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ARTICLE

Targeting PFKFB3 sensitizes chronic myelogenous leukemia cells to tyrosine kinase inhibitor

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Abstract

Resistance to the *BCR-ABL* tyrosine kinase inhibitor (TKI) remains a challenge for curing the disease in chronic myeloid leukemia (CML) patients as leukemia cells may survive through *BCR-ABL* kinase activity-independent signal pathways. To gain insight into BCR-ABL kinase activity-independent mechanisms, we performed an initial bioinformatics screen and followed by a quantitative PCR screen of genes that were elevated in CML samples. A total of 33 candidate genes were identified to be highly expressed in TKIs resistant patients. Among those genes, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*PFKFB3*), controlling the limiting step of glycolysis, was found to be strongly associated with TKIs resistance. *PFKFB3* knockdown or pharmacological inhibition of its kinase activity markedly enhanced the sensitivity of CML cells to TKIs. Furthermore, pharmacological inhibition of *PFKFB3* inhibited CML cells growth and significantly prolonged the survival of both allograft and xenograft CML mice. ChIP-seq data analysis combined with subsequent knockdown experiment showed that the Ets transcription factor *PU.1* regulated the elevated expression of *PFKFB3* in TKIs-resistant CML cells. Therefore, our results showed that targeting *PFKFB3* sensitizes CML cells to TKIs and *PFKFB3* may be a potential *BCR-ABL* kinase activity-independent mechanism in CML.

Introduction

Chronic myeloid leukemia (CML) is a hematopoietic malignancy characterized by an uncontrolled increase of myeloid cells and their accumulation in bone marrow and peripheral blood [1, 2]. The Ph^+ CML is characterized by the Philadelphia chromosome, which generated from a reciprocal translocation between chromosomes 9 and 22 [3, 4]. The t(9;22) translocation results in a new fusion gene *BCR-ABL*, which expressed the constitutive activated tyrosine kinase *ABL* [3, 4]. *BCR-ABL* transformed hematopoietic stem cells and myeloid progenitor cells, and promoted leukemogenesis by activating its downstream cell signaling pathways including *RAS*/mitogen-activated protein kinase (*RAF/MEK/ERK*) [5], phosphatidylinositol 3-kinase/*AKT* (*PI3K/AKT*) [6, 7], and *JAK/STAT* signaling cascades [8, 9].

Tyrosine kinase inhibitors (TKIs) such as Imatinib mesylate (IM) were the current first-line treatment for CML, which bound to the *ABL* kinase domain and inhibited its kinase activity [1]. TKIs have markedly improved the survival of CML patients in the chronic phase and advanced stage of the disease. However, CML cannot be cured by

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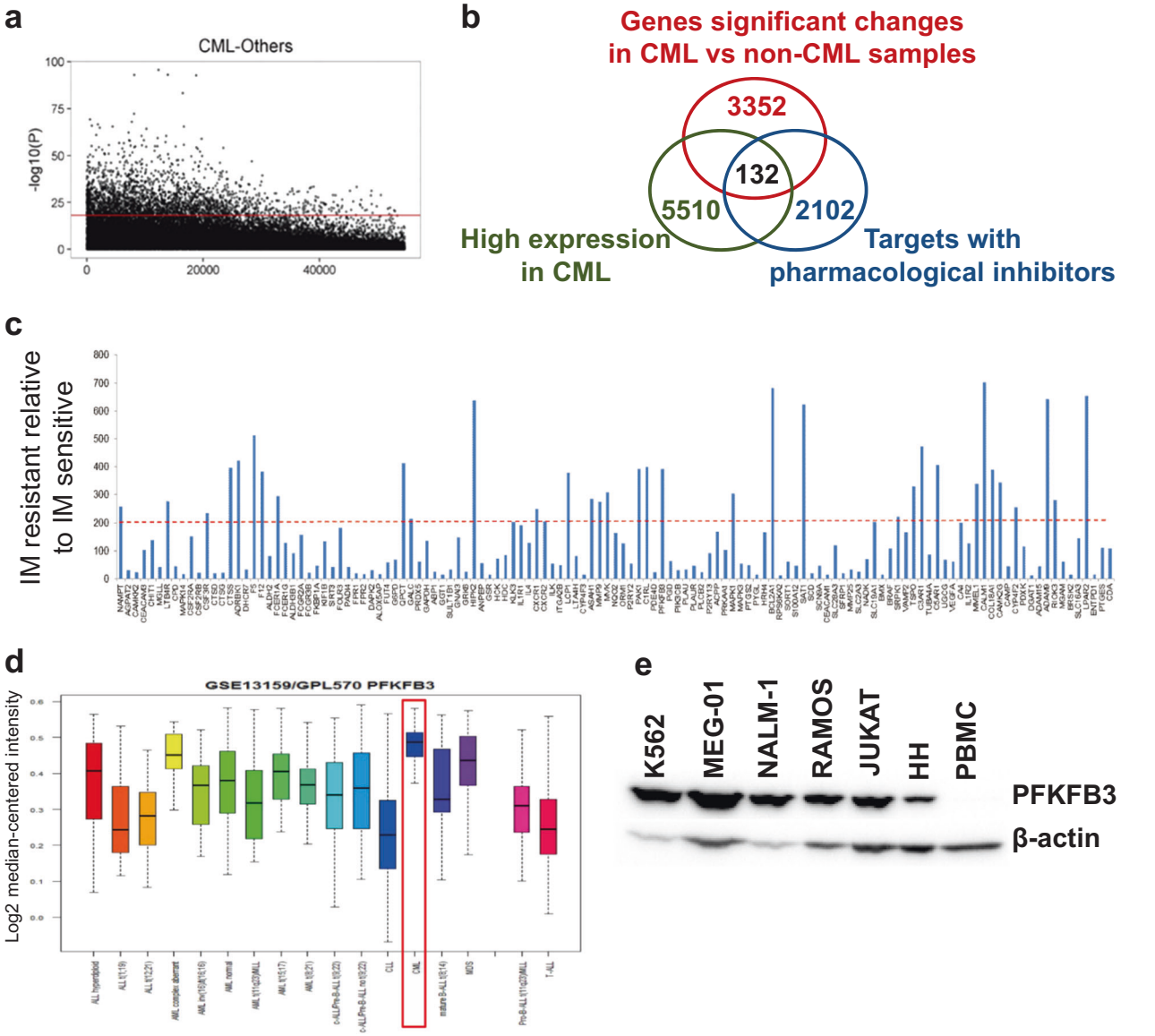


Fig. 1 *PFKFB3* expression is significantly elevated in CML. **a** Statistics of differential gene expression in CML samples vs. non-CML samples. Plot is represented with negative log10 of adjusted *P* value for every probe of the whole genome. The red line represents the adjusted *P* value as 10^{-20}. **b** Venn diagram of the intersection of 3352 genes that are statistically differentially expressed in CML samples, 5510 genes that are top ranked by their expression level in CML, and 2102 genes that have their chemical inhibitors commercially available. The intersection of the three parts represents 132 candidate genes. **c** Reverse transcription and quantitative real-time PCR of the selected 132 candidate genes from (**b**) were performed on both Imatinib-sensitive and -resistant CML patient samples. The expression bars above the red dashed line represent the final 33 genes that were expressed at least twofold higher in Imatinib-resistant CML patients than in Imatinib-sensitive CML patients. **d** *PFKFB3* mRNA level was shown in samples of individual type of leukemia. **e** Western blot of *PFKFB3* in multiple human leukemia cell lines and health human PBMCS

TKIs although the *BCR-ABL* kinase activity was fully inhibited in CML patients. Furthermore, the TKIs resistance developed, especially in advanced-stage disease, and led to disease relapse and progression [10, 11]. Many CML patients' resistance to TKIs resulted from the acquisition of point mutations in *BCR-ABL* kinase domain that interfered with TKIs binding and kinase inhibition [12–14]. However, half of the TKIs-resistant CML patients did not carry any mutations in *BCR-ABL* kinase domain and the resistance of CML cells may be due to the *BCR-ABL* kinase activity-independent mechanism [15, 16]. For instance, elevated expression of *LYN/HCK* leads to some CML patients resistant to TKIs without mutations in *BCR-ABL* [17–19]. In addition, by using the CML mouse model, multiple metabolic genes including *Alox5* or *Alox15* have been shown to be the potential *BCR-ABL* kinase activity-independent mechanism in leukemic stem cells (LSCs) [20, 21]. Those studies suggested that other kinases signal pathways and

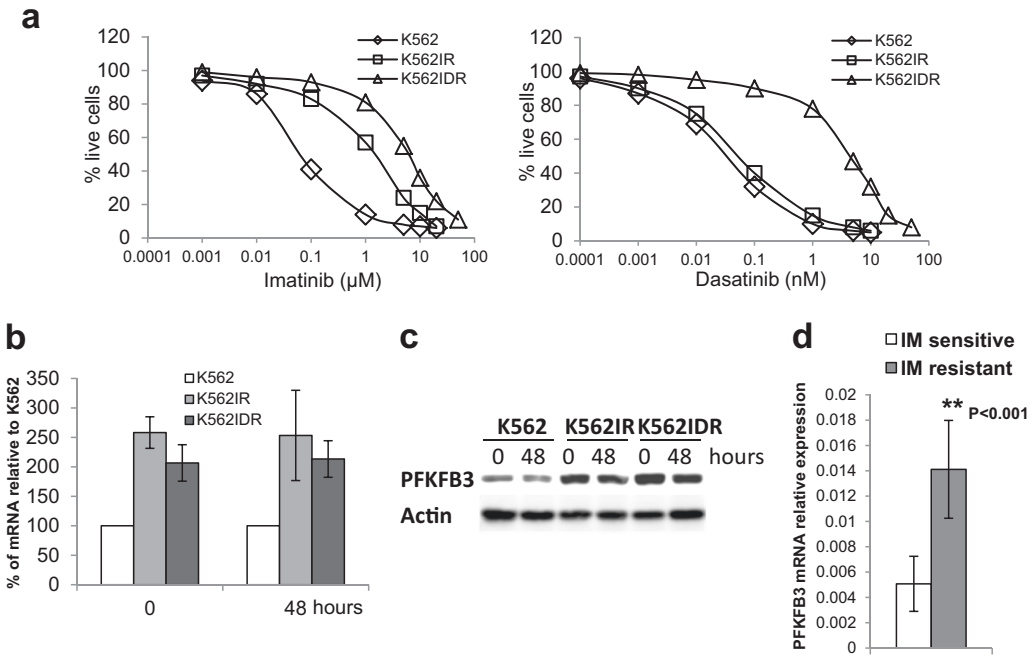


Fig. 2 *PFKFB3* expression elevates in TKI-resistant CML cell lines or CML patients. **a** Relative cell viability of K562, K562IR, and K562IDR cultured in the presence of Imatinib (left) or Dasatinib (right) at different concentrations. **b** *PFKFB3* mRNA levels in K562, K562IR, and K562IDR cells were relative to the level in K562 cells. **c** Protein levels in K562, K562IR, and K562IDR cells. **d** *PFKFB3* mRNA level was shown in Imatinib-resistant CML patients and in Imatinib-sensitive CML patients

cancer metabolic signal pathways contributed to the response of CML cells to TKIs [20, 21].

In this study, we performed in silico screen of genes whose expression was significantly elevated in CML patients and further screened those candidate genes among TKIs-resistant CML patients by quantitative real-time PCR to identify potential genes regulating the leukemia cells in response to acquired TKIs resistance. We showed that 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (*PFKFB3*) was among the most upregulated candidate genes in TKIs-resistant CML samples. *PFKFB3* was required for TKIs-resistant CML to compensate the TKIs-inhibited proliferation of leukemia cells and the increase of leukemia cell apoptosis. In addition, we found that *PFKFB3* was upregulated by transcription factor *PU.1* in TKI-resistance CML cells. Our findings reveal a novel metabolic survival pathway that regulated *BCR-ABL* kinase activity-independent TKIs resistance and may also contribute to the TKIs resistance of CML stem cells.

Results

Gene expression analysis reveals that *PFKFB3* expression is significantly elevated in CML

We performed differential expression analysis of the Haferlach leukemia data sets [22], including 2096 leukemia patient samples (GSE13159) to compare changes of gene expression in CML patients vs. non-CML patients (other leukemia patients from the Haferlach leukemia data sets), and generated a list of 3352 genes with statistically significant changes in CML samples (Fig. 1a, $P < 10^{-15}$). Each gene was ranked by the levels of the expression in all 17 types of leukemia and the top ranked expression of 5510 genes in CML were selected. Among these candidate genes, we further selected the final 132 genes that overlapped with the list of 2102 targets with the small molecule pharmacological inhibitors currently available (Fig. 1b). To identify candidate genes associated with TKIs resistance, we performed quantitative real-time PCR screen on the cDNAs from 15 Imatinib-sensitive patients and 9 Imatinib-resistant patients (Supplemental Table S1). The expression of 33 candidate genes was elevated at least twofold in the Imatinib-resistant CML samples in comparison with Imatinib-sensitive CML samples (Fig. 1c). We found that *PFKFB3* was among the final candidates list and its elevated expression was associated with TKIs-resistance in CML patients. *PFKFB3* encodes the protein phosphofructokinase-2 that catalyze the conversion of 6-phospho-fructose (*F6P*) into 2, 6-biphospho-fructose (*F2,6 BP*) [23, 24]. *PFKFB3* is the key enzyme that regulates glycolysis in the control of tumor cell proliferation, migration, and apoptosis [25–28]. The glycolysis pathway has been known to regulate the apoptosis of leukemia cells and associate with poor prognosis in patients with

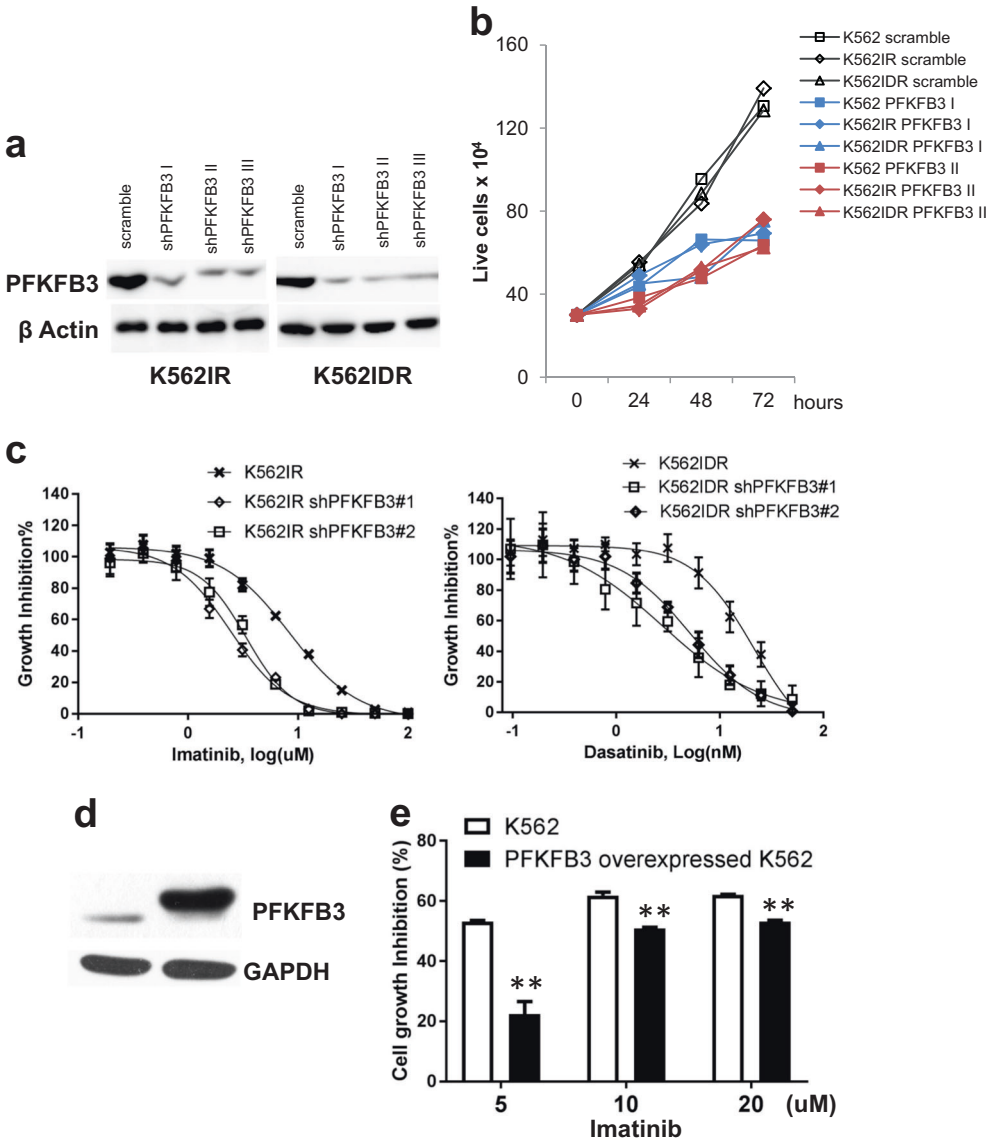
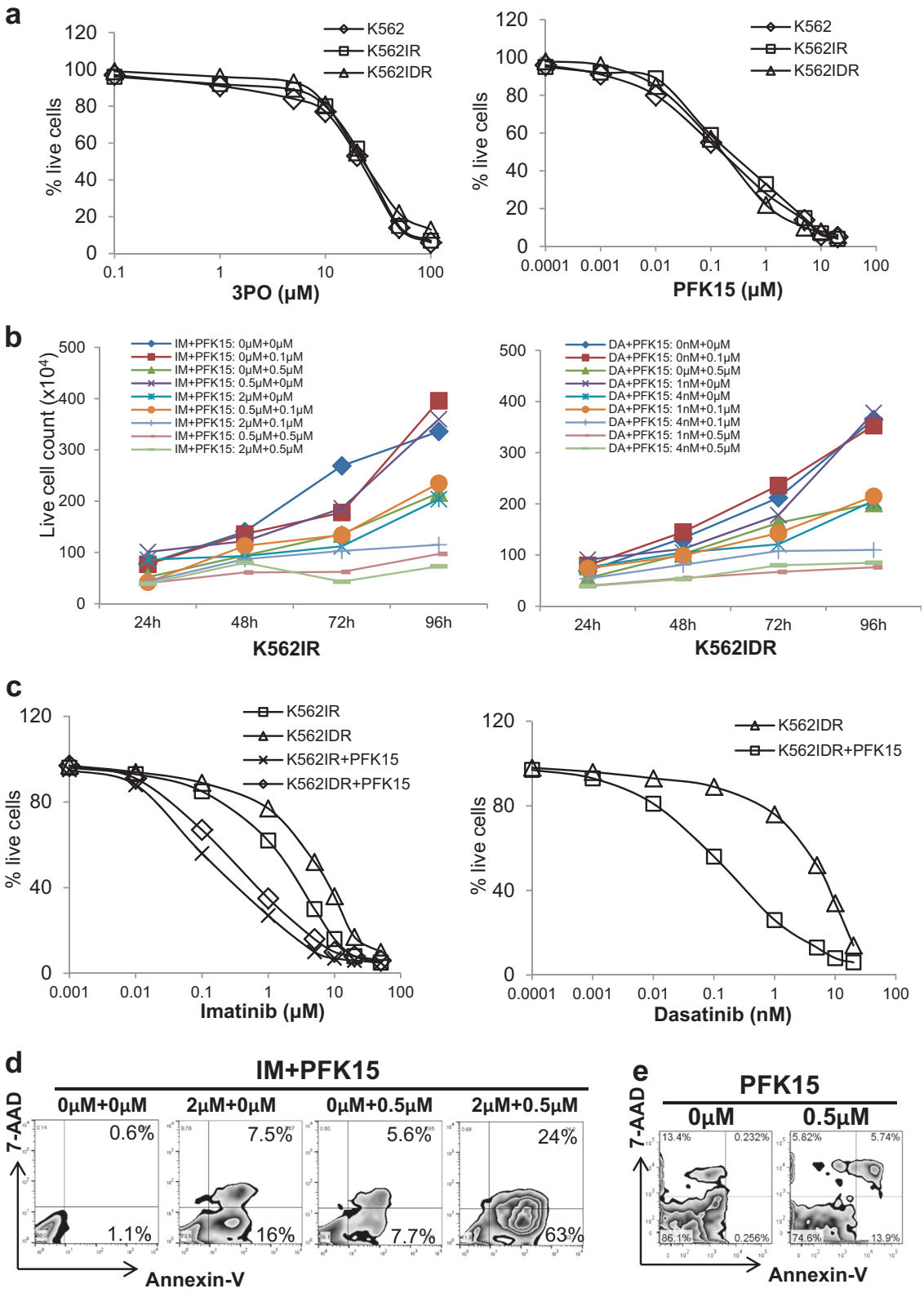


Fig. 3 Loss of *PFKFB3* sensitizes CML cells to TKI inhibition. Protein levels (**a**) and cell growth (**b**) of K562, K562IR, and K562IDR cells transduced with lentivirus bearing scramble shRNA, or shRNAs specifically against *PFKFB3* (shRNAI, shRNAII, and shRNAIII). **c** Dose response of Imatinib or Dasatinib in K562, K562IR, and K562IDR cells transduced with scramble shRNA, or sh*PFKFB3*. Leukemia cells were treated with DMSO or serial dilutions of Imatinib or Dasatinib for 48 h. Relative cell growth was measured by CTG. **d** Western blotting analysis of *PFKFB3* expression in K562 cells with overexpression of *PFKFB3*. **e** Dose response of Imatinib in K562- and *PFKFB3*-overexpressed K562 cells. Leukemia cells were treated with DMSO or three dilutions of Imatinib for 48 h. Relative cell growth was measured by CTG

cytogenetically normal acute myeloid leukemia [29]. The expression of *PFKFB3* was much higher in CML patients than any other leukemia patients (Fig. 1d). To validate the expression of *PFKFB3*, we measured the expression of *PFKFB3* among multiple human leukemia cell lines and health human peripheral blood mononuclear cell (PBMCs). We showed that the expression of *PFKFB3* was higher in human CML cell lines than other leukemia cell lines (Fig. 1e). In addition, the expression of *PFKFB3* was not detected in healthy human PBMCs. Those results suggested that *PFKFB3* may play an important role in human CML and TKIs-resistant CML.

PFKFB3 expression elevates in TKI-resistant CML cell lines or CML patients

To investigate the potential function of *PFKFB3* in human CML and TKI-resistant CML, we compared the expression of *PFKFB3* among K562 cells, the K562-derived TKIs-resistant CML cell lines K562IR and K562IDR. K562IR


a
K562
K562IR
K562IDR
% live cells
3PO (μM)
PFK15 (μM)
b
IM+PFK15: 0μM+0μM
IM+PFK15: 0μM+0.1μM
IM+PFK15: 0μM+0.5μM
IM+PFK15: 0.5μM+0μM
IM+PFK15: 2μM+0μM
IM+PFK15: 0.5μM+0.1μM
IM+PFK15: 2μM+0.1μM
IM+PFK15: 0.5μM+0.5μM
IM+PFK15: 2μM+0.5μM
DA+PFK15: 0nM+0μM
DA+PFK15: 0nM+0.1μM
DA+PFK15: 0μM+0.5μM
DA+PFK15: 1nM+0μM
DA+PFK15: 4nM+0μM
DA+PFK15: 1nM+0.1μM
DA+PFK15: 4nM+0.1μM
DA+PFK15: 1nM+0.5μM
DA+PFK15: 4nM+0.5μM
Live cell count (x10⁴)
24h
48h
72h
96h
K562IR
K562IDR
c
K562IR
K562IDR
K562IR+PFK15
K562IDR+PFK15
% live cells
Imatinib (μM)
Dasatinib (nM)
d
IM+PFK15
0μM+0μM
2μM+0μM
0μM+0.5μM
2μM+0.5μM
7-AAD
Annexin-V
0.6%
1.1%
7.5%
16%
5.6%
7.7%
24%
63%
e
PFK15
0μM
0.5μM
13.4%
0.232%
86.1%
0.256%
5.82%
5.74%
74.6%
13.9%

◀ **Fig. 4** Inhibition of *PFKFB3* in combination with BCR-ABL kinase inhibitor suppresses the growth of TKIs-resistant CML cells in vitro. **a** Relative cell viability of K562, K562IR, and K562IDR cultured in the presence of *PFKFB3* kinase inhibitor 3-PO (left) or PFK15 (right) at different concentrations. **b** Cell growth of K562IR (left) and K562IDR (right) cells cultured with Imatinib, PFK15, alone or combined at different concentrations. **c** Relative cell viability of K562IR and K562IDR cultured with 5 nM PFK15 and Imatinib (left) or Dasatinib (right) at different concentrations. **d** Flow cytometry analysis of K562IR cells treated with Imatinib, PFK15, or both at different concentrations for Annexin-V and 7-AAD stained cells. **e** Flow cytometry analysis of human primary TKI-resistant CML cells treated with PFK15 for Annexin-V and 7-AAD stained cells

acquired resistance to Imatinib by overexpression of SRC-family kinase *LYN/HCK*. K562IR is sensitive to Dasatinib (Fig. 2a), the second generation of TKIs that inhibited both *BCR-ABL* and *LYN/HCK* kinases [17, 18]. K562IDR cells were overexpressed with *LCK*, *MAPK*, and *AKT* genes to bypass the first and second generations of TKIs [6, 30]. Quantitative real-time PCR and immunoblotting showed the significant increase of *PFKFB3* in TKIs-resistant K562 cells at both mRNA and protein levels (Fig. 2b, c). In addition, we also showed that the mRNA level of *PFKFB3* expression was significantly elevated in the bone marrow cells from Imatinib-resistant patients (Fig. 2d).

Loss of *PFKFB3* sensitizes CML cells to TKI inhibition

To investigate whether *PFKFB3* affected the TKIs resistance in CML, we used small hairpin RNA interfering (shRNA) to knockdown *PFKFB3* in Imatinib- and Dasatinib-resistant leukemia cell line: K562IR and K562IDR (Fig. 3a). After comparing the retroviral transducing leukemia cells with scramble shRNAs or *shPFKFB3*, the protein levels of *PFKFB3* were significantly decreased in both K562IR and K562IDR cells (Fig. 3a). *PFKFB3* knockdown alone significantly reduced the proliferation rate of K562, K562IR, and K562IDR, suggesting its crucial role of biological energy metabolism in promoting the proliferation of leukemic cells (Fig. 3b). Next, we further tested whether *PFKFB3* knockdown may affect the response of TKI-resistant leukemia cells to TKIs treatment. Treatment of these *shPFKFB3*-transduced leukemic cells with TKIs additionally enhanced the response of these TKI-resistant leukemia cells to TKIs in vitro (Fig. 3c). Furthermore, we decided to validate whether *PFKFB3* overexpression may lead to leukemia cell more resistant to Imatinib treatment. *PFKFB3* was overexpressed in K562 cells and the overexpression of *PFKFB3* was shown by western blot (Fig. 3d). Overexpression of *PFKFB3* led K562 cells to be more resistant to Imatinib treatment at different doses (Fig. 3e). Therefore, we showed that *PFKFB3* is required for the proliferation of CML cells. More importantly, *PFKFB3* knockdown in combination with TKIs markedly sensitized the response of TKIs-resistant leukemia cells to TKI treatment, which suggested that *PFKFB3* may be one of the crucial factors for overcoming TKIs resistance in CML.

Inhibition of *PFKFB3* in combination with BCR-ABL kinase inhibitor suppresses the growth of TKIs-resistant CML cells in vitro

To test whether kinase activity of *PFKFB3* is crucial for the TKIs-resistant CML cells, we applied *PFKFB3* inhibitors 3PO and PFK15 to K562-derived TKIs-sensitive or -resistant CML cells. We tested the effect of *PFKFB3* inhibitor alone on the proliferation of TKI-sensitive or -resistant K562 cells. The IC_{50} of 3PO is one magnitude higher than that of PFK15, both of which were similar for all the tested K562 cell lines, which was consistent with the decrease of proliferation of CML cells after genetic knockdown of *PFKFB3* (Fig. 4a). Next, we selected PFK15, the more efficient *PFKFB3* inhibitor for further analysis. TKI-sensitive CML cell lines, K562 and MEG-01, showed the similar response to TKI alone or TKI combined with PFK15. In contrast, T-cell leukemia cell line JURKAT did not show any response to TKI alone or TKI combined with PFK15 (Supplemental Figure S1). TKIs-resistant K562IR and K562IDR cells were cultured with Imatinib or Dasatinib alone, or combined with PFK15 at different concentrations, and live cells were counted for up to four consecutive days. The proliferation of K562IR was completely inhibited by 2 μM Imatinib and 0.5 μM PFK15, 0.5 μM Imatinib and 0.5 μM PFK15, or 2 μM Imatinib and 0.1 μM PFK15 (Fig. 4b). Similarly, the growth of K562IDR cells was significantly inhibited by 4 nM Dasatinib and 0.5 μM PFK15, 1 nM Dasatinib and 0.5 μM PFK15, or 4 nM Dasatinib and 0.1 μM PFK15 (Fig. 4b). Treatment of TKIs-resistant CML cells with Imatinib and 0.5 μM PFK15, or Dasatinib and 0.5 μM PFK15, markedly decreased their IC_{50} to the levels of TKIs-sensitive K562 cells (Fig. 4c). Annexin-V staining of drug-treated K562IR cells followed showed that combination of PFK15 and TKIs treatment induced the increased apoptosis of TKIs-resistant CML cells in comparison with leukemia cells treated with PFK15 or TKIs alone (Fig. 4d). In addition, we also tested the effect of *PFKFB3* inhibitor on human primary TKI-resistant CML cells and we showed that PFK15 treatment induced increased apoptosis of TKIs-resistant CML cells significantly in vitro (Fig. 4e). Thus, *PFKFB3* kinase inhibition abolished TKIs resistance of CML cells, and TKIs in combination with PFK15 efficiently inhibited the leukemia growth and induced the increase of cell apoptosis in TKIs-resistant CML cells. In addition, we also tested whether *PFKFB3* inhibition may affect the expression of *PFKFB* genes. We showed that Imatinib treatment enhanced the

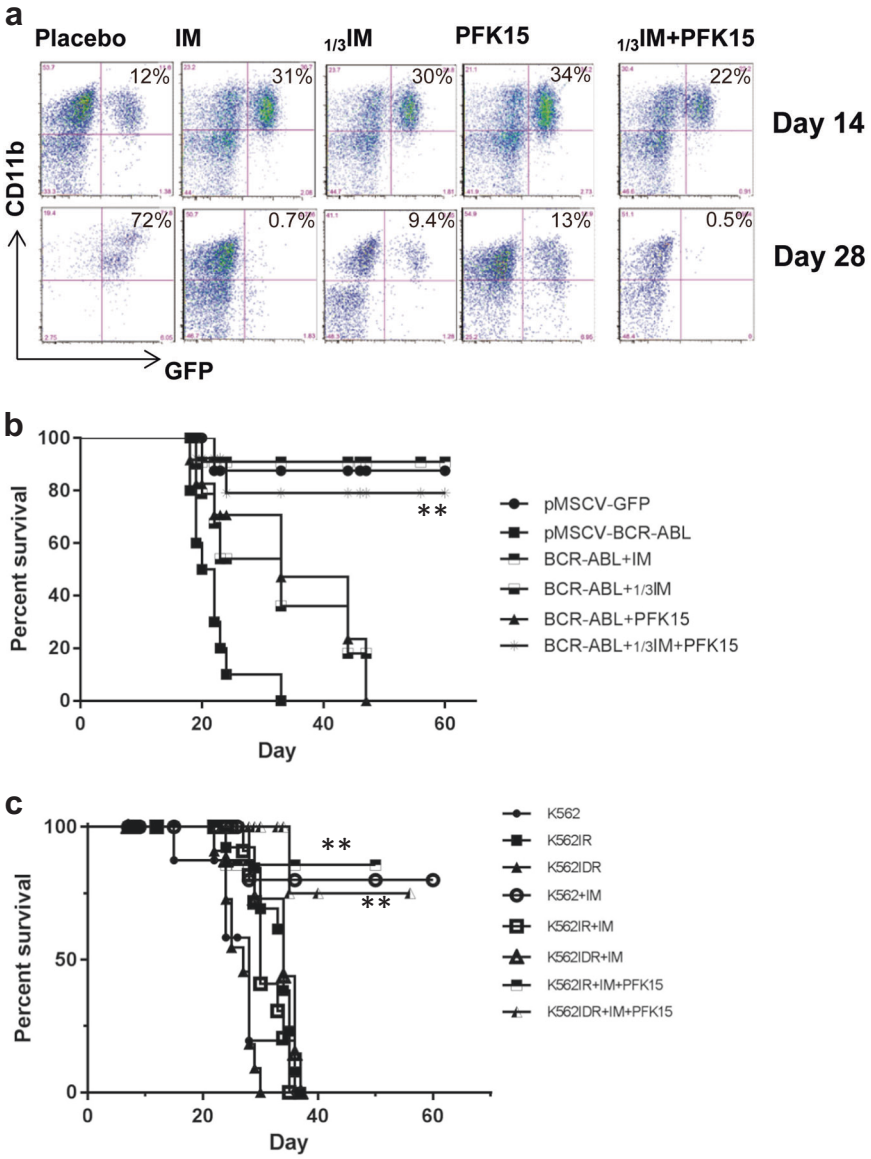


Fig. 5 Inhibition of *PFKFB3* in combination with TKI suppresses TKIs-resistant CML cells in vivo and prolongs the survival of allo-transplanted and xeno-tranplanted CML mice. **a** Flow cytometry analysis of the peripheral blood from the *BCR-ABL*-transduced bone marrow cells-transplanted mice for the percentages of $CD11b^{+}GFP^{+}$ cells. **b** Kaplan−Meier survival curves of recipient mice transplanted with bone marrow cells transduced with MSCV retrovirus bearing *GFP* alone or *BCR-ABL-GFP*. Mice received *BCR-ABL-GFP*-transduced cells were grouped and treated with either full dosage of Imatinib (100 mg/kg/day), one third of the full Imatinib dosage (33 mg/kg/day), or PFK15 (10 mg/kg/day), or the combination of PFK15 and 33 mg/kg/day of Imatinib. **c** Kaplan−Meier survival curves of recipient NOD/SCID/IL2Rg (NSG) mice transplanted with K562, K562IR, or K562IDR cells and treated with Imatinib at full dosage, PFK15, or the combination of both Imatinib and PFK15

mRNA expression of *PFKFB1* and *PFKFB2*, which were partially reversed by *PFKFB3* inhibitor: PFK15 (Supplemental Figure S2).

Inhibition of *PFKFB3* in combination with TKI suppresses TKIs-resistant CML cells in vivo and prolongs the survival of allo-transplanted and xeno-tranplanted CML mice

To test the combinational effects of PFK15 with TKIs on CML development in vivo, we generated allograft CML mice with retroviral transduced *BCR-ABL* expression in murine hematopoietic stem cells followed by bone marrow transplantation into semi-lethal irradiated wild-type recipient mice. Mice transplanted with *BCR-ABL*-expressed bone marrow cells developed CML with increased percent of GFP^{+} and $CD11b^{+}$ cells in peripheral blood and died from 2−4 weeks after transplantation (Fig. 5a, b). CML mice treated with full dosage of Imatinib showed the decrease of GFP^{+} and $CD11b^{+}$ cells and survived more than 60 days (Fig. 5a, b). Treatment of CML mice with sub-optimal dosage of Imatinib, or PFK15 alone only slightly decreased CML cell growth and modestly prolonged their survival (Fig. 5a, b). In contrast, combined treatment of CML mice with sub-optimal dosage of Imatinib and PFK15 eliminated most of the GFP^{+} and $CD11b^{+}$ cells (Fig. 5a). These mice survived significantly longer that was comparable to the full dosage of Imatinib-treated CML mice ($^{*}P < 0.05$, $^{**}P < 0.01$) (Fig. 5b). Thus, inhibition of *PFKFB3* in combination with sub-optimal

dosage Imatinib efficiently prevented CML development in CML mice.

To confirm the synergistic effects of PFK15 with TKIs on human CML cells developed in vivo, we generated xenograft CML mouse model by transplantation of TKIs-sensitive or -resistant K562 cells into NOD/SCID/IL2Rg (NSG) mice. NSG mice lack mature T cells, B cells, or functional NK cells and showed the better engraftment of human cells than any other published mouse strain [31]. As shown in Fig. 5c, NSG mice transplanted with K562, K562IR, or K562IDR cells without TKI treatment died within 40 days following transplantation. Imatinib treatment of mice transplanted with K562 cells survived more than 60 days. But Imatinib-treated mice transplanted with K562IR, or Dasatinib-treated mice transplanted with K562IDR failed to survive longer than the untreated comparable recipients. In contrast, PFK15 combined with Imatinib treatment of mice transplanted with K562IR, or PFK15 combined with Dasatinib treatment of mice transplanted with K562IDR survived much longer than the comparable recipients with Imatinib or Dasatinib treatment alone ($*P < 0.05$, $**P < 0.01$) (Fig. 5c). Thus, inhibition of *PFKFB3* in combination with TKIs efficiently suppresses TKIs-resistant human CML development in CML xenograft mouse model.

The Ets transcription factor *PU.1* upregulates the expression of *PFKFB3* in TKIs-resistant CML cells

As *PFKFB3* mRNA transcripts were significantly elevated in TKIs-resistant CML cells, we next sought to identify the transcription factors that might control the *PFKFB3* expression in TKIs-resistant CML cells. We analyzed ENCODE ChIP-seq data on K562 cells with UCSC genome browser and collected a list of 71 transcription factors that bound to the proximal regions (within 30 Kb flanking either end of *PFKFB3* genomic sequence hg19) of *PFKFB3* gene (Supplemental Table S2). These binding sites were also clustered and co-localized with histone methylation (H3K4Me3, H3K4Me1) and acetylation (H3K27Ac) (Fig. 6a). We retrieved the expression data for these transcription factors from the Haferlach leukemia data sets including 2096 leukemia patient samples (GSE13159), and performed differential expression analysis to compare changes of gene expression in CML patients vs. non-CML patients, and selected five transcription factors (represented by seven probes) with statistically significant, 30% or more elevated expression in CML samples (Fig. 6b, $P < 0.01$ and log2 (fold Change > 0.4)). Among these candidate genes, *ZBTB7A* was not top ranked for the expression in CML in comparison with other types of leukemia (Supplemental Figure S3). Although *GATA-1*, *GATA-2,* and *NFE2* passed the screening criteria, those transcription factors were major transcription factors regulating erythroid cell development (Supplemental Figures S4−S6). Thus, we only focused on *SPI1* (the gene nomenclature of *PU.1*) because it is the master transcription factor for myeloid progenitor cell development [32, 33] and its expression level was top ranked in CML patient samples (Fig. 6b). We performed quantitative real-time PCR to reveal the higher level expression of *PU.1* in TKIs-resistant CML patients in comparison with the level in TKIs-sensitive patients (Fig. 6c and Supplemental Table S1). The protein level of *PU.1* was also compared between TKIs-resistant K562IR cells and TKIs-sensitive K562 cells and *PU.1* was expressed much higher in TKIs-resistant K562IR cells than in TKIs-sensitive K562 cells (Fig. 6d). The correlation of transcription factor *PU.1*expression to *PFKFB3* in TKIs-resistant CML cells also suggested that *PU.1* may be responsible for the elevated expression of *PFKFB3*in TKIs-resistant CML cells although we did not observe a correlation between *PU.1* and *PFKFB3* among the whole population of primary CML patients (Supplemental Figures S7). To test whether *PU.1* regulated *PFKFB3* expression in TKIs-resistant CML cells, we knocked down *SPI1* expression with shRNA specifically against *SPI1* gene in K562IR. *PU.1*protein levels were shown to be significantly decreased in CML cells transduced with *SPI1*-specific shRNA in comparison to CML cells transduced with scrambled shRNA (Fig. 6e).The knockdown of *SPI1* gene led to the corresponding decrease in the mRNA and protein level of *PFKFB3* in K562IR cells (Fig. 6e, f). Therefore, the Ets transcription factor *PU.1* is required for the elevated expression of *PFKFB3* in TKIs-resistant K562 CML cells.

Discussion

In this study, we performed the bioinformatics screen of genes that overexpressed in CML patient in comparison to non-CML patient samples. The genes with FDA-approved pharmacological inhibitors or tool inhibitors available were selected. Then, mRNA levels of those candidate genes in CML patients resistant to TKI treatment were measured to identify the final list of genes with elevated expression in response to TKIs. Among those candidate genes, *PFKFB3* controlled the limiting step of glycolysis and its expression was significantly enhanced in TKI-resistant CML cells [34]. Therefore, we knocked down *PFKFB3* or used small molecule to inhibit its kinase activity and observed the *PFKFB3* knockdown or inhibition markedly enhanced the response of resistant CML cells to TKIs. In addition, we also observed that TKI in combination with *PFKFB3* inhibitor, PFK15, significantly inhibited the proliferation of CML cells in both allograft and xenograft CML mouse models.

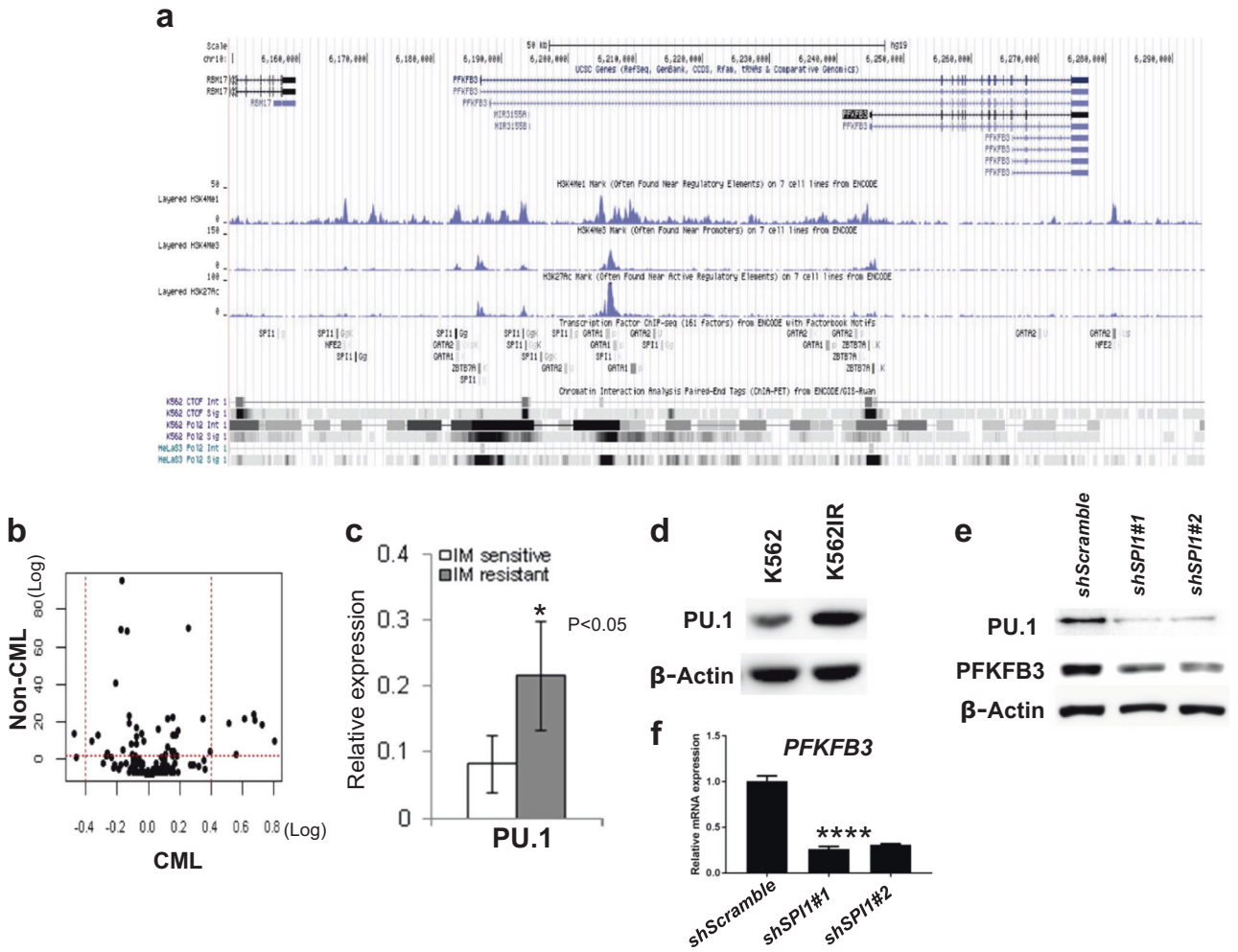


Fig. 6 The Ets transcription factor *PU.1* upregulates the expression of *PFKFB3* in TKIs-resistant CML cells. **a** Diagram generated from UCSC genome browser demonstrates the human *PFKFB3* genomic region. Selected transcription factors binding to this region according to the existing ChIP-seq data are illustrated in the diagram. **b** Volcano plot showed the differential expression of the *PFKFB3* binding transcription factors in CML patient samples in comparison to any other type of leukemia. Only the transcription factors that are statistically overexpressed in CML samples are shown in (**a**). **c** *PU.1* mRNA level was shown in Imatinib-resistant CML patients and in Imatinib-sensitive CML patients. **d** *PU.1* protein levels were shown in K562 and K562IR cells. **e** *PU.1* and *PFKFB3* protein levels were shown in K562IR cells transduced with lentivirus bearing scramble shRNA, or shRNAs specifically targeted to *PU.1* gene (shRNA1 and shRNA2). **f** The mRNA expression of *PFKFB3* was measured in K562IR cells transduced with lentivirus bearing scramble shRNA, or sh*PU.1*. Total RNA was collected and real-time PCR was performed (**$P < 0.01$)

PFKFB3 is crucial for the regulation of glycolysis in the control of cancer cell proliferation, migration, and apoptosis [25–28]. Clem et al. synthesized the small molecule inhibitors of *PFKFB3*, 3-PO and more efficient PFK15, and showed that *PFKFB3* inhibition led to the decrease of *F2,6BP*, lactate, ATP, NAD/NADH and glucose uptake in Jurkat T-cell leukemia cells, and cell cycle arrest in the G2/M phases [35, 36]. Moreover, *PFKFB3*-overexpressed Jurkat cells showed the increase of intracellular *F2,6 BP* concentration and the increased resistance to 3-PO treatment [37, 38].

In this study, we showed that *PFKFB3* played an important role in regulating the TKIs-resistance in human CML. The significantly increased mRNA and protein levels of *PFKFB3* were found in TKIs-resistant K562 cells. This upregulation of *PFKFB3* expression was further validated in the bone marrow cells from Imatinib-resistant patients. By knocking down *PFKFB3* or using the *PFKFB3* inhibitors, 3-PO and PFK15, the response of resistance leukemia cells to TKIs were significantly enhanced. In addition, TKI in combination with *PFKFB3* inhibitor can extend the survival much longer than the comparable recipients with Imatinib or Dasatinib treatment alone. Therefore, our study suggested that *PFKFB3* is important for TKI-sensitive (K562) or -resistant (K562IR and DR). Our findings revealed a novel metabolic survival pathway and a

therapeutically targetable mechanism of *BCR-ABL*-independent TKIs resistance in human CML.

Beside TKIs-resistance CML cells, *PFKFB3* may also regulate the proliferation of CML cell or other type of leukemia cells. Our study showed that *PFKFB3* had a high expression among other leukemia cell lines, but not in human normal blood cells. *PFKFB3* inactivation by shRNA or small molecule compounds led to growth inhibition in both TKI-sensitive (K562) or -resistant (K562IR and DR), which suggested that *PFKFB3* may also have important role in CML cell or other leukemia cells. Furthermore, *PFKFB3* could be a potential target in other human cancer types. For example, 3-PO significantly reduced the tumor size in BALB/c nude mice with intraperitoneal injection of HL-60 myeloid leukemia cells [35]. The *PFKFB3* inhibitor, PFK15, significantly inhibited the tumor growth in a couple of mouse lung, colon and pancreatic tumor models and showed the comparable anti-tumor effects with cytotoxic anti-tumor reagents irinotecan or gemcitabine [37, 39–42]. In this study, we also investigated the potential mechanism of *PFKFB3* upregulation. We identified the Ets transcription factor *PU.1* as the potential regulator of *PFKFB3* through ChIP-seq data from K562 cells. *PU.1* has been known as a key protein to regulate the development of B-lymphocytes and myeloid cells [43]. The findings from Iwasaki et al. showed that loss of *PU.1* in HSCs leads to cell exhaustion and *PU.1* is a commonly deregulated transcription factor in human myeloid leukemia cells [22]. In this study, we found that upregulated *PU.1* is crucial to control the mRNA and protein expression of *PFKFB3* in TKIs-resistant CML cells, which suggested that *PU.1* is involved into the development of TKIs-resistant CML cells. However, the mechanisms of *PU.1* regulating the expression of *PFKFB3* gene or other metabolic genes in leukemia cells upon TKI treatment is still not clear and more efforts are needed to understand whether *PU.1* directly binds to the *PFKFB3* locus and activates transcription in the future. Moreover, inhibition of *PU.1* activity was developed as a therapeutic strategy in AML recently [44]. It may also be worth to test whether PU.1 inhibition could enhance the response of CML resistant cells to TKIs.

The function of *PFKFB3* in human hematologic malignancies remained unknown, although the anti-tumor effects of *PFKFB3* had been reported in human solid tumors [38]. Here, we showed that *PFKFB3* expression was elevated and potentially upregulated by the Ets transcription factor *PU.1* in TKI-resistant CML cells. The overexpression of *PFKFB3* was crucial for regulating CML cells resistance to TKIs. Therefore, our findings revealed a novel TKI-resistant pathway independent of *BCR-ABL* signaling, and may provide an alternative chemotherapeutic target that help to circumvent TKI-resistant CML and eradicate CML stem cells.

Materials and methods

Reagent

The following reagents were purchased from the companies indicated: Imatinib (Catalog No. S2475), Dasatinib (Catalog No. S1021), and PFK15 (Catalog No. S7289) were purchased from SelleckChem. 3PO (Catalog No. 525330) was purchased from EMD Millipore. Anti-*PFKFB3* antibody (Catalog No. ab181861) was purchased from Abcam. Anti-*PU.1* (Catalog No. sc-352) was purchased from Santa Cruz Biotechnology. These reagents were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA), distributed into small aliquots, and stored at −20 °C.

Bioinformatics data analysis

Microarray data sets were downloaded from NCBI GEO sessions and analyzed with R/RStudio programming scripts. R packages including GEO query and limma were used for differential analysis. The original data sets were log2 transformed and median-centered before comparison. The drug target list files were downloaded from www.guidetopharmacology.org and combined as unique target list. The transcription factor list was generated from ENCODE ChIP-Seq data sets via UCSC genome browser.

Patient samples

Institutional Review Board (IRB) approval for the acquisition of CML patient samples was obtained from the Department of Hematology, the First Affiliated Hospital of Nanjing Medical University, and written informed consent was obtained in accordance with Declaration of Helsinki principles. Human CML samples were selected on the basis of sample availability and a requirement to achieve statistical significance.

Cell culture

JURKAT, MEG-01, NALM-1, RAMOS, HH, and K562 leukemia cell lines were obtained from American Type culture Collection (ATCC). K562IR and K562IDR were kindly provided by Nicholas J. Donato, University of Michigan. Those leukemia cells were grown in RPMI 1640 medium containing 10% fetal bovine serum, 4 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cell growth was evaluated by viable cell count with trypan blue exclusion or celltiter glo (CTG). These cells were derived and characterized. They have been tested for mycoplasma contamination.

Mice

Six to 8-week-old female C57BL/6J and NOD/SCID/IL2Rg (NSG) mice were obtained from The Jackson Laboratory. Mice were maintained in a temperature- and humidity-controlled environment and given unrestricted access to a 6% chow diet and acidified water.

CML mice study

The study consisted of a series of controlled laboratory experiments and measured multiple parameters including gene expression, cell proliferation, survival, apoptosis, and leukemic progression as described below. For animal experiments, mice were randomly allocated to each group for drug treatment after bone marrow transplantation, and were subsequently analyzed in a non-blinded fashion. Briefly, to model CML, BM from 5-FU–treated (200 mg/kg) donor mice was transduced twice with *BCR-ABL* retrovirus by co-sedimentation in the presence of IL-3, IL-6, and stem cell factor. WT recipient mice received 1100 cGy gamma irradiation (given by two split 550-cGy doses), and 0.5×10^6 (CML) cells were transplanted into the recipient mice via tail vein injection. Animal sample sizes were selected based on precedent established from previous publications and an understanding that at least $n = 4$ is generally required to achieve statistical significance. Survival was represented with Kaplan−Meier survival curves and statistical significance was called based on log ranks of Cox's proportional-hazards model.

shRNA knockdown and *PFKFB3* overexpression

The shRNA constructs were generated by ligating the synthesized oligonucleotides (either the non-specific (NC) scrambled control or the specifically targeted to *PFKFB3* or *PU.1*) into the RNAi Consortium (TRC) lentiviral human short hairpin RNA (Open Biosystems/Thermo Scientific). Knockdown efficiency was calculated relative to that obtained with a control NS shRNA. Full length *PFKFB3* was cloned into lentiviral vector. Lentiviral supernatants were generated and K562 cells were infected.

Quantitative real-time PCR

Total RNA was isolated from cells using Trizol Reagent (Invitrogen) followed by treatment with turbo DNase to remove contaminating genomic DNA. Reverse transcription was performed using MMLV reverse transcriptase (New England Biotechnology) followed by quantitative PCR with Fast SYBR Green Master Mix (Applied Biosystems). The expression of each gene was normalized to that of β-ACTIN or GAPDH.

Immunoblotting

After treatment, cells were harvested and lysed with radioimmunoprecipitation assay (RIPA) buffer (10×200 mM HEPES pH 6.8, 1400 mM NaCl, 25 mM $MgCl_2$, 25 mM $CaCl_2$, 10% NP40, and 5% sodium deoxycholate) plus phosphatase (Sigma) and protease inhibitors (Roche). Blots were probed with the following primary antibodies: anti-*PFKFB3* (Abcam), anti-*PU.1* (Santa Cruz Biotechnology), and anti-β-Actin (Sigma Aldrich). Primary Abs were diluted in 5% BSA at 1:1000. Blots were developed with Pico/Femato super signal (Sigma) and visualized using autoradiography or a Bio-Rad ChemiDoc MP Imaging System.

Flow cytometry

For apoptosis staining, 5 µl Annexin-V antibody (eFluor450-conjugated Annexin V, eBioscience) was added to each sample after the last wash and incubated for 20 min in the dark at room temperature. Samples were washed once, and 2 µl 7-aminoactinomycin D (7AAD) (eBioscience) was added within 4 h before FACS analysis. Bone marrow cells were flushed out of mouse femur and tibia bones with RBC lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA), spun down at 1000 rpm for 10 min, and washed once with phosphate-buffered saline (PBS). $2–5\times10^6$ cells from each sample were aliquoted for staining. Fluorescent-tagged primary antibodies for CD11b or Gr-1 (eBioscience) was added to each sample in a total volume of 100 µl, incubated on ice for 30 min, washed once by adding PBS (1 ml) and spin down at 1000 rpm for 10 min. FACS analysis was performed immediately using an LSR II flow cytometer and Flowjo software (BD Biosciences).

Statistics

Statistical analyses were performed using a one-tailed Student's *t*-test ($^*P < 0.05$; $^{**}P < 0.01$) and GraphPad Prism software, version 5.01 for Windows (GraphPad Software). A P value less than 0.05 was considered statistically significant.

Study approval

All animal protocols were approved by the Institution Animal Care and Use Committee (IACUC) at the First Affiliated Hospital of Nanjing Medical University or by the IACUC of the University of Massachusetts Medical School. CML patient samples were taken at the time of diagnosis of CML, and written informed consent was obtained in accordance with Declaration of Helsinki principles and with IRB approval from the First Affiliated Hospital of Nanjing Medical University.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Rosti G, Castagnetti F, Gugliotta G, Baccarani M. Tyrosine kinase inhibitors in chronic myeloid leukaemia: which, when, for whom? Nat Rev Clin Oncol. 2016;14:141–54.
2. Schütz C, Inselmann S, Sausslele S, Dietz C, Mü Ller M, Eigendorff E, et al. Expression of the CTLA-4 ligand CD86 on plasmacytoid dendritic cells (pDC) predicts risk of disease recurrence after treatment discontinuation in CML. Leukemia. 2017;31:829–36.
3. Holyoake TL, Vetrie D. The chronic myeloid leukemia stem cell: stemming the tide of persistence. Blood . 2017;129:1595–606.
4. Gotlib J. How I treat atypical chronic myeloid leukemia. Blood. 2017;129:838–45.
5. Steelman LS, Franklin RA, Abrams SL, Chappell W, Kempf CR, Bäsecke J, et al. Roles of the Ras/Raf/MEK/ERK pathway in leukemia therapy. Leukemia. 2011;25:1080–94.
6. Bibi S, Arslanhan MD, Langenfeld F, Jeanningros S, Cerny-Reiterer S, Hadzijusufovic E, et al. Co-operating STAT5 and AKT signaling pathways in chronic myeloid leukemia and mastocytosis: possible new targets of therapy. Haematologica . 2014;99:417–29.
7. Quentmeier H, Eberth S, Romani J, Zaborski M, Drexler HG. BCR-ABL1-independent PI3Kinase activation causing imatinib-resistance. J Hematol Oncol. 2011;4:6.
8. Yamada O, Kawauchi K. The role of the JAK-STAT pathway and related signal cascades in telomerase activation during the development of hematologic malignancies. Jak-Stat. 2013;2: e25256.
9. Bar-Natan M, Nelson EA, Xiang M, Frank DA. STAT signaling in the pathogenesis and treatment of myeloid malignancies. Jak-Stat. 2012;1:55–64.
10. Zhou H, Mak PY, Mu H, Mak DH, Zeng Z, Cortes J et al. Combined inhibition of β-catenin and Bcr–Abl synergistically targets tyrosine kinase inhibitor-resistant blast crisis chronic myeloid leukemia blasts and progenitors in vitro and in vivo. Leukemia. 2017. https://doi.org/10.1038/leu.2017.87.
11. Cang S, Liu D. P-loop mutations and novel therapeutic approaches for imatinib failures in chronic myeloid leukemia. J Hematol Oncol. 2008;1:15.
12. Han SH, Kim S-H, Kim H-J, Lee Y, Choi S-Y, Park G, et al. Cobll1 is linked to drug resistance and blastic transformation in chronic myeloid leukemia. Leukemia. 2017;31:1532–9.
13. Chen Z, Shao C, Wang W, Zuo Z, Mou X, Hu SJ, et al. Cytogenetic landscape and impact in blast phase of chronic myeloid leukemia in the era of tyrosine kinase inhibitor therapy. Leukemia. 2017;31:585–92.
14. Eadie L, Dang P, Saunders V, Yeung D, Osborn M, Grigg A, et al. The clinical significance of ABCB1 overexpression in predicting outcome of CML patients undergoing first-line imatinib treatment. Leukemia. 2016;31:75–82.
15. Wagle M, Eiring AM, Wongchenko M, Lu S, Guan Y, Wang Y, et al. A role for FOXO1 in BCR–ABL1-independent tyrosine kinase inhibitor resistance in chronic myeloid leukemia. Leukemia. 2016;30:1493–501.
16. Lee C-R, Kang J-A, Kim H-E, Choi Y, Yang T, Park S-G. Secretion of IL-1β from imatinib-resistant chronic myeloid leukemia cells contributes to B*CR* - A*BL* mutation-independent imatinib resistance. FEBS Lett. 2016;590:358–68.
17. Okabe S, Tauchi T, Ohyashiki K. Characteristics of dasatinib- and imatinib-resistant chronic myelogenous leukemia cells. Clin Cancer Res. 2008;14:6181–6.
18. Pene-Dumitrescu T, Smithgall TE. Expression of a Src family kinase in chronic myelogenous leukemia cells induces resistance to imatinib in a kinase-dependent manner. J Biol Chem. 2010;285:21446–57.
19. Sweet K, Zhang L, Pinilla-Ibarz J. Biomarkers for determining the prognosis in chronic myelogenous leukemia. J Hematol Oncol. 2013;6:54.
20. Chen Y, Peng C, Abraham SA, Shan Y, Guo Z, Desouza N, et al. Arachidonate 15-lipoxygenase is required for chronic myeloid leukemia stem cell survival. J Clin Investig. 2014;124:3847–62.
21. Chen Y, Hu Y, Zhang H, Peng C, Li S. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. Nat Genet. 2009;41:783–92.
22. Haferlach T, Kohlmann A, Wieczorek L, Basso G, Te Kronnie G, Béné MC, et al. Clinical utility of microarray-based gene expression profiling in the diagnosis and subclassification of leukemia: report from the international microarray innovations in leukemia study group. J Clin Oncol. 2010;28:2529–37.
23. Cantelmo AR, Conradi LC, Brajic A, Goveia J, Kalucka J, Pircher A, et al. Inhibition of the glycolytic activator PFKFB3 in endothelium induces tumor vessel normalization, impairs metastasis, and improves chemotherapy. Cancer Cell. 2016;30:968–85.
24. Yamamoto T, Takano N, Ishiwata K, Ohmura M, Nagahata Y, Matsuura T, et al. Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway. Nat Commun. 2014;5:1–16.
25. Cruys B, Wong BW, Kuchnio A, Verdegem D, Cantelmo AR, Conradi L-C, et al. Glycolytic regulation of cell rearrangement in angiogenesis. Nat Commun. 2016;7:12240.
26. Yang Z, Goronzy JJ, Weyand CM. The glycolytic enzyme PFKFB3/phosphofructokinase regulates autophagy. Autophagy. 2014;10:382.
27. Doménech E, Maestre C, Esteban-Martínez L, Partida D, Pascual R, Fernández-Miranda G, et al. AMPK and PFKFB3 mediate glycolysis and survival in response to mitophagy during mitotic arrest. Nat Cell Biol. 2015;17:1304–16.
28. De Bock K, Georgiadou M, Schoors S, Kuchnio A, Wong BW, Cantelmo AR, et al. Role of PFKFB3-driven glycolysis in vessel sprouting. Cell. 2013;154:651–63.
29. Qian S, Li J, Hong M, Zhu Y, Zhao H, Xie Y, et al. TIGAR cooperated with glycolysis to inhibit the apoptosis of leukemia cells and 3 acute myeloid leukemia. J Hematol Oncol. 2016;9:128.
30. Oaxaca DM, Yang-Reid SA, Ross JA, Rodriguez G, Staniswalis JG, Kirken RA. Sensitivity of imatinib-resistant T315I BCR-ABL CML to a synergistic combination of ponatinib and forskolin treatment. Tumor Biol. 2016;37:12643–54.
31. Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, et al. Human lymphoid and myeloid cell development in NOD/

LtSz-scid IL2R null mice engrafted with mobilized human hemopoietic stem cells. J Immunol. 2005;174:6477–89.
32. Ray D, Culine S, Tavitain A, Moreau-Gachelin F. The human homologue of the putative proto-oncogene Spi-1: characterization and expression in tumors. Oncogene. 1990;5:663–8.
33. Zhang P, Behre G, Pan J, Iwama A, Wara-aswapati N, Radomska HS, et al. Negative cross-talk between hematopoietic regulators: GATA proteins repress PU.1. Proc Natl Acad Sci USA. 1999;96:8705–10.
34. Ganapathy-Kanniappan S, Geschwind, J-FH. Tumor glycolysis as a target for cancer therapy: progress and prospects. Mol Cancer. 2013;12:152
35. Clem B, Telang S, Clem A, Yalcin A, Meier J, Simmons A et al. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. Mol Cancer Therapeut. 2008. https://doi.org/10.1158/1535-7163.MCT-07-0482.
36. Yalcin A, Clem BF, Imbert-Fernandez Y, Ozcan SC, Peker S, O'Neal J, et al. 6-Phosphofructo-2-kinase (PFKFB3) promotes cell cycle progression and suppresses apoptosis via Cdk1-mediated phosphorylation ofp27. Cell Death Dis. 2014;5:e1337.
37. Coller HA. Is cancer a metabolic disease? Am J Pathol. 2014;184:4–17.
38. Clem BF, O'Neal J, Tapolsky G, Clem AL, Imbert-Fernandez Y, Kerr DA, et al. Targeting 6-Phosphofructo-2-Kinase (PFKFB3) as a therapeutic strategy against cancer. Mol Cancer Ther. 2013;12:1461–70.
39. Li T, Kon N, Jiang L, Tan M, Ludwig T, Zhao Y, et al. Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. Cell. 2012;149:1269–83.
40. Rodriguez OC, Choudhury S, Kolukula V, Vietsch EE, Catania J, Preet A, et al. Dietary downregulation of mutant p53 levels via glucose restriction: mechanisms and implications for tumor therapy. Cell Cycle. 2012;11:4436–46.
41. Moon SH, Prives C. Mutant p53 succumbs to starvation. Cell Cycle. 2013;12:867–8.
42. Yalcin A, Solakoglu TH, Ozcan SC, Guzel S, Peker S, Celikler S, et al. 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase-3 is required for transforming growth factor β1-enhanced invasion of Panc1 cells in vitro. Biochem Biophys Res Commun. 2017;484:687–93.
43. DeKoter RP. Regulation of B lymphocyte and macrophage development by graded expression of PU.1. Science. 2000;288:1439–41.
44. Antony-Debré I, Paul A, Leite J, Mitchell K, Kim HM, Carvajal LA, et al. Pharmacological inhibition of the transcription factor PU.1 in leukemia. J Clin Investig. 2017;127:4297–4313.