ABSTRACT

Tyrosine kinase inhibitors (TKI) have revolutionized chronic myelogenous leukemia (CML) management. Disease eradication, however, is hampered by innate resistance of leukemia-initiating cells (LIC) to TKI-induced killing, which also provides the basis for subsequent emergence of TKI-resistant mutants. We report that EZH2, the catalytic subunit of Polycomb Repressive Complex 2 (PRC2), is overexpressed in CML LICs and required for colony formation and survival and cell-cycle progression of CML cell lines. A critical role for EZH2 is supported by genetic studies in a mouse CML model. Inactivation of Ezh2 in conventional conditional mice and through CRISPR/Cas9-mediated gene editing prevents initiation and maintenance of disease and survival of LICs, irrespective of BCR–ABL1 mutational status, and extends survival. Expression of the EZH2 homolog EZH1 is reduced in EZH2-deficient CML LICs, creating a scenario resembling complete loss of PRC2. EZH2 dependence of CML LICs raises prospects for improved therapy of TKI-resistant CML and/or eradication of disease by addition of EZH2 inhibitors.

SIGNIFICANCE:

This work defines EZH2 as a selective vulnerability for CML cells and their LICs, regardless of BCR–ABL1 mutational status. Our findings provide an experimental rationale for improving disease eradication through judicious use of EZH2 inhibitors within the context of standard-of-care TKI therapy. Cancer Discov; 6(11): 1237–47. © 2016 AACR.

See related article by Scott et al., p. 1248.

INTRODUCTION

Chronic myelogenous leukemia (CML) is a stem cell–driven malignancy induced by the Philadelphia chromosome that generates the fusion oncoprotein BCR–ABL1 (1, 2), which harbors constitutive tyrosine kinase activity. Tyrosine kinase inhibitor (TKI) drugs are highly effective, especially in chronic phase, the early stage of the disease. Nevertheless, disease eradication is challenged by the relative resistance of leukemia-initiating cells (LIC) to cell killing and the emergence of...
EZH2 target genes were enriched in LICs compared with phases of disease (Supplementary Fig. S1A). Meanwhile, excluded from statistical analysis. H, (CML LICs with normal HSCs (Lin− CD34+). Our findings suggest that EZH2 is overexpressed in LICs at all three phases of disease (Supplementary Fig. S1A). Meanwhile, EZH2 target genes were enriched in LICs compared with HSCs in gene set enrichment analysis (GSEA; ref. 22), suggesting enhanced EZH2 activities in LICs (Supplementary Fig. S1B). Moreover, expression of EZH2, but not other PRC2 components such as EED, appeared dependent on BCR–ABL1 signaling in CML cells, as administration of TKIs (imatinib or dasatinib) reduced EZH2 protein (Supplementary Fig. S1C). Collectively, these findings led us to hypothesize that CML LICs, and perhaps their respective LICs, might require, or display “addiction” to, PRC2.

To interrogate the hypothesis, we first tested the response of human CML cells to shRNAs directed to EZH2. Two hairpins directed to EZH2, which reduced EZH2 protein levels by approximately 70% to 90% (Supplementary Fig. S1D), inhibited the growth of K562 cells (Supplementary Fig. S1E), a consequence of apoptosis and disruption of normal cell cycle (Supplementary Fig. S1F and S1G). Although current TKIs are effective in managing CML in chronic phase, some BCR–ABL1 mutants, such as the “gatekeeper” T315I mutant, present a therapeutic challenge (23). Of note, BCR–ABL1 mutational status does not change the dependence of CML cells on EZH2, as shRNA knockdown also inhibited growth of K562 cells engineered to express T315I mutant BCR–ABL1 (K562-T315I; Supplementary Fig. S1E).

**RESULTS**

**EZH2 Is Overexpressed in CML LICs, and Its Inactivation Inhibits Cell Growth of Human CML Cell Lines**

In a search for potential therapeutic targets essential for CML LICs, we compared gene expression profiles of human CML LICs with normal HSCs (Lin−CD34+CD38−CD45RA−CD90+; ref. 21), EZH2 was upregulated in LICs at all three phases of disease (Supplementary Fig. S1A). Meanwhile, EZH2 target genes were enriched in LICs compared with HSCs in gene set enrichment analysis (GSEA; ref. 22), suggesting enhanced EZH2 activities in LICs (Supplementary Fig. S1B). Moreover, expression of EZH2, but not other PRC2 components such as EED, appeared dependent on BCR–ABL1.

**Figure 1.** Ezh2 inactivation inhibits initiation/progression of CML and establishment of secondary leukemia from existing LICs. A, protocol for induction of Ezh2 deleted (Ezh2 KO) or control (Con) CML in mice. B, FACS plots showing percentages of leukemic cells in peripheral blood of control and Ezh2 KO-T315I CML mice at indicated days after BMT. C, percentage of T315I CML cells, as shown in B, n = 10 for both control and Ezh2 KO CML mice. P = 0.21 for day 6, and P = 0.0001 for days 9, 12, and 14, as determined by the Student t test. D, peripheral WBC of control (n = 5) and Ezh2 KO CML mice (n = 5), determined at day 14 after BMT. P < 0.0001 as determined by the Student t test. E, survival curve of control (n = 10) and Ezh2 KO CML mice (n = 10). P < 0.0001, as determined by Gehan-Breslow-Wilcoxon test. F, percentage and cell number (right) of LICs (EYFP−Lin−/Kit+/Sca1+) in control (n = 9) and Ezh2 KO (n = 21) CML mice, measured at day 14 after BMT. P = 0.0001 for the percentage and P = 0.003 for the cell number of LICs, as determined by the Student t test. G, experimental protocol for assessing impact of Ezh2 loss in LICs for leukemia development in secondary recipients. H, FACS plots showing percentages of Cre+ (GFP-EYFP+) and Cre− (GFP-EYFP−) secondary leukemic cells in peripheral blood of control and Ezh2 KO CML mice at days 7, 11, and 14 after BMT. Samples analyzed right before second BMT were designated as day 0, and ratio of Cre+ (GFP-EYFP+) versus Cre− (GFP-EYFP−) secondary leukemic cells, as shown in H, at the indicated days after second BMT in control or Ezh2 KO 2nd CML mice. Only one sample each for day 0 was analyzed and thus was excluded from statistical analysis. P = 0.0001 for days 7, 11, and 14 and P = 0.04 for day 17, as determined by the Student t test. I, survival curve of control (n = 6) and Ezh2 KO (n = 5) secondary CML mice. P = 0.036, as determined by Gehan-Breslow–Wilcoxon test. Results shown in B–J are representative of at least two repeat experiments.
CML LICs Require EZH2

A 5-FU BCR-ABL1-Cre virus or T315I mutant
Ezh2KO BM cells Recipient

B Day 6 Day 9 Day 12 Day 14 Day 19
Con T315I Ezh2 KO T315I

C % Leukemic cells % LIC (of total BM)

D WBC (10^3/µL)

E Percent survival

F % LIC (of total BM) Total LICs per mouse (>10,000 cells)

G 5-FU BCR-ABL1-GFP virus

H Day 0 Day 7 Day 11 Day 14
Con Ezh2 KO Ezh2 KO

I Ratio GFP-EYFP vs. GFP+EYFP

J % Survival

Published OnlineFirst September 14, 2016; DOI: 10.1158/2159-8290.CD-15-1439
Together, these data demonstrate that leukemia initiation and development are dependent on EZH2.

To assess whether EZH2 is also required for T315I mutant CML, we induced CML with T315I mutant BCR–ABL1 (Fig. 1A). CML progressed rapidly in control T315I CML mice and culminated with heavy leukemia load and death of animals at approximately 2 weeks after BMT. In contrast, leukemia development was markedly blunted in Ezh2 KO CML mice, and survival was greatly extended (Fig. 1B–E). Thus, the requirement for EZH2 in CML appears independent of the mutational status of BCR–ABL1.

**EZH2 Is Required for Maintenance and Function of CML LICs**

CML is a stem-cell disorder in which disease initiation and maintenance rely on LICs, a population of cells enriched in the Lin−c-Kit−Sca1+ population in CML mice (24, 25). To assess the impact of EZH2 loss on CML LICs, we first determined the frequency and total cell number of LICs in control or Ezh2 KO CML mice. Loss of EZH2 greatly reduced LIC frequency, as well as cell number, in Ezh2 KO CML mice, compared with control CML mice, indicating sensitivity of LICs to EZH2 loss (Fig. 1F). In the experimental setting depicted in Fig. 1A where Cre-expressing virus is transduced into normal HSCs to inactivate EZH2, it is not possible to distinguish between requirements for EZH2 and requirements for EZH2 in transforming HSCs into LICs by BCR–ABL1.

To interrogate more directly the requirement of LICs for EZH2, we determined the consequences of inactivating Ezh2 in preexisting LICs. To this end, control or Ezh2fl/fl LICs from primary CML mice, induced by BCR–ABL1-IRES-GFP virus, were isolated by fluorescence-activated cell sorting (FACS), transduced with Cre virus, and then transplanted into secondary recipients (Fig. 1G). Control CML LICs, with (GFP+EYFP) or without (GFP+EYFP) Cre virus transduction, contributed comparably to the development of secondary leukemia (Fig. 1H and I). In contrast, uninfected Ezh2fl/fl LICs (GFP+EYFP) greatly outcompeted Ezh2 KO LICs (GFP+EYFP) in the secondary leukemia, and the percentage of Ezh2 KO LIC-derived secondary leukemic cells decreased over time (Fig. 1H and I). Accordingly, deletion of Ezh2 in CML LICs prolonged survival of secondary CML mice (Fig. 1J). Notably, of the 2 Ezh2 KO secondary CML mice that died in this study, 1 died of GFP+EYFP (Cre negative) CML and the second was euthanized for conditions unrelated to CML. In contrast, biopsy showed that all control secondary CML mice died of CML. Taken together, these results indicate that CML LICs require EZH2 for their maintenance and ability to initiate leukemia on transplantation.

**Catalytic Activity of EZH2 Is Required for CML Cells**

Genetic study in the mouse CML model demonstrated that EZH2 is required for CML and its LICs, and therefore suggests that EZH2, a histone methyltransferase, might be targeted for CML therapy. The findings in Cre conditional mice, in which intact protein is eliminated, fail to exclude a noncatalytic role of EZH2 for CML cells (26). We next determined whether catalytic activity of EZH2 is required for CML cells. Exogenous EZH2, either wild-type or a mutant with three substitutions (F677I, H689A, and R727K) that result in loss of catalytic activity (27, 28), was expressed in K562 cells. Endogenous EZH2 was then deleted by CRISPR/Cas9-mediated gene editing (Fig. 2A). Exogenous wild-type EZH2 rescued growth and survival of cells with homozygous loss of endogenous EZH2. In contrast, exogenous mutant EZH2 expression failed to rescue cells lacking a functional endogenous EZH2 allele (Fig. 2B). These data provide strong genetic evidence that the catalytic activity of EZH2 is required for viability of CML cells.

We next tested several small-molecule catalytic inhibitors of EZH2: GSK126 (29), UNC1999 (30, 31), and JQEZ5 (32) as well as an inactive analogue of JQEZ5, JQEZ23 (Supplementary Fig. S3A). The inhibitors, but not the inactive analogue, effectively reduced the global level of the PRC2-associated mark, H3K27me3, in K562 cells (Supplementary Fig. S3B) and suppressed cell growth in a dose-dependent manner in K562 and KBM5 CML cells, as well as corresponding T315I derivative lines (Fig. 2C and Supplementary Fig. S3C and S3D). These results provide further evidence that human CML cells rely on EZH2, and more specifically its enzymatic activity, to support cell survival and growth, independent of the mutational status of BCR–ABL1.

To assess EZH2 dependence of primary human CML stem/progenitor cells, we performed colony formation assays in the presence of EZH2 inhibitors. JQEZ5 and UNC1999 inhibited colony formation of primary human CD34+ CML stem/progenitor cells (Fig. 2D and E and Supplementary Fig. S3E and S3F). JQEZ5 did not appreciably affect colony formation of normal human CD34+ HSPCs, although at higher concentrations, UNC1999 modestly inhibited their colony formation (Fig. 2D and E and Supplementary Fig. S3E and S3F). This is likely due to moderate inhibition of EZH1 by UNC1999 (30). The inhibitors also reduced colony formation of primary CML cells with the T315I mutation (Fig. 2E and Supplementary Fig. S3F). Together, these results demonstrate that colony formation of human CML stem/progenitor cells is sensitive to EZH2 inhibition. This conclusion is compatible with our observations presented below regarding the dependence of mouse CML LICs, but not HSCs (20), on EZH2.

**EZH2 Inactivation in CML LICs Creates a Context Resembling Total Loss of PRC2**

To probe potential molecular mechanisms for the dependence of CML LICs on EZH2, gene expression profiles of Ezh2 KO and Ezh2 wild-type CML LICs, together with Ezh2 KO, EED KO, and wild-type HSCs, were compared. Ezh1 mRNA expression decreased in LICs, in comparison with HSCs, both in mouse and in human (Fig. 3A and Supplementary Fig. S4A). Of note, the level of Ezh1 was further reduced in CML LICs lacking Ezh2 (Fig. 3A). A striking similarity in gene expression changes was observed on comparison of Ezh2 KO LICs and EED KO HSCs. For example, gene expression changes between Ezh2 KO in LICs and EED deletion in HSCs were more similar than between Ezh2 deletion and EED deletion in HSCs (Fig. 3B). In addition, we found that a subset of genes upregulated in Ezh2 KO LICs as compared with control LICs was more enriched in EED KO HSCs than in Ezh2 KO HSCs in comparison with wild-type HSCs, as revealed by GSEA analysis (Fig. 3C). Ezh2 KO in CML LICs

[1240] | CANCER DISCOVERY  NOVEMBER 2016  www.aacrjournals.org
CML LICs Require EZH2

**Figure 2.** EZH2 catalytic activity is required for CML cells and their stem/progenitor cells. **A,** experimental protocol for rescue of CML cells with either wild-type (WT) or catalytically inactive EZH2. The endogenous EZH2 was inactivated by CRISPR/Cas9-mediated gene editing. **B,** table presenting numbers and percentage of single-cell clones with 0, 1, or 2 alleles of endogenous EZH2 deleted. **C,** growth curve of K562 cells treated with EZH2 catalytic activity inhibitors UNC1999 and JQEZ5 or an inactive analogue, JQEZ23. Cells were cultured with compounds at the indicated concentrations for 6 days, and cell proliferation was determined by WST-1 cell proliferation assay every other day. Absorbance (A 450 –A 690 ) of DMSO-treated samples was set as 1, and the ratios of absorbance in EZH2 inhibitors–treated samples relative to DMSO-treated samples were plotted. Data shown as mean ± SD. Three biological replicates were performed for each sample, and two repeat experiments were performed. **D** and **E,** relative colony numbers of human BM CD34 + CML stem/progenitor cells (**D**) or total primary CML BM cells (**E**), along with their respective normal cells (Con) plated in media with DMSO or with different concentrations of JQEZ5. Colonies were enumerated at day 9 after plating. The number of colonies in the DMSO-treated group was set as 1, and ratios of colonies of the JQEZ5-treated group relative to DMSO-treated group were plotted. Each sample was plated in duplicate, and two repeat experiments were performed. Data shown as mean ± SD. P values for JQEZ3 and DMSO comparison are 0.30, 0.34, 0.23, and 0.21 for a concentration of 0.5, 1, 2, and 5 μmol/L respectively; for JQEZ5 and DMSO comparison the P values are 0.002, 0.003, 0.002, and 0.001 for a concentration of 0.5, 1, 2, and 5 μmol/L, respectively; for UNC1999 and DMSO comparison, these values are 0.003, 0.003, 0.006, and 0.004 for a concentration of 0.5, 1, 2, and 5 μmol/L, respectively.

induced changes in the expression of many genes belonging to different functional groups, or gene sets. A substantial portion (143 of a total of 353) of these gene sets, enriched in EZH2 KO LICs as compared with control LICs, were also enriched in EED KO HSCs as compared with wild-type HSCs, but not in EZH2 KO HSCs as compared with their wild-type counterparts (Fig. 3D and Supplementary Table S1). Taken together, these findings indicate that the impact of EZH2 loss in LICs resembles, in part, EED loss in HSCs. EZH2, or its close homolog EZH1, together with EED and SUZ12 comprise the core subunits of PRC2 complexes. Inactivation of EED disrupts the integrity of the PRC2 complexes and completely disables their functions. These data strongly suggest that inactivation of EZH2 in LICs creates a situation mimicking complete loss of PRC2.

Enhanced repression of PRC2 target genes is a common feature of many cancers with overexpressed PRC2 components. EZH2 overexpression in CML LICs, compared with normal HSCs, was associated with downregulation of 263 genes (at 2-fold cutoff). Twenty-seven of these 263 genes were
upregulated in EZH2 KO CML LICs, comprising about 1/3 of the total upregulated genes in EZH2 KO LICs compared with control LICs (at 2-fold cutoff; Fig. 4E and Supplementary Table S2). Among these 27 “derepressed” genes, Trp53inp1, Phlp1, H19, and Cdkn2c are associated with apoptosis or inhibition of cell proliferation (33–36). Accordingly, we observed enrichment of gene sets including p53 targets, p53 pathways, and MDM4 targets in EZH2 KO LICs compared with control LICs, as revealed by GSEA analysis. Conversely, gene sets that favor cell growth were enriched in control CML LICs (Supplementary Fig. S4B and data not shown). Collectively, these data suggest that PRC2 suppresses apoptosis.

Figure 3. EZH2 inactivation in CML LICs creates a context mimicking complete loss of PRC2. A, relative abundances of Ezh1 mRNA transcripts in normal LKS cells [Lin − c-Kit+Sca1 −] (Con HSC), EZH2 wild-type (WT) primary CML (CML LIC), and EZH2 KO primary CML LICs (Ezh2KO LIC), determined at day 14 after BMT. Value of Ezh1 mRNA transcripts in normal LKS cells was set to 1, and ratios of Ezh1 mRNA transcripts in control CML LICs or Ezh2KO LICs relative to normal LKS cells were plotted. Data shown as mean ± SD. P = 0.038 for wild-type LKS cells vs. control CML LICs and P = 0.002 for control CML vs. Ezh2KO CML LICs. B, heat maps comparing expression of genes, upregulated in Eed KO vs. wild-type HSCs (4-fold cutoff, 239 probes for 193 genes), among Eed KO, EZH2 KO, and WT HSCs, as well as between Ezh2 KO vs. control CML LICs. C, genes upregulated in Ezh2 KO vs. control CML LICs (4-fold cutoff) are enriched in Eed KO (left) but not in Ezh2KO HSCs (right), in comparison with wild-type HSCs, as assessed by GSEA. D, Venn diagram showing overlap of gene sets enriched in different comparisons. Affymetrix array expression values of Eed KO vs. WT HSCs, Ezh2KO vs. WT HSCs, and Ezh2 KO vs. control CML LICs were analyzed in GSEA for the enrichment of gene sets in C2 (curated gene sets), C4 (computational gene sets), C6 (oncogenic signatures), and C7 (immunologic signatures) from Molecular Signature Database (MSigDB). Gene sets enriched in KO cells, for each comparison group, were filtered for FDR q < 0.01, and the results plotted in this Venn diagram. E, Venn diagram depicting the correlation of the following 4 gene sets: (1) upregulated (UP) genes in control CML LICs vs. normal HSCs (LKS cells), depicted as the blue oval; (2) downregulated (DN) genes in control CML LICs vs. normal HSCs, depicted as the yellow oval; (3) upregulated genes in Ezh2 KO LICs vs. control CML LICs, depicted as the green oval; (4) downregulated genes in Ezh2 KO LICs vs. control CML LICs, depicted as the red oval. A fold-change cutoff of ±2.0 and FDR < 0.05 was applied to all comparison groups.
and proliferation inhibitor genes in CML LICs to support cell survival and growth. Consequently, inactivation of EZH2 is associated with increased expression of these genes, and subsequent growth inhibition and cell death.

**EZH2 Inactivation in Existing Disease Induces Disease Regression**

To be considered further as a target for CML treatment, manipulation of EZH2 in existing disease should lead to clearance or regression of disease. We explored this possibility by inactivating Ezh2 through CRISPR/Cas9-mediated gene editing in mice with preexisting CML. To achieve temporal and highly efficient gene inactivation, we employed a doxycycline-inducible Cas9-expressing mouse line developed in our lab (to be described elsewhere). For efficient editing and inactivation of EZH2 catalytic function in the in vivo setting, we chose an Ezh2 guide RNA (gRNA) that targets the SET domain (37). This gRNA achieves greater than 85% allele editing after Cas9 induction in a B-cell line transformed from the doxycycline-inducible Cas9 strain (Supplementary Fig. S5A). CRISPR/Cas9-mediated gene editing of the Ezh2 locus significantly reduced global 3mH3K27, although seemingly without an appreciable change in total EZH2 protein (Supplementary Fig. S5B). This is likely due to inability of this SDS-PAGE Western blot analysis to resolve wild-type full-length EZH2 (746aa) from mutant EZH2 proteins caused by indels that are just 50 to 60aa shorter.

To test the role of EZH2 in established disease, we generated primary CML in mice with BCR–ABL1-GFP virus plus Ezh2 gRNA virus or an empty virus. CML stem/progenitor cells were then transplanted into recipients to generate mice with secondary leukemia. Upon establishment of secondary leukemia, recipients were treated with doxycycline to induce Cas9 expression and observed over time (Fig. 4A). Ezh2–CRISPR/Cas9-mediated gene editing in mice with established CML slowed disease progression, as compared with the empty virus–transduced cohort also treated with doxycycline. Of special note, disease regression subsequently occurred in all CML mice expressing Ezh2 gRNA (Fig. 4B–D and Supplementary Fig. S5C). Consequently, CRISPR/Cas9-mediated Ezh2 inactivation in CML mice greatly extended survival, as compared with the empty virus cohort (Fig. 4E).

Leukemic cells were sorted at different times, and the frequency of indels was determined by Tracking of Indels by DEComposition (TIDE) analysis (ref. 38; Supplementary Fig. S5C). The overall dynamics of indels at the population level are likely complex, as cells with inactivation of both EZH2 alleles are presumably eliminated, whereas those with one or two active alleles remain and may sustain editing events in the presence of continued Cas9 expression. Accordingly, indel frequency of leukemic cells is predicted to rise to approximately 50% and plateau, at which point all leukemic cells have one edited allele and the other is inactivated in those cells destined for elimination.

**DISCUSSION**

TKI therapy has transformed CML management, turning a lethal disorder into a chronic condition. However, challenges remain in conventional TKI therapy for CML, including the relative resistance of CML LICs to TKIs and the appearance of TKI-resistant BCR–ABL1 mutants. Candidate targets of alternative pathways have been explored, but none have been validated in clinical trials. Recently, a PPARγ agonist was reported to sensitize LICs to TKIs, likely by coaxing LICs into cycle, and thereby raised the possibility of combination therapy for disease eradication (39). Simultaneous targeting of p53 and c-MYC has recently been reported as another strategy (40).

Here, we have identified and validated EZH2 as a selective vulnerability for CML LICs. Inactivation of Ezh2 in the engineered mouse model of CML blocks leukemia initiation/development and impairs CML LICs. More importantly, CRISPR/Cas9 domain–directed editing to inactivate Ezh2 in mice with existing disease leads to disease regression and a marked improvement in survival. The latter also suggests that EZH2 catalytic activity is required for CML cells in vivo, which is further supported by the rescue experiment where wild-type EZH2, but not a catalytically inactive mutant, rescues CML cells with homozygous loss of endogenous EZH2. In addition to providing additional in vivo genetic evidence in support of a critical role for EZH2 in CML maintenance, our dox-inducible CRISPR/Cas9 gene-editing experimental system should have broad applicability in other cancer models.

EZH2 inactivation in human CML cells leads to cell growth inhibition and inhibition of colony formation by primary CML stem/progenitor cells. Of note, dependence on EZH2 is independent of the mutational status of BCR–ABL1. EZH1 expression is reduced in CML LICs upon Ezh2 inactivation, which may lead to impairment of PRC2 function and failure of LIC maintenance or survival. The consequence of Ezh2 loss in the context of normal HSCs differs, in that EZH1 expression is maintained at a higher level. Although the mechanistic basis for this difference is unclear, the presence of EZH1 in HSCs partially compensates for Ezh2 loss, preserves PRC2 function, and protects cells from the deleterious effects of complete PRC2 loss. The dependence of CML LICs on Ezh2 appears quite selective in comparison with several other proposed targets for enhanced CML therapy (5), the majority of which are required for normal hematopoiesis and/or maintenance of HSCs. Such selectivity predicts existence of a therapeutic window for the administration of EZH2 inhibitors to eliminate CML LICs without adversely affecting overall hematopoiesis. Nevertheless, EZH2 inactivation may also be tumorigenic (14, 17, 41) in some circumstances. Although transient pharmacologic inhibition of EZH2 is unlikely to mimic genetic loss of EZH2, caution would need to be taken to monitor any undesired effects of EZH2 inhibitors in therapy.

In conclusion, we have genetically defined a dependence of CML LICs on EZH2, thereby establishing a potential target for improved therapy. Given the current clinical development of EZH2 inhibitors, our findings provide an experimental rationale for incorporating these agents into therapy of CML directed at disease eradication. We propose that inhibition of EZH2 should be considered as an additional strategy to circumvent intrinsic resistance to TKIs and eliminate CML LICs.

In an accompanying article in this issue, Scott and colleagues independently identify the dependence of CML on Ezh2 (42).
Figure 4. CRISPR/Cas9-mediated Ezh2 inactivation in existing CML induces disease regression and extends survival. A, protocol for generation of Ezh2 gRNA and empty-vector control CML followed by doxycycline-induced Cas9 expression and Ezh2 inactivation. B, FACS plots showing percentages of leukemic cells within Mac1+ population in peripheral blood of Ezh2 gRNA or empty-vector control CML mice at different times after BMT. C, percentage of CML cells in peripheral blood of Ezh2 gRNA or empty-vector control CML mice. n = 5 for both Ezh2 gRNA and empty-vector control CML mice. P = 0.06 for day 6, and P < 0.001 for days 15 and 20 as determined by the Student t test. Only one Empty CML mouse survived at day 26 and was excluded for statistical analysis. D, peripheral WBC of Ezh2 gRNA (n = 5) and Empty CML mice (n = 5), determined at day 20 after BMT. P = 0.001 as determined by the Student t test. E, survival curve of Ezh2 gRNA (n = 5) and Empty CML mice (n = 5). Of note, CML is unlikely to be the primary cause of death for the 2 mice in the Ezh2 gRNA group that succumbed, as CML cells were only 34.2% and 0.2% of total white blood cells respectively at the time of death. P = 0.003, as determined by Gehan–Breslow–Wilcoxon test.
METHODS

Experimental Animals

C57BL/6 and Rosa26-EYFP+/− (43) lines were obtained from The Jackson Laboratory. 129/B6 mice were purchased from Taconic. The doxycycline-inducible Cas9-expressing line was created in the Orkin laboratory and will be described elsewhere. Ezh2 conditional KO mice have been described (44) and were maintained on a C57BL/6 background. Animals were maintained in the animal facility at Boston Children’s Hospital. The Institutional Animal Ethics Committee approved all animal experiments.

Cell Lines

KB5 and KB5-T315I cells were generously provided by Dr. Ralph B. Arlinghaus (The University of Texas MD Anderson Cancer Center) in 2013 without further authentication. K562 cells were obtained from the ATCC (CCL-243; ATCC) in 2012 without further authentication. The T315I BCR-ABL1-IRES-Cre derivative line was generated by expansion of FACS-sorted GFP+ cells from BCR-ABL1(T315I)-IRES-GFP retrovirus–transduced K562 cells. All cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) media supplemented with 10% FBS, 50 μmol/L 2-mercaptoethanol, and 100 U/mL penicillin-streptomycin. Cells were tested by PCR-based assay to verify that they were free of Mycoplasma contamination.

Rescue of CRISPR Inactivated Endogenous EZH2 with Exogenous EZH2 Enforced Expression

EZH2 #2 and #3 were: CCGGGCTAGGTTAATTGGGACCAAACTCAGATTGCTGTCCCAAATTAATCTCGAGATTTGGTCCATCTATGTTGGTTCG, respectively. LZ-EZH2 gRNAs were cloned into CRISPR Nuclease Mission shRNA collection (Sigma-Aldrich). The sequences of shRNA E2B γ and #3 were: CCGGGCTAGGTTAATTGGGACCAAACTCGATTGTGTCCCAAATTAATCTCGAGATTTGGTCCATCTATGTTGGTTCG, respectively.

Bone Marrow Transduction and Transplantation

BM transduction and transplantation procedures have been described (45). Eight-to-10-week-old donor mice were primed by intravenous injection of 5-fluorouracil (200 mg/kg; Sigma) 5 days before harvest. BM cells were plated at 2 × 10^5 cells per 10-cm plate in prestimulation medium (DMEM, 15% FBS, 100 ng/mL recombinant murine SCF, 10 ng/mL recombinant murine IL3, 10 ng/mL recombinant murine IL6, 50 μmol/L 2-mercaptoethanol, and 100 U/mL penicillin-streptomycin). Cytokines were purchased from R&D systems. Cells were incubated overnight at 37°C. Virus transduction was performed by suspending cells in prestimulation media containing 5% virus supernatant and supplemented with 10 μg/mL polybrene (Millipore). Cells were then spun for 60 minutes at 700 g and incubated at 37°C for 2 to 4 hours before transfer to fresh media, and then incubated at 37°C overnight. A second round of transduction was performed, and the cells were collected 3 hours later, washed once in Hank’s Balanced Salt Solution (HBSS), and counted. Cells (0.5 × 10^6) in 0.4 mL HBSS were injected via tail vein to the recipient mice (congenic, 8–10 weeks old), which had received two doses of 400-cGy gamma irradiation separated by at least 3 hours. All recipient mice were mixed after secondary irradiation and randomly separated into two groups, one of which received BCR-ABL1-IRES-Cre virus–transduced Rosa-EYFP+/− BM cells, and the other of which received the same virus-transduced Ezh2fl/fl;Rosa-EYFP+/− BM cells.

Retrovirus Transduction of LICs and Secondary Transplants

To assess dependence of LICs on EHz2, primary LICs (GFP+Lin−c-Kit+Sca1+) were purified by FACS sorting from CML induced by BCR-ABL1-IRES-GFP virus–transduced Ezh2fl/fl;Rosa-EYFP+/− or Rosa-EYFP+/− BM cells, 12 days after BMT. Primary LICs were transduced with Cre retrovirus for two rounds. Thirty-six hours after second round Cre virus transduction, 0.2 × 10^6 cells were transplanted into second recipients, which had received two doses of 400-cGy gamma irradiation separated by at least 3 hours, together with 0.5 × 10^6 C57BL/6 BM cells. Leukemia progression was determined by percentage of leukemic cells in the blood by flow cytometry.

CRISPR Inactivation of Ehz2 in Existing Mouse CML

Ezh2 gRNA targeting the SET domain of Ezh2 has been previously described (37). Primary CML was derived as described above, using doxycycline-inducible Cas9-expressing mice as BM donors (B6/129 background). Lin GFP−BFP leukemia stem/progenitor cells were FACS-sorted from BM and spleen of primary CML. Half a million sorted cells were mixed with 2 million B6/129 BM cells and transplanted into secondary recipients (B6/129). Four days after secondary BMT, peripheral blood was assessed by FACS to confirm the establishment of leukemia, and mice were fed with doxycycline food from day 5 after BMT and thereafter. Mac1+BFP+GFP+ cells were sorted from each secondary CML mouse at different times, genomic DNA extracted, and DNA sequences flanking Cas9 cutting site amplified by PCR, which were sequenced and percentages of edited Ezh2 alleles were calculated by TIDE analysis.

Lentiviral Vectors and Viral Infection

shRNAs, constructed in pLKO.1-puro lentiviral vector, targeting human EZH2 (RefSeq ID: NM_004456) were obtained from the Mission shRNA collection (Sigma-Aldrich). The sequences of shRNA E2B γ and #3 were: CCGGGCTAGGTTAATTGGGACCAAACTCGATTGTGTCCCAAATTAATCTCGAGATTTGGTCCATCTATGTTGGTTCG, respectively.

Flow Cytometry

For assessing leukemic cells in the blood, erythrocyte-lysed blood samples were suspended in FACS staining buffer (PBS supplemented with 4% FBS) and incubated with Fc-Block (purified CD16/CD32, clone 93) for 10 minutes on ice before addition of APC-Cy7-conjugated B220 (RA3-6B2) and APC-conjugated CD11b (M1/70) antibodies, and incubated on ice for 20 minutes. A second wash with FACS staining buffer, cells were incubated with PerCP-Cy5.5-conjugated streptavidin, PE-Cy7-conjugated B220, and APC-conjugated c-Kit (2B8) on ice for 20 minutes. Cells were then washed and suspended in FACS staining buffer with 0.1 μg/mL of DAPI (Invitrogen).

For analysis or prospective isolation of phenotypic LICs, BM cells were suspended in FACS staining buffer and first incubated with Fc-Block for 10 minutes on ice, and then with biotin-conjugated lineage marker antibodies (CD3e (145-2C11), CD4 (GK1.5), CD5 (53-73), CD18a (53-6.7), CD11b (M1/70), B220 (RA3-6B2), Gr1 (RB6-8C5), and Ter119 (TER119)) on ice for 20 minutes. After a wash with FACS staining buffer, cells were incubated with PerCP-Cy5.5-conjugated streptavidin, PE-Cy7-conjugated Sca1 (D7), and APC-conjugated c-Kit (2B8) on ice for 20 minutes. Cells were then washed, and suspended in FACS staining buffer with 0.1 μg/mL of DAPI (Invitrogen).

For analysis of apoptotic cells, cells were washed and suspended in Annexin V staining buffer (10 mmol/L M HEPES, pH 7.4; 140 mmol/L NaCl, 2.5 mmol/L CaCl2) before addition of APC-conjugated Annexin V (B23036APC/100; ebioscience) and incubation for 30 minutes at room temperature. Annexin V-positive cells were then washed and suspended in Annexin V staining buffer supplemented with 0.1 μg/mL DAPI (Invitrogen).

All samples were filtered through 40 μm mesh (BD Bioscience) before running on LSR II/LSR Fortessa (BD Bioscience) for analysis or on FACSaria II SORP (BD Bioscience) for cell sorting. Fluorescence compensation or spillover was calculated automatically using
single color controls and Diva software. FACS data were analyzed with FlowJo software (Tree Star).

**BrdUrd Incorporation and Cell-Cycle Analysis**

Determination of cell-cycle distribution by flow cytometry detection of BrdUrd pulse-labeled cells was performed with the APC BrdU Flow Kit (BD Biosciences) following the manufacturer’s instructions. Briefly, cells were incubated with BrdUrd (final concentration 10 mM/L) containing media for 18 minutes. After a PBS wash, cells were fixed with paraformaldehyde, permeabilized, and treated with DNase I to expose BrdUrd, incorporated in DNA. After washing, cells were incubated with APC-conjugated BrdUrd antibody for 20 minutes on ice. Stained cells were washed and suspended in FACS staining buffer with 0.1 mg/mL of DAPI for flow cytometry.

**Global Gene Expression Profiling**

Phenotypic CML LICs (EYFP-Lin+c-Kit+Scal+) were purified from E2B KO or control CML mice, 2 weeks after BMT. Each sample was sorted from BM cells pooled from 4 to 6 CML mice and double-sorted to increase the purity of the designated cell population. During the second sort, target cells were directly deposited into a 1.5 mL DNA LoBind tube (Eppendorf) filled with 350 μL RLT PLUS buffer (Qiagen) supplemented with 2-Mercaptoethanol (Sigma). Total RNA was isolated using the RNeasy Micro Plus Kit (Qiagen), reverse transcribed, amplified with the WT-Ovation Pico RNA Amplification System V2 (NuGen Technologies), labeled (NuGen; Encore Biotin Module), and hybridized to Affymetrix mouse 430A 2.0 arrays. Data were normalized and analyzed with GenePattern and GSEA. Gene expression data have been deposited in the Gene Expression Omnibus (GEO) under accession numbers GSE85744 and GSE51084.

Published data sets (21) deposited on GEO (GSE47927) were used for comparative analysis of gene expression between human CML LICs and normal HSCs.

**Western Blot Analysis**

Whole-cell lysates were prepared in RIPA buffer and proteins separated on SDS-PAGE gel. Proteins were then detected with anti-H3 (Ab1791; Abcam), anti-H3K27me3 (07-449; Millipore), anti-c-ABL (sc-131; Santa Cruz Biotechnology), anti-β-Actin (Ab8226; Abcam), and anti-GAPDH (#2118S; Cell Signaling Technology) primary antibodies plus proper horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and ECL reagents.

**Human Samples**

Primary human CML samples were obtained from the Pasquarrello Tissue Bank of Dana-Farber Cancer Institute. Patient samples were collected under Dana-Farber/Harvard Cancer Center Protocol 01-206 with consent of patients and their families. Primary human adult CD34+ BM HSPCs were purchased from Stem Cell Technology.

**Colony Formation Assay**

Note that 1 × 10^3 human CD34+ normal BM HSPCs or CD34+ human CML stem/progenitor cells or 5 × 10^3 primary human BM CML cells were suspended in 100 μL of IMDM with 2% FBS, 0.1% ciprofloxin, and 100 U/mL Pen/Strep and added to 1 mL of Methocult H4435 supplemented with 0.1% ciprofloxin and 100 U/mL Pen/Strep. DMSO or EHZ2 catalytic activity inhibitors were added to the mixture and cell plated onto 35-mm dishes and incubated in 37°C for 9 days before colonies were enumerated.

**Statistical Analysis**

Statistical analysis was performed by the Student t test, unless otherwise indicated. Results were considered significant if the P value was < 0.05.

**Disclosure of Potential Conflicts of Interest**

J.E. Bradner is President, NIBR at Novartis. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: H. Xie, C. Peng, W. Kim, S.H. Orkin

Development of methodology: H. Xie, C. Peng, W. Kim, S. Li, S.H. Orkin

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Xie, C. Peng, B.E. Li, E.C. Smith, G. Cheloni, P.P. Das, M. Nguyen, S. Li, S.H. Orkin

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Xie, C. Peng, J. Huang, B.E. Li, P.P. Das, S. Li, J.E. Bradner, S.H. Orkin

Writing, review, and/or revision of the manuscript: H. Xie, C. Peng, J. Qi, P.P. Das, S. Li, J.E. Bradner, S.H. Orkin

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Peng, B.E. Li, Y. Fujisawa, J. Qi, J.E. Bradner

Study supervision: H. Xie, C. Peng, S. Li, S.H. Orkin

**Acknowledgments**

We are grateful to the members of our laboratory for discussions and technical help. We thank Dr. Jerome Ritz and Doreen Hearsey (Dana-Farber Cancer Institute), Dr. Frank C. Kuo (Brigham and Women’s Hospital), Dr. Richard Stone, and Ilene Ann Galinsky (Dana-Farber Cancer Institute) for primary CML patient samples.

**Grant Support**

This work was funded in part by NIH U01 CA105423 (to S.H. Orkin), a Scholar Award from Hyundai Hope on Wheels, and the Harvard Stem Cell Institute Blood Program. H. Xie was a Leukemia and Lymphoma Society fellow. E.C. Smith is supported by a Jane Coffin Childs Memorial Fund for Medical Research Fellowship and a Burroughs Wellcome Fund Postdoctoral Enrichment Program Award.

Received December 7, 2015; revised August 22, 2016; accepted August 25, 2016; published OnlineFirst September 14, 2016.

**REFERENCES**


CML LICs Require EZH2


Chronic Myelogenous Leukemia—Initiating Cells Require Polycomb Group Protein EZH2

Huafeng Xie, Cong Peng, Jialiang Huang, et al.

Cancer Discov 2016;6:1237-1247. Published OnlineFirst September 14, 2016.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-15-1439

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2016/09/13/2159-8290.CD-15-1439.DC1

Cited articles
This article cites 43 articles, 15 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/6/11/1237.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/6/11/1237.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerdiscovery.aacrjournals.org/content/6/11/1237.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.