulation of *PRAME* after the *BCR–ABL* transfection. (**d**) Relative expression of *PRAME* in healthy individuals (control) and CML patients in chronic (CP), accelerated (AP), blastic phase (BP), and in patients who achieved complete cytogenetic remission (CCyR). The lines represent the median values. (**e**) Relative expression of *EZH2* in healthy individuals (control) and CML patients in chronic (CP), accelerated (AP), blastic phase (BP), and in patients who achieved complete cytogenetic remission (CCyR). (**f**) Correlation between *PRAME* and *BCR–ABL* in all CML patients. Spearman's $R = 0.6117$, $P < 0.0001$. $^{**}P < 0.01$.

pRS-PRAME significantly reduced endogenous *PRAME* mRNA (Figure 2c) and protein (Figure 2d) in K562 cells. As expected, knocking down *PRAME* significantly increased the levels of *TRAIL* found in wild-type K562 cells (Figure 2e). This result is in agreement with a direct role of PRAME as a repressor of *TRAIL* in BCR–ABL-positive leukemia cell lines.

EZH2 is required for PRAME-mediated repression of TRAIL

Despite the fact that the expression of EZH2 is not modulated by BCR–ABL, EZH2 was shown to be required for the transcriptional repression activity of PRAME (Epping *et al*., 2005). Therefore, we tested the functional relevance of EZH2 in PRAME-mediated repression of *TRAIL*. We inhibited endogenous *EZH2* in K562 cells using two specific short hairpin RNA vectors (Figures 3a and b) and obtained K562.EZH2^{KD} cells. As a result of EZH2 downregulation, *TRAIL* expression increased significantly (Figure 3c). Importantly, EZH2 knockdown does not lead to PRAME destabilization, nor does PRAME knockdown lead to EZH2 destabilization (Supplementary Figure 3). These data argue that although expression of EZH2 does not seem to be changed in the presence of BCR–ABL, it is still an important mediator of PRAME-dependent repression of *TRAIL*.

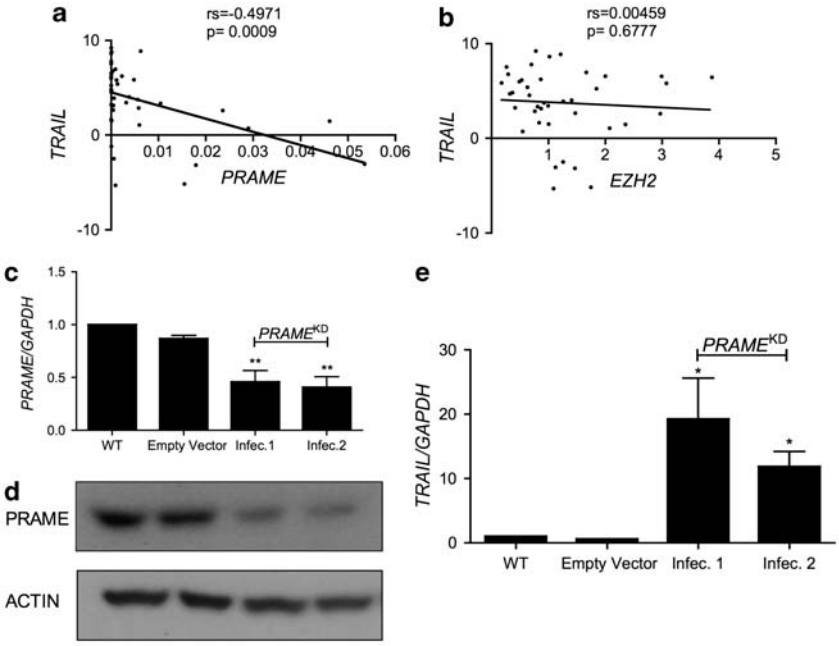

Figure 2 PRAME is responsible for knocking down *TRAIL* expression in CML. Correlation between *PRAME* and *TRAIL* (**a**) and *EZH2* and *TRAIL* (**b**) absolute expression in all CML patients shown in Figure 1d. RT–qPCR (**c**) and western blot (**d**) analysis of *PRAME* expression in K562 wild type (WT) or stably transfected with pRS (empty vector), or with two infections with pRS-PRAME to knock down endogenous *PRAME* (indicated as $PRAME^{KD}$). (**e**) RT–qPCR analysis of *TRAIL* mRNA in K562 cell lines before and after *PRAME* knockdown. $*P<0.05$; $**P<0.01$.

In order to gain a better understanding of the role of EZH2 in PRAME-mediated repression of TRAIL, we performed chromatin immunoprecipitation assay using an antibody against EZH2. We found that in the K562 cell line wherein PRAME is highly expressed, EZH2 is enriched at the TRAIL promoter (Figure 4b). We hypothesized that aberrant binding of EZH2 to the TRAIL promoter is dependent on PRAME activity. To test this, we compared by chromatin immunoprecipitation assay the binding of EZH2 to TRAIL promoter in the presence and absence of PRAME. As expected, there is a significant loss of EZH2 from the TRAIL promoter in K562.$PRAME^{KD}$ cells (Figure 4b). Taken together, our data clearly show that the high levels of PRAME, mediated by BCR–ABL signaling, is responsible for the aberrant targeting of the endogenous EZH2 to the TRAIL promoter and, consequently, silencing of this tumor suppressor gene.

PRAME or EZH2 knocking down enhances Imatinib sensitivity in a CML cell line

We next sought to investigate whether this novel regulatory mechanism could be used as a secondary target for CML treatment. We treated K562.vector, K562.$PRAME^{KD}$ or K562.$EZH2^{KD}$ with increasing doses of Imatinib and measured cell death after 48 h by propidium iodide incorporation into the DNA and cell viability by Trypan blue exclusion. We found a statistically significant increase in apoptosis and a concomitant decrease in cell viability in K562.PRAME^{KD} and K562.$EZH2^{KD}$ cells treated with Imatinib when compared with the empty vector control (Figures 5a and b). To confirm that TRAIL, instead of other putative PRAME targets, is responsible for the enhanced susceptibility of K562.$PRAME^{KD}$ and K562.$EZH2^{KD}$ cells to Imatinib, we treated every cell line with Imatinib for 72 h in the presence or absence of TRAIL R2-Fc (a protein able to inhibit TRAIL-induced apoptosis). The increased apoptosis observed in Imatinib-treated K562.$PRAME^{KD}$ and K562.$EZH2^{KD}$ was not seen when cells were treated in the presence of TRAIL R2-Fc (Figure 5c). Our results suggest that therapeutic strategies targeting PRAME and/or EZH2, and consequently restoring TRAIL expression, can be an interesting avenue for increasing Imatinib efficiency in CML.

TRAIL re-expression after PRAME or EZH2 knockdown can kill TRAIL-sensitive cells

In order to investigate whether the re-expression of TRAIL in K562.$PRAME^{KD}$ or K562.$EZH2^{KD}$ (Figures 2e and 3c) is functionally active, we co-cultured the K562 cell lines with the TRAIL-sensitive, carboxyfluorescein diacetate succinimidyl ester-labeled Jurkat cells, in the ratio of 3:1. First, we showed that Jurkat cells are indeed TRAIL-sensitive, by treating the cells with different concentrations of isoleucine-zipper-TRAIL, a version of recombinant TRAIL. As shown in Figure 6a, apoptosis can be significantly induced in Jurkat cells with doses as low as 40 ng/ml of isoleucine-zipper-TRAIL. Second, we found that Jurkat cells underwent significantly more apoptosis when co-cultured

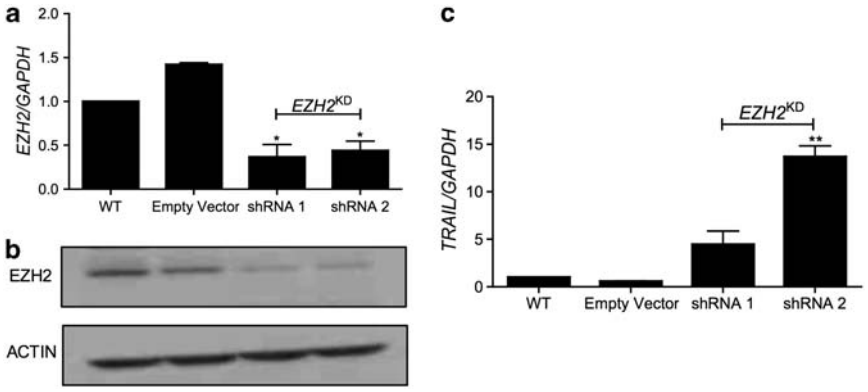

Figure 3 EZH2 knockdown restores TRAIL expression. RT–qPCR (**a**) and western blot (**b**) analysis of *EZH2* expression in K562 wild type (WT) or stably transfected with pRS (empty vector) or pRS-EZH2 #3 (shRNA 1) or pRS-EZH2 #4 (shRNA 2), or with two different shRNAs to knock down endogenous *EZH2* (indicated as $EZH2^{KD}$). (**c**) RT–qPCR analysis of *TRAIL* mRNA in K562 cell lines before and after *EZH2* knockdown. $*P<0.05$; $**P<0.01$.

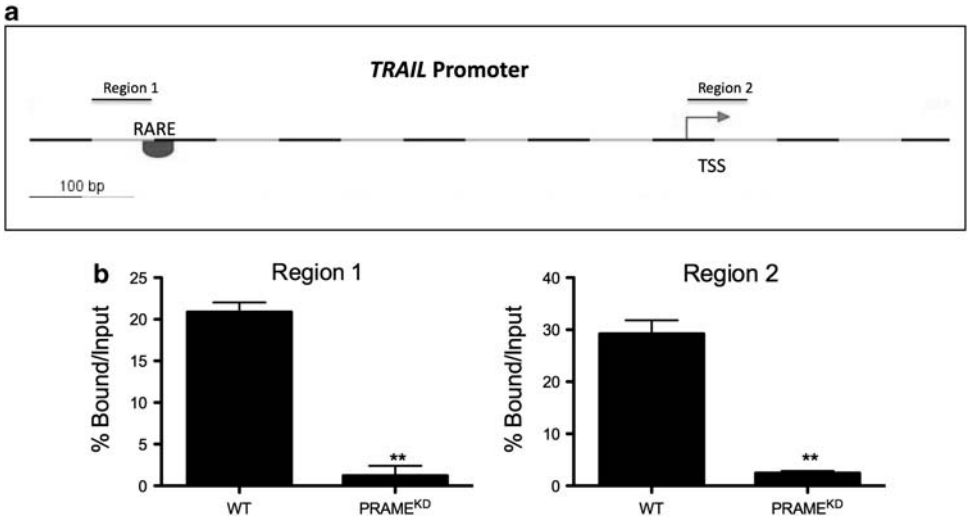

Figure 4 PRAME-mediated EZH2 binding to TRAIL promoter. (**a**) Schematic representation of the TRAIL promoter using the Gene2Promoter software from Genomatix (http://www.genomatix.de). (**b**) ChIP analysis of EZH2 binding to TRAIL promoter in K562 wild type (WT) and K562, wherein endogenous PRAME cells were knocked down (indicated as $PRAME^{KD}$). Bound and unbound fractions were quantified and used to calculate bound/input ratio. $**P<0.01$. RARE, retinoic acid responsive element; TSS, transcript start site.

with K562.$PRAME^{KD}$ or K562.$EZH2^{KD}$ cells compared with K562.vector cells (Figure 6b).

Inverse correlation between PRAME and TRAIL in different tumors

Finally, we addressed whether the downregulation of TRAIL by PRAME is a general phenomenon in cancer patients. We performed a meta-analysis using the Oncomine platform (http://www.oncomine.org). By assessing six studies and more than 200 samples of lung adenocarcinoma, melanoma, breast carcinoma and matched healthy tissues (Figure 7), we could find several situations wherein the expression of *PRAME* was inversely correlated with the expression of *TRAIL*, suggesting that *TRAIL* could indeed be a target for transcriptional regulation by *PRAME* in a variety of cancer.

Discussion

Increasing evidences show that TRAIL can act as a tumor suppressor. First, *TRAIL* knockout mice are more susceptible to tumorigenesis induced by chemical carcinogens and metastasis (Cretney *et al*., 2002; Sedger *et al*., 2002). Second, experiments that used neutralizing antibodies against TRAIL demonstrated that it could significantly increase liver metastases in mice (Cretney *et al*., 2002; Clarke *et al*., 2004). Indeed, several expression-profiling surveys in cancer have shown that TRAIL expression is lost upon cancer progression (Daniels *et al*., 2005; Popnikolov *et al*., 2006; Vigneswaran *et al*., 2007) and there are diverse studies demonstrating that *TRAIL* expression is a central mediator of apoptosis in some cancer treatments such as RA, histone deacetylase inhibitors and interferon (Altucci *et al*., 2001; Clarke *et al*., 2004; Insinga *et al*.,

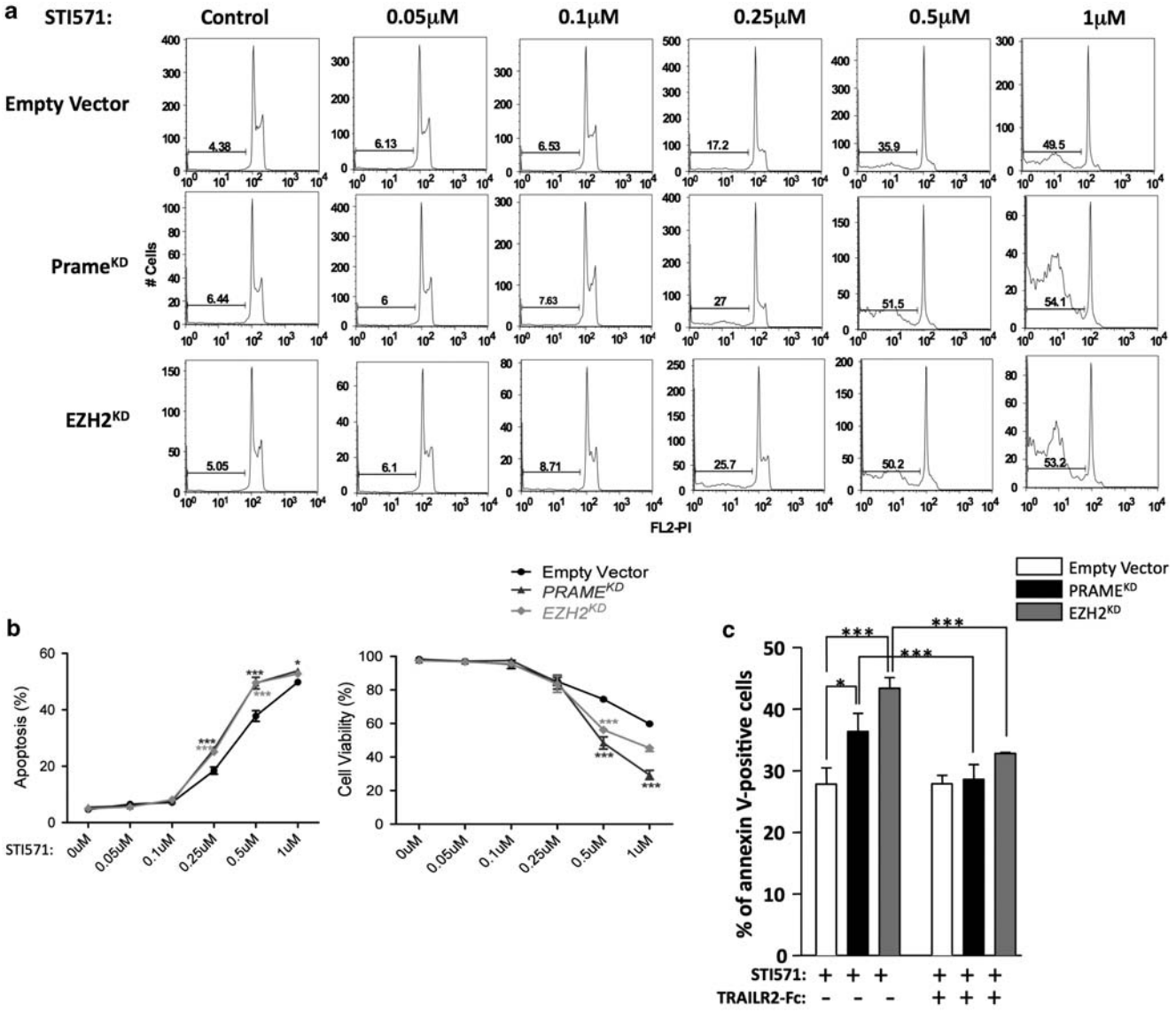

Figure 5 PRAME^{KD} and EZH2^{KD} enhance Imatinib-mediated cell death in K562 cell line in a TRAIL-dependent way. (**a**) Cell cycle analysis after 48-h treatment with indicated doses of Imatinib. Apoptosis was estimated by measuring the number of cells with sub-diploid DNA content. The figure shows representative data of three independent experiments. (**b**) Graphical representation of the three experiments in **a** (left panel). Cell viability was measured by Trypan Blue exclusion (right panel). (**c**) Percentage of Annexin V-positive cells after treatment with TRAIL R2-Fc, STI571 or both for 72 h. Annexin V staining shows the percentage of apoptotic cells. $*P<0.05$; $***P<0.001$.

2005; Nebbioso *et al*., 2005). These data suggest that *TRAIL* downregulation has a central role in tumor initiation and/or progression, and that *TRAIL* re-expression is a promising therapeutic avenue for killing transformed cell lines without affecting normal cells.

PRAME is frequently expressed in many different cancers (Epping and Bernards, 2006), including BCR–ABL-positive leukemia (Watari *et al*., 2000; Radich *et al*., 2006; Yong *et al*., 2008). We confirmed that PRAME is a target for BCR–ABL tyrosine-kinase signaling pathways and showed that *PRAME* expression correlates well with *BCR–ABL* in CML patients, in particular, as a marker of disease progression. Furthermore, our results substantiate the suggestion that *PRAME* over-expression is involved in tumorigenesis, possibly by suppressing the RA signaling pathway (Epping *et al*., 2005). PRAME can bind to the RA receptor in the presence of RA and prevent receptor activation and target gene transcription. This mechanism seems to require the histone methyltransferase activity of the polycomb protein, EZH2, which forms a complex with PRAME and mediates the epigenetic silencing of the RA target genes (Epping *et al*., 2005). As a result, PRAME may be able to confer growth or survival advantages to cancer cells. However, this mechanism has been well established only in melanoma and, presently, it is not clear whether PRAME inhibits RA signaling in other forms of cancer. In fact, PRAME expression was not found to be associated with the downregulation of RA signaling in acute myeloid

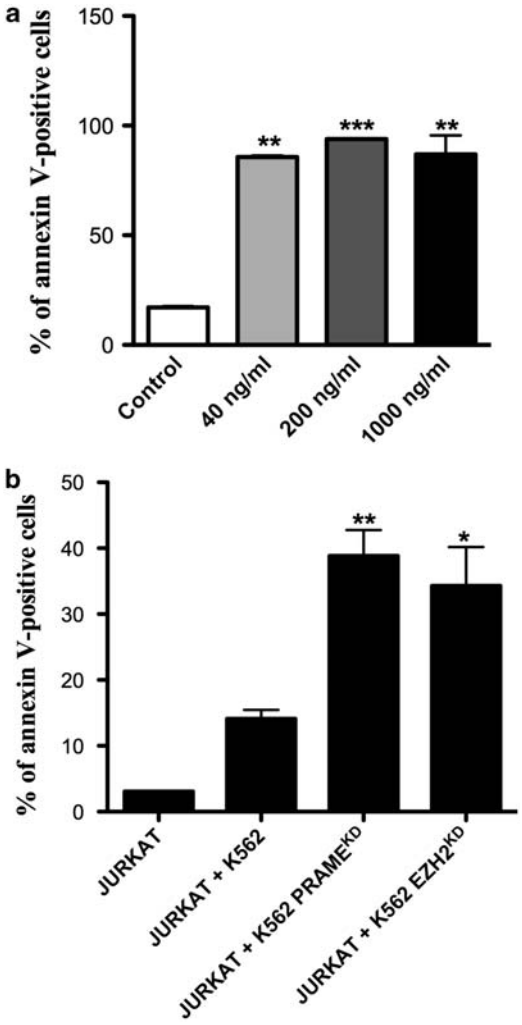

Figure 6 K562.PRAME^{KD} or K562.EZH2^{KD} can kill TRAIL-sensitive cells. (**a**) Jurkat cells are sensitive to isoleucine-zipper-TRAIL, a recombinant version of TRAIL. (**b**) K562.PRAME^{KD} or K562.EZH2^{KD} can induce significantly more apoptosis in TRAIL-sensitive JURKAT cells than K562 empty vector. $*P<0.05$; $**P<0.01$; $***P<0.001$.

leukemia (Steinbach *et al*., 2007). Interestingly, although PRAME is an independent prognostic marker of poor clinical outcome in different solid tumors (van't Veer *et al*., 2002; Oberthuer *et al*., 2004), in childhood acute myeloid leukemia patients, PRAME was rather suggested to be a marker of favorable prognosis (Tajeddine *et al*., 2008).

Another important point that remains obscure is the nature of the target genes that are repressed by the PRAME–EZH2 complex and are vital for more than one aspect of tumorigenesis. At the moment, only the *p21* and *RARβ* expression levels were shown to be downregulated in melanoma cells in a PRAME-dependent manner (Epping *et al*., 2005). We hypothesize that *TRAIL* expression could be regulated by PRAME. First, PRAME is a dominant repressor of RA receptor signaling (Epping *et al*., 2005), whereas *TRAIL* is a gene inducible by RA treatment. Second, PRAME is capable of inhibiting the induction of apoptosis by histone deacetylase inhibitors in a concentration-dependent manner (Epping *et al*., 2007), an event shown to be modulated, at least in leukemia, by induction of *TRAIL* expression (Insinga *et al*., 2005; Nebbioso *et al*., 2005). Indeed, our results clearly showed that in the CML line K562, the inhibition of TRAIL expression is mediated by PRAME and this process relies on EZH2 binding to the TRAIL promoter. We specifically chose this cell line because of its relative resistance to TRAIL-mediated cell death, allowing cells to survive after *TRAIL* re-expression. In comparison, we were not able to select viable cells when we tried to stably knock down PRAME or EZH2 in TRAIL-sensitive cell lines (data not shown). We are currently verifying whether this effect is indeed mediated by upregulation of TRAIL in these cells.

It is known that TRAIL frequently induces apoptosis in BCR–ABL-positive leukemia cells (Uno *et al*., 2003) and it was suggested that TRAIL production can overcome Imatinib mesylate resistance in CML cell lines (Kikuchi *et al*., 2007). This information is in agreement with our data showing that K562 cells with an artificial reduction in *PRAME* or *EZH2* expression are more sensitive to Imatinib treatment owing to increased *TRAIL* expression. As the anti-apoptotic protein c-FLIP is known to participate in the regulation of Imatinib-induced cell death (Nimmanapalli *et al*., 2001), we assessed whether knocking down *PRAME* or *EZH2* could lead to destabilization of c-FLIP. We did not observe downregulation of c-FLIP in K562.PRAME^{KD} and K562.EZH2^{KD} cells (Supplementary Figure 3), suggesting that TRAIL is indeed the major PRAME target responsible for enhanced susceptibility to Imatinib.

Taken together, our results open new therapeutic possibilities for combined treatment of CML patients. In fact, co-treatment with Imatinib mesylate enhances TRAIL-induced apoptosis of BCR–ABL-positive cell lines, even in K562, a TRAIL-resistant cell line (Nimmanapalli *et al*., 2001). Therefore, interference in the expression of PRAME or in the activity of histone methyltransferase of EZH2 may be a good alternative or co-adjuvant strategy for CML.

Finally, by analyzing the expression changes in cancer microarray databases, using the Oncomine platform, we found that several forms of cancer, such as lung adenocarcinomas, melanomas and breast carcinomas, show a higher *PRAME* expression associated with a lower *TRAIL* expression when compared with matched healthy tissues. These data correlate with our *in vitro* knockdown results suggesting that PRAME may be involved in the tumorigenic process in a wide range of cancers, at least in part by blocking the tumor suppressor pathway mediated by TRAIL expression.

Materials and methods

Patient samples

All samples were collected, under the protocols approved by all Hospitals and Institutional Ethics Committees, after obtaining patients' written informed consent. Peripheral blood was collected from 10 healthy individuals and 40 CML

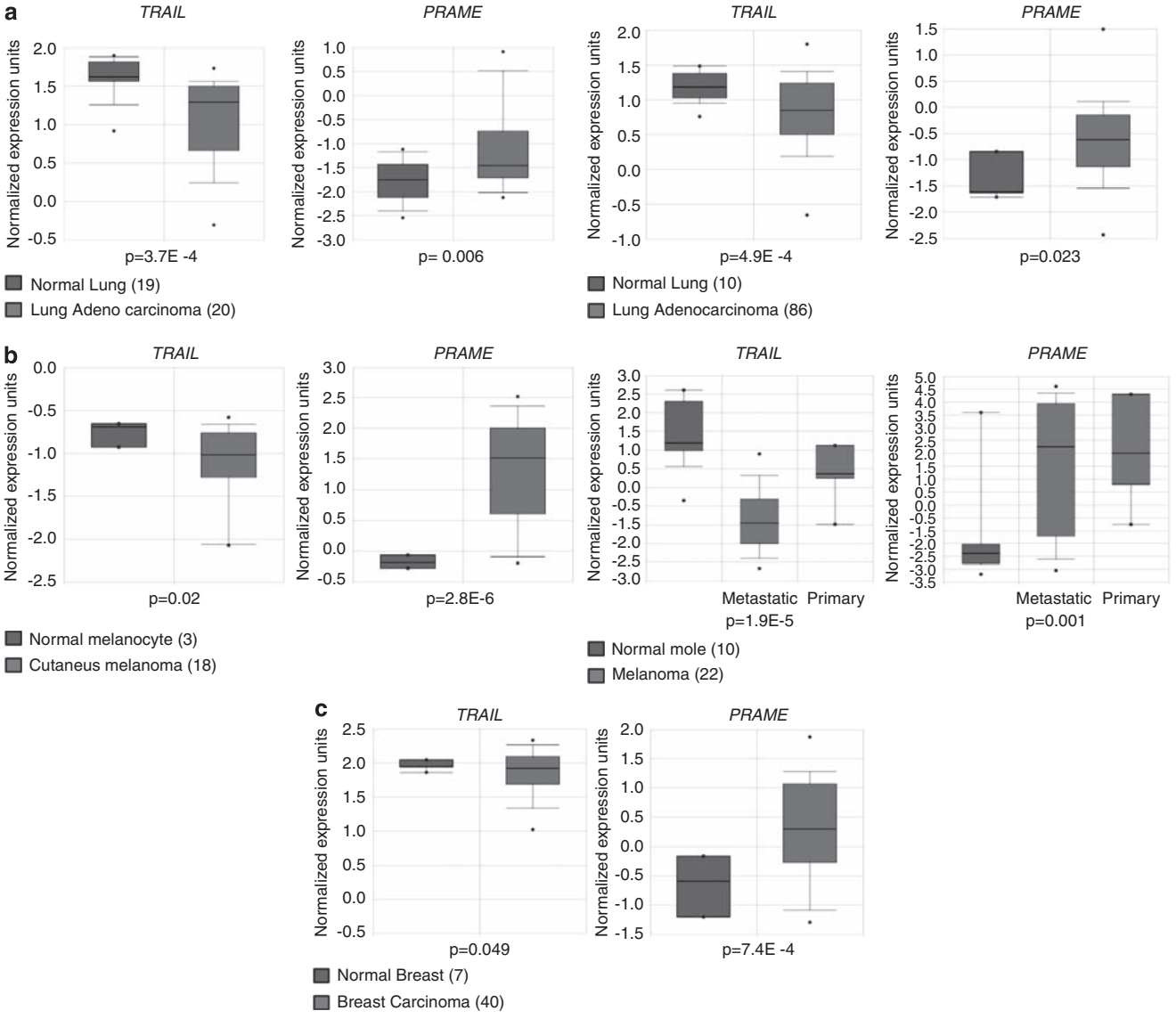

Figure 7 *TRAIL* is downmodulated in lung adenocarcinoma, melanoma and breast carcinoma, while *PRAME* is upregulated. Box plots showing decreased expression of *TRAIL* and increased expression of *PRAME* on datasets performed in lung adenocarcinoma (Beer *et al*., 2002; Stearman *et al*., 2005) (**a**); melanoma (Haqq *et al*., 2005; Hoek *et al*., 2006) (**b**); and breast carcinoma (Richardson *et al*., 2006) (**c**). The *y*-axis represents normalized expression. Shaded boxes represent the interquartile range (25th–75th percentile). Whiskers represent the 10th–90th percentile. The bars denote the median.

patients (11 chronic phase, 10 accelerated phase, 10 blastic phase and 9 with complete cytogenetic remission (CCyR) post-Imatinib mesylate (Gleevec, Novartis, Basel, Switzerland) (400 mg/day)). CML diagnosis of the patients enrolled in this study was confirmed by Philadelphia chromosome and *BCR–ABL* rearrangement detection. Peripheral blood mononuclear cells from patients and controls were isolated according to standard protocol with the Ficoll-Hypaque 1077 density technique.

Reagents

PRAME and *EZH2* short hairpin RNA vectors were kind gifts of Dr R Bernards (Amsterdam, The Netherlands) and were generated as previously described (Epping *et al*., 2005). Antibodies against EZH2 (4905) and phospho-FOXO3a (9465) were from Cell Signaling, Danvers, MA, USA. Anti-PRAME was from Abcam (ab32185) (Cambridge, MA, USA) and anti-ACTIN was from ICN Biomedicals Inc. (C4) (Aurora, OH, USA). TRAIL R2-Fc and isoleucine-zipper-TRAIL recombinant proteins were generously provided by Dr Henning Walczak (Imperial College, London, UK).

Cell culture and cell lines

All cells were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum, 25 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, except for Phoenix packaging cell, which was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 25 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and the KBM7 cell line, which was grown in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum, 25 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. HL-60 and K562 cells were obtained from

ATCC, Manassas, VA, USA. LAMA-84 was kindly provided by James Griffin (Dana-Farber Cancer Institute, Boston, MA, USA). HL-60.Bcr-Abl cells were derived from wild-type HL-60 by retroviral transfection with pSRαMSVp185$^{bcr\text{-}abl}$tkneo (Amarante-Mendes *et al*., 1998; Bueno-da-Silva *et al*., 2003). SKW6.4 cells were kindly provided by Dr Henning Walczak (Imperial College). Phoenix packaging cells (kindly provided by Dr Gary Nolan, Stanford University, CA, USA) were transfected with retroviral plasmids to generate amphotropic retroviruses. K562 cells were infected with pRS (empty vector) or pRS-PRAME or pRS-EZH2 #3 or pRS-EZH2 #4 retrovirus and selected for puromycin resistance.

Western blot
Protein samples were resolved under reducing conditions as previously described (Souza-Fagundes *et al*., 2003). Separated proteins were transferred onto polyvinylidene difluoride membranes and reactions were detected with a suitable secondary antibody conjugated to horseradish peroxidase (Jackson Laboratory, Bar Harbor, ME, USA and Amersham, Arlington, IL, USA) using enhanced chemiluminescence (Pierce, Rockford, IL, USA).

ChIP assay
Chromatin from the K562 cell lines was fractionated by incubation of purified nuclei with micrococcal nuclease (MNase) (Umlauf *et al*., 2004). Chromatin immunoprecipitation with EZH2 antibody was performed as previously described (Umlauf *et al*., 2004) and DNA extractions from bound fractions were performed following the Abcam (www.abcam.com) protocol. The immunoprecipitated DNA was analyzed by quantitative PCR. For a negative control we performed chromatin immunoprecipitation assay using only PTN A.

Quantitative PCR
Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). RNA concentration and purity were determined spectrophotometrically by measuring fluorescence at 260 and 280 nm. Three micrograms of RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) transcription reagents according to the manufacturer's instructions. After obtaining the cDNA, gene expression was quantified by quantitative PCR using Platinum SYBRGreen Kit (Invitrogen) in Mx3005P detector equipment (Stratagene, Santa Clara, CA, USA).

The following primers were used: *BCR–ABL* 5′-GGGGTC CAGCGAGAAGGTT-3′ (forward) and 5′-GCATTCCGCTG ACCATCAAT-3′ (reverse); *GAPDH* 5′-GGAGAAGGCTGG GGCTCAT-3′ (forward) and 5′-GTCCTTCCACGATAC CAAAGTT-3′ (reverse); *TRAIL* 5′-AAGGCTCTGGGCCGC AAAATAAAC-3′ (forward) and 5′-GCCAACTAAAAAG GCCCCGAAAAA-3′ (reverse); *EZH2* 5′-AGGACGGCTCC TCTAACCAT-3′ (forward) and 5′-CTTGGTGTTGCACT GTGCTT-3′ (reverse). Results were given as relative expression in terms of the amplicon ratio: investigated gene/*gapdh* housekeeping gene. The PRAME quantitative PCR was performed using TaqMan PCR master mix as previously described (Proto-Siqueira *et al*., 2006).

The following primers were used for TRAIL promoter. Region 1: 5′-TCCAGCCTAACACACAGGCA-3′ (forward) and 5′-GCAAACCTTCAGACACAAATTCTAATC-3′ (reverse); region 2: 5′-GGACAGACCTGCGTGCTGA-3′ (forward) and 5′-ACACGTAAGTTACAGCCACACAGAG-3′ (reverse).

Assessment of apoptosis
Apoptosis was quantified using a fluorescence-activated cell sorter (FACS) calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA), by flow-cytometric analysis of DNA content as described earlier (Amarante-Mendes *et al*., 1997). The results represent the average ± s.d. in triplicate samples. Every experiment was repeated at least three times. The samples were also analyzed by Trypan blue exclusion to access cell viability.

Meta-analysis
Patient information and gene expression data were obtained from and analyzed using Oncomine. The microarray meta-analysis algorithms and statistical analysis used were as previously described (Rhodes *et al*., 2004). *P*-values were calculated as described at http://www.oncomine.org, using adjustment for multiple testing and false discovery (Rhodes *et al*., 2004).

Statistical analysis
Experiments were performed always in triplicate and at least three times. Kruskall–Wallis followed by Dunn's multiple comparison tests were used to compare gene expression data between controls and CML patients in different phases of the disease. Associations between *BCR–ABL* and *PRAME* or between *PRAME* and *TRAIL* mRNA levels were assessed through non-parametric Spearman's rank correlation test. One-way ANOVA and Tukey's post-test were used to compare gene expression data from knockdown cell lines. Differences between experimental groups were considered significant when $P<0.05$. All statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA).

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)