shRNA screening identifies JMJD1C as being required for
leukemia maintenance

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Abstract

Epigenetic regulatory mechanisms are implicated in the pathogenesis of acute myeloid and lymphoid leukemia (AML and ALL). Recent progress suggests that proteins involved in epigenetic control are amenable to drug intervention, but little is known about the cancer-specific dependency on epigenetic regulators for cell survival and proliferation. We used a mouse model of human AML induced by the MLL-AF9 fusion oncogene, and an epigenetic shRNA library to screen for novel potential drug targets. As a counter-screen for general toxicity of shRNAs, we used normal mouse bone marrow cells. One of the best candidate drug targets identified in these screens was Jmjd1c. Depletion of Jmjd1c impairs growth and colony formation of mouse MLL-AF9 cells in vitro, as well as establishment of leukemia after transplantation. Depletion of JMJ D1C impairs expansion and colony formation of human leukemic cell lines, with the strongest effect observed in the MLL-rearranged ALL cell line, SEM. In both mouse and human leukemic cells, the growth defect upon JMJ D1C depletion appears to be primarily due to increased apoptosis, which implicates JMJ D1C as a potential therapeutic target in leukemia.
Introduction

Translocations involving mixed lineage leukemia (MLL) gene occur frequently in acute leukemia, especially in childhood and therapy-related leukemia. Leukemias with MLL translocations are associated with higher resistance to chemotherapy and lower survival rates than other types of leukemia. In recent years, the understanding of the molecular basis of leukemogenesis driven by MLL fusions has greatly improved: MLL is a H3K4 methyltransferase and is required for transcription of 2% of mammalian genes, including many Hox genes and Wnt regulated genes. In MLL fusions, the H3K4 methyltransferase activity of MLL is lost and the mechanism of MLL-fusion-driven leukemogenesis depends on the identity of a fusion partner, most commonly AF4, AF9 and ENL. These recruit MLL into several protein complexes associated with transcriptional elongation, such as the elongation assisting protein (EAP) complex, the AF4/ENL/P-TEFb (AEP) complex, the super elongation complex (SEC) and the DOT1L complex (DotCom) (reviewed in)

In search for targeted therapy in MLL-rearranged leukemia, several chromatin-associated proteins were found to be required for survival of MLL-fusion-driven leukemia. These include: H3K79 methyltransferase DOT1L; histone demethylase LSD1; bromodomain-containing 4, BRD4; MLL binding partner menin; PRC2 complex components, EZH1/EZH2, EED and SUZ12; PRC1 complex member, CBX8; H2B ubiquitin ligase RNF20; and methylcytosine dioxygenase TET1. Small molecule inhibitors to some of these have been published, such as JQ1 and I-BET151, inhibiting the interaction of BRD4 with histones; EPZ004777, inhibiting H3K79 methylation by DOT1L; MI-2 and MI-3, inhibiting menin-MLL interaction; GSK126, EPZ-6438 and EI1, inhibiting H3K27 methylation by EZH2; and ORY-1001, inhibiting H3K4 demethylation by LSD1.

Pooled shRNA screens have been successfully employed to identify novel oncogenes and tumor suppressors, e.g. in liver cancer and lymphoma. Two shRNA
screens in MLL-AF9 leukemia identified potential therapeutic targets: An *in vitro* screen with shRNAs targeting 243 chromatin-associated factors resulted in the identification of Brd4 as a promising drug target\(^\text{11}\), and an *in vivo* screen with a library of shRNAs targeting 268 established and putative cancer-associated genes revealed integrin beta 3 (Itgb3) as critical for maintenance of MLL-AF9 AML\(^\text{28}\). Here, we present an shRNA screen in primary mouse MLL-AF9 AML cells, accompanied by a counter-screen in c-Kit\(^+\)-enriched mouse bone marrow (BM) cells using an shRNA library targeting 319 known and candidate epigenetic regulators.

One potential drug target candidate identified through our screening approach was *Jmjd1c*. Interestingly, it has previously been described as a target of MLL-AF9 and MLL-AF4 fusion proteins in mouse and human leukemic cells\(^\text{7,29,30}\). JMJD1C was originally identified as a ligand-dependent thyroid receptor interacting protein\(^\text{31}\) and an androgen receptor co-activator\(^\text{32}\). It was reported to be an H3K9me2/me1 demethylase and transcriptional activator\(^\text{33}\), however two recent studies failed to observe any JMJD1C histone demethylase activity, following extensive cellular and biochemical assays\(^\text{34,35}\). In this study, we validate and characterize a role for JMJD1C in maintenance of leukemia.
Methods

Generation of pMLS library
shRNAs were subcloned from pGIPZ (Open Biosystems) into pMLS (MSCV-LTRmir30-SV40-GFP) vector. Sequences of shRNA hairpins are listed in Table S1.

Pooled shRNA screening
All mouse work was approved by the Danish Animal Ethical Committee (“Dyreforsøgsstilsynet”). Mouse MLL-AF9 or c-Kit+-enriched BM cells were transduced with the shRNA library and FACS sorted two days later. Genomic DNA was extracted from the reference (day 0) and samples cultured for 14 days, and shRNA hairpins were PCR-amplified with oligos carrying Illumina adaptors and barcodes. Illumina HiSeq sequencing was performed at Danish National High-throughput DNA Sequencing Centre, University of Copenhagen. Sequencing results were de-multiplexed and mapped to the shRNA library using barcodes with checks for cross alignments between barcodes. Alignment was performed with bowtie on an shRNA library pseudo-genome where up to 2 mismatches were accepted within the trimmed reads. A sum of ranked standardization scores was then calculated for each gene. See Supplementary Methods.

Mouse transplantation

C-Kit+-enriched BM cells from B6 (CD45.2+) donor mice were transduced with MSCV-MLL-AF9-neo. After two days, cells were plated into methylcellulose media (M3534, STEMCELL Technologies) with G418. Following 2 rounds of re-plating pre-leukemic cells were transplanted into lethally irradiated (900cGy) B6.SJL (CD45.1+) recipient mice at 1x10⁶ cells per recipient. 2x10⁵ whole B6.SJL BM cells were co-injected as a support. Primary leukemic cells from BM and spleen of sick mice were harvested, analyzed by flow cytometry and frozen.
For secondary transplants, pMLS-transduced MLL-AF9 spleen leukemic cells were FACS sorted and injected into sub-lethally irradiated (450cGy) B6.SJL recipient mice at 1x10^4 cells per recipient.

**Virus production**

For retrovirus production, Phoenix-Ecotropic cells were co-transfected with pMLS or pMSCV vectors and pCL-Eco using calcium phosphate transfection method. For lentivirus production, 293FT cells were co-transfected with: pLKO.1-puro, pLKO.1-GFP or pLKO-puro-IPTG-3xLacO (Sigma), and pAX8 and pCMV-VSV, using calcium phosphate transfection method.

**Generation of JMJD1C antibody**

The 1-289aa coding sequence of human JMJD1C variant 2 was transferred into pET-28 vector (Novagen) and expressed in Rosetta 2 (DE3) cells (Novagen). The recombinant protein was purified using TALON metal affinity resin (Clontech), and Superdex 200 HR 10/30 gel filtration column (GE Healthcare). JMJD1C polyclonal antibodies were generated by immunizing rabbits with the purified recombinant antigen, and the antibodies were affinity purified using the antigen (GenScript).

**IPTG inducible system**

Cells were transduced with pLKO-puro-IPTG-3xLacO lentiviral vectors and selected with 2 μg/ml puromycin (Sigma). For shRNA expression, culture medium was supplemented with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma).

**mRNA expression analysis**

RNA was purified using RNeasy Plus RNA kit (Qiagen) and reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems). RT-qPCR was performed
with LightCycler® 480 SYBR Green I Master and LightCycler®480 System (Roche). Expression was normalized to RPLP0. Primer sequences are listed in Supplementary Methods.

Expression microarray

RNA was extracted with RNeasy Plus RNA kit (Qiagen). For SEM cells, RNA was hybridized on Affymetrix Human Gene 2.0 ST arrays by the RH Microarray Center at Rigshospitalet, Copenhagen. For MLL-AF9 cells, RNA was hybridized on Agilent SurePrint G3 Mouse GE 8x60K arrays according to the manufacturer’s protocol. (Microarray accession numbers: GSE54311 [mouse] and GSE50048 [human].)

GSEA

Murine gene names were mapped to their orthologous human HUGO Gene Nomenclature Committee (HGNC) approved gene names using the Mouse Genome Informatics Mouse/Human Orthology dataset (HMD_HumanPhenotype.rpt, download from ftp://ftp.informatics.jax.org/pub/reports/index.html#homology, 14/07/2013), previously published gene sets, and the VLOOKUP function in Microsoft Excel 2011. Gene Set Enrichment Analysis (http://www.broadinstitute.org/gsea/index.jsp) was performed on KD vs. SCR triplicate expression files. Gene sets used, references and statistics are listed in Table S2 and (http://www.broadinstitute.org/gsea/msigdb/index.jsp). For all gene sets, 1000 permutations and the Signal2noise metric were used. Permutations by gene sets were conducted to assess statistical significance.

Supplementary Methods are listed in the Supplemental Data file.
Results

Pooled shRNA screens reveal Jmjd1c as a potential drug target in MLL-AF9 leukemia

To identify new epigenetic factors involved in AML, we generated a mouse model of human AML induced by the MLL-AF9 fusion oncogene, using a protocol described previously. Mice transplanted with pre-leukemic MLL-AF9 cells developed AML with a median latency of 70 days (Figure S1A) and of expected myeloid immunophenotype: Mac1+ Gr1+ c-Kit+/− CD3− and B220− (Figure S1B and C). We generated a retroviral shRNA library targeting epigenetic factors (epi-library), by sub-cloning selected shRNAs from pGIPZ (Open Biosystems) into pMLS vector (Figure 1A). The library contained 898 constructs targeting 319 genes belonging to all major chromatin-associated gene families (Figure 1B and Table S1).

To identify potential drug targets in AML, we performed an in vitro screen revealing shRNAs inhibiting growth of MLL-AF9 cells, and an independent screen in c-Kit+−enriched mouse BM cells. The latter was used as a counter-screen to exclude generally toxic shRNAs. In both screens the epi-library retrovirus was titrated to ensure transduction efficiency of less than 30%, with the aim of reducing the number of cells infected by more than one shRNA. Transduced cells were FACS sorted and maintained in culture for 14 days (Figure 1C). shRNA hairpins were amplified from genomic DNA isolated from cells at the beginning and at the end of the culture, and abundance of each shRNA was quantified by high-throughput sequencing.

We observed a good correlation between two replicates of the MLL-AF9 in vitro screen, results are therefore presented as an average of two experiments (Figure 1D and Table S3). Control shRNAs inhibiting growth of MLL-AF9 cells such as shRNA targeting Myb, a gene critical for MLL-AF9 leukemia maintenance, and shRNAs targeting essential genes, Rpa3, PcnA and Polr2b, were strongly depleted at day 14 of MLL-AF9 cell culture compared to day 0. In contrast, three non-targeting controls (Scrambled, Scr)
were neither strongly depleted nor enriched (Figure 1D). In contrast to the MLL-AF9 screen, the correlation between two replicates of the screen in c-Kit+ BM cells was not high, most likely due to heterogeneity of the c-Kit+ cell population (Figure 1E, Table S3).

To select hits with the strongest drug-target potential, genes were ranked according to the combined performance of their shRNAs in both screens (Table S4). Genes with strongest depletion in MLL-AF9 cells, but little depletion in cKit+ BM obtained the highest score. Some genes showing a strong depletion in the MLL-AF9 screen Myb, Tapbp and Hdac3, show strong depletion in cKit+ BM resulting in a relatively low combined score (Table S4). Brd4, which is known as required for survival of MLL-AF9 leukemia cells11 ranks 29th. Jmjd1c is the highest scoring gene. Lack of other known required genes for leukemic cell proliferation such as Ezh2 or Dot1l amongst the top ranking hits could be due to poor knock-down efficiencies of their shRNAs, as the library is not validated.

**Leukemic cells are more sensitive to Jmjd1c depletion than normal BM cells**

As a first line of validation, we tested the effect of Jmjd1c knock-down on the proliferation of MLL-AF9 AML and c-Kit+ BM cells in liquid culture. Control cells (shScr) did not show proliferative advantage or disadvantage compared to untransduced cells, while MLL-AF9 cells expressing shRNAs targeting Jmjd1c (shJmjd1c_867 or shJmjd1c_868) were gradually depleted over time (Figure 2A). In contrast, c-Kit+ BM cells transduced with shJmjd1c_867 or shJmjd1c_868 were not out-competed by untransduced cells (Figure 2B). Importantly, Jmjd1c transcript levels were depleted to a similar extent in MLL-AF9 and c-Kit+ BM cells upon knock-down (Figure 2C and 2D), while MLL-AF9 AML cells have higher basal Jmjd1c expression levels compared to c-Kit+ cell population (Figure 2E). Similarly to the results in liquid culture, Jmjd1c-depleted MLL-AF9 cells formed significantly less colonies than control cells (Figure 2F) and Jmjd1c-depleted c-Kit+ cells were largely unaffected compared to control BM cells.
Together, these data suggest that \textit{Jmjd1c} expression is required for survival of MLL-AF9 AML cells, while \textit{Jmjd1c} depletion has no immediate negative effect on normal BM cells in \textit{in vitro} culture.

To determine whether depletion of \textit{Jmjd1c} also has an effect on AML maintenance \textit{in vivo}, we transplanted $10^4$ control or \textit{Jmjd1c}-depleted primary MLL-AF9 leukemic cells into secondary recipients. 6 out of 7 mice transplanted with control cells succumbed to short latency leukemia. In contrast, mice transplanted with \textit{Jmjd1c}-depleted cells either did not show any symptoms or developed leukemia with a significantly longer latency compared to the control mice ($p=0.003$, Figure 2H). At the end of the experiment, BM and spleen cells from the remaining mice were GFP negative (data not shown), i.e. they have lost the shRNA expressing cells. These data indicate that \textit{Jmjd1c} plays a role in AML maintenance both \textit{in vitro} and \textit{in vivo}.

\textbf{Mouse LSK cells exhibit only mild phenotype after \textit{Jmjd1c} knock-down}

Since silencing of \textit{jmjd1c} in zebrafish results in impaired erythrocyte and megakaryocyte development\textsuperscript{32} and inhibition or knockout of several genes involved in MLL-rearranged leukemia affect function of HSCs and/or erythroid progenitors, e.g. \textit{Lsd1}\textsuperscript{39} and \textit{Myb}\textsuperscript{43}, we tested the effect of \textit{Jmjd1c} depletion on survival and function of Lin\textsuperscript{-} Sca-1\textsuperscript{+} c-Kit\textsuperscript{+} (LSK) cells, with a particular focus on erythroid potential. To this end, we transduced LSK cells with \textit{Jmjd1c}-targeting shRNA or control shRNA and followed proliferation of transduced cells for 14 days in media supporting self-renewal of HSCs (Figure 3A). \textit{Jmjd1c} depletion was assessed using Lin\textsuperscript{-} Sca-1\textsuperscript{-} c-Kit\textsuperscript{-} cells sorted in parallel to the LSK cells used in the experiment (Figure 3B). We did not observe differences in growth rate, colony size and number between \textit{Jmjd1c}-depleted and control cells (Figure 3 C-D).

To also test the quality of \textit{Jmjd1c}-depleted cells after 14 days of culture, we analyzed them for expression of Sca-1, c-Kit and lineage markers, and plated them in media supporting growth of erythroid progenitors. We did not detect notable
differences in immunophenotype at day 14 (Figure 3E) or in the number of erythroid colonies formed by Jmjd1c-depleted and control cells (Figure 3F). However, Jmjd1c-depleted erythroid colonies were generally smaller than control colonies (Figure 3G), despite they contained similar percentages of Ter119+ erythroid cells (Figure 3H). Together, these data show that Jmjd1c depletion does not have a major impact on hematopoietic progenitors, at Jmjd1c knock-down efficiency similar to MLL-AF9 transformed hematopoietic cells.

**Human leukemic cells are sensitive to depletion of JMD1C**

To understand if JMJD1C is required for human leukemia, we first analyzed the expression of JMJD1C in 5 MLL-rearranged and 7 non-MLL-rearranged human leukemic cell lines. In agreement with studies on primary patient samples44-48, leukemic cell lines with MLL rearrangements had significantly higher JMJD1C mRNA levels compared to cell lines with other mutations (Figure 4A and 4B). Despite these differences, both cell lines carrying MLL-AF9 or AF4 rearrangements and leukemic cells lacking MLL fusions were sensitive to JMJD1C knock-down (Figure 4C and Figure S2). Similarly, colony forming potential of all the cell lines tested was reduced upon JMJD1C depletion (Figure 4D). Importantly, growth of human osteosarcoma cell line U2OS was not affected by JMJD1C knock-down (Figure S3). Together, these data show that JMJD1C plays an important function in both human MLL-rearranged and non-MLL-rearranged leukemic cells. Since the strongest effect of JMJD1C depletion was observed in the human MLL-AF4 ALL cell line SEM, we chose this cell line for characterization of the JMJD1C knock-down phenotype. To this end, we generated an inducible system, where the expression of JMJD1C shRNAs is induced by IPTG.

**Growth defect upon JMJD1C depletion is primarily due to increased apoptosis**

To investigate the growth defect caused by JMJD1C depletion, we measured the fraction of apoptotic, annexin V-stained cells by flow cytometric analysis (Figure 5A and 5B).
Mouse MLL-AF9 cells with \textit{jmjd1c} knock-down displayed increasing apoptotic percentages over time as compared to shScr. In SEM cells, induction of \textit{JMJD1C} knock-down had a similar effect approximately 1 day after detectable reduction of protein levels by immunoblot (Figure 5B and 5C). Onset of apoptosis was accompanied by cleavage of Caspase 3 and PARP in these cells. These pro-apoptotic events were detectable at rates inversely correlating with \textit{JMJD1C} protein levels (Figure 5C) and cell proliferation (Figure 5D). We nonetheless observed no cell cycle progression defects prior to the onset of apoptosis, as there was no accumulation of cells in S phase, G0/1 or G2/M transitions (Figure 5E). This suggests that apoptosis is not a result of cell cycle arrest, but rather a direct effect of \textit{JMJD1C} depletion in SEM cells.

We also explored the possibility that cells were being induced to differentiate. Mouse MLL-AF9 cells were stained with antibodies to c-Kit and Mac1 and analyzed by flow cytometry. The hematopoietic stem and progenitor cell (HSPC) marker c-Kit was slightly decreased in the \textit{jmjd1c} depleted cells in comparison to the control cells (Figure 5F). Mac1 on the other hand was increased in the sh\textit{jmjd1c}-transduced versus the shScr-transduced cell population. In addition, RT-qPCR analysis revealed up-regulation of \textit{Mac1}, \textit{Csfr} and \textit{lysozyme 2} transcript levels in \textit{jmjd1c}-depleted cells (Figure 5G). However, we did not observe striking differences in morphology between shScr- and sh\textit{jmjd1c}-transduced cells (Figure 5H). Together, these results indicate that \textit{jmjd1c} depletion triggers concomitant up-regulation of myeloid differentiation markers and down-regulation of the HSPC marker c-Kit in murine MLL-AF9 cells, suggestive of altered gene expression programs.

**Early gene expression changes after \textit{JMJD1C} knock-down in SEM cells**

To assess the effect of \textit{JMJD1C} depletion on transcription, we compared the transcriptome of human SEM and murine MLL-AF9 cells expressing sh\textit{JMJD1C} or shScr. To ensure detecting early changes, 48h was selected as the earliest timepoint displaying \textit{JMJD1C} depletion and detectable phenotype as monitored by PARP and Caspase 3
cleavage in SEM cells (Figure 5C). A total of 138 transcripts were detected as changing between the two conditions in SEM cells (FDR<0.05) (Figure S4A-B, Table S5) and 451 transcripts in MLL-AF9 cells (FDR<0.25) (Figure S5A-C, Table S6). Classification of genes into Gene Ontologies (www.pantherdb.org) revealed a high percentage of changes in genes related to the same top three biological process categories: cell communication, cellular and metabolic processes, as well as molecular function, with genes coding for products with putative binding, catalytic and receptor activities (Figure 6A-B). Few of the changes related to cell cycle and apoptosis associated genes, thus confirming the data previously shown regarding cell cycle progression. Apoptotic related genes were not expected to be enriched in this analysis, as this process is primarily regulated on a signaling-cascade level.

To explore the potential perturbation of gene expression programs upon JMJ D1C knock-down, we performed Gene Set Enrichment Analysis (GSEA) using previously published gene sets. While the vast majority of these gene sets were not significantly enriched with JMJ D1C knock-down, several oncogenic and pluripotent programs were revealed to be dependent on JMJ D1C expression levels (FDR<0.05: 11.6%, Figure 6C-D, Table S2), including a leukemic stem cell (LSC) maintenance signature, genes down-regulated in CD133+ hematopoietic stem cells (HSC) compared to CD133− cells, gene sets up-regulated by induction of c-MYC expression in human myelogenous and lymphoma cells, and genes defining a “Myc Core Module” in mouse embryonic stem cells. A gene set defined upon suppression of Myb in murine MLL-AF9 cells is also enriched in the knock-down of JMJ D1C in SEM and MLL-AF9 cells. Moreover, genes that are down-regulated in murine hematopoietic precursor cells conditionally expressing Hoxa9 and Meis1 are up-regulated both in SEM and MLL-AF9 cells with JMJ D1C knock-down, thus suggesting that JMJ D1C levels are important for maintenance of these transformation programs in both species.
However, down-regulation of JMJD1C increased expression of genes up-regulated in pediatric AML with rearranged MLL compared to AML cases without MLL rearrangements\textsuperscript{46}, and genes associated with MLL fusions irrespective of the lineage of the pediatric acute leukemia\textsuperscript{46}, suggesting an MLL-rearrangement independent role of JMJD1C. Taken together, these results suggest that by hindering one or several of its functions, JMJD1C suppression perturbs the leukemic expression programs irrespective of lineage and MLL-rearrangement status in mouse and human leukemia.

**Ectopic expression of Myb or Myc partially rescues jmjd1c knock-down phenotype**

Since the gene expression changes following jmjd1c knock-down strongly resemble changes after Myb depletion, we tested whether ectopic expression of Myb or its transcriptional target Myc can rescue the growth defect associated with depletion of jmjd1c. To this end, we co-transduced murine MLL-AF9 cells with pMLS-YFP carrying either shScr or shjmjd1c and one of the pMSCV-GFP vectors: empty, Myb or Myc, and followed the percentage of GFP$^+$YFP$^+$ cells over 2 weeks after transduction. While not fully rescuing jmjd1c-depleted cells, over-expression of Myb or Myc provided a growth advantage over cells co-transduced with an empty vector (Figure 7A-B, S6).

Because the expression of MYB or MYC does not change upon JMJD1C knock-down, we looked at the expression of several genes potentially contributing to the leukemic phenotype over a JMJD1C knock-down time-course. Expression of Src family tyrosine kinase, LYN, and CSK (c-Src tyrosine kinase)-binding protein, PAG1, was down-regulated at 24h after induction, coinciding with the earliest decrease in JMJD1C mRNA levels (Figure 7C). Expression of Grb2-binding adaptor protein, GAPT, which was reported to inhibit B cell proliferation\textsuperscript{57}, was increased starting from 36h after induction. CD300LF was up-regulated in the mouse and human knock-down cells (Figures S4A and S5A,C), in MLL-AF9 cells with Myb knock-down\textsuperscript{41}, and was shown to mediate cell death in myeloid cells\textsuperscript{58}. Moreover, Cd300lf levels are partly restored with Myb or Myc over-expression in jmjd1c-depleted cells, further implicating this gene in a
Myb-Myc-Jmjd1c network (Figure 7B). However, the CD300LF up-regulation was apparent already before IPTG induction in SEM cells (Figure 7C), which could be due to a potential leakiness of the inducible system and high sensitivity of CD300LF to JMJ1C depletion.
Discussion

In this study, we identify JMJD1C as exerting a key role in leukemia maintenance by using a focused shRNA library in a genetically defined mouse model of human MLL-AF9 leukemia. *JMJD1C* is a common MLL-AF4 and -AF9 target and is 1.6-3.2 fold upregulated in MLL-rearranged versus non-MLL-rearranged leukemias (Figure 4B). Higher expression in murine MLL-AF9 versus c-Kit-enriched cells (Figure 2E) and association with HSC self-renewal and MLL-AF9 transformation might suggest a proto-oncogene role of Jmjd1c in transformed blood cells. In contrast, *Jmjd1c* is not differentially expressed between high versus low LSC frequency MLL leukemia. Due to the large size of the *JMJD1C* coding sequence, we have not been able to ectopically express it in blood cells and address its transformation capability in different genetic backgrounds.

Our data indicates that depletion of *Jmjd1c* leads to differential growth-impairment of normal hematopoietic and leukemic cells (Figure 1D-E, 2A-G and Figure 3). Lack of effect on non-leukemic cells is in agreement with a recent study reporting lack of overt phenotype in *Jmjd1c* knock-out mice and suggests that Jmjd1c is a potential clinically relevant drug target. We performed a panel of assays that revealed apoptosis as being the most prominent effect of *Jmjd1c* depletion in mouse AML and human ALL cells. No effect on cell cycle progression was observed prior to onset of apoptosis and we detected only mild downregulation of HSCP marker c-Kit in murine MLL-AF9 cells. We observed no increase in differentiated cell frequencies with *Jmjd1c* knock-down, thus precluding that the observed growth defect is due to cells terminally differentiating and exiting the cell cycle. Upregulation of myeloid differentiation markers was however apparent as measured by RT-qPCR, which implies that in addition to apoptosis onset, self-renewal transcription programs are being lost by the reduction of Jmjd1c levels (Figure 5).

Gene expression analysis in human SEM and murine MLL-AF9 cells enabled the detection of genes de-regulated upon *JMJD1C* depletion. Importantly, these changes
strongly correlated with the effect of the suppression of the key leukemia-promoting gene Myb. Correlation of \(JMJD1C\) knock-down with loss of the MLL-rearranged LSC signature and the CD133+ HSC and c-MYC signatures (Figure 6C-D), implicates \(JMJD1C\) as having a role in promoting self-renewal and transformation. Although the expression of Myb and Myc does not change upon \(jmjd1c\) knock-down, the overexpression of either of them partially rescues \(jmjd1c\)-depleted cells (Figure 7A-B), functionally confirming the link between \(jmjd1c\) and Myb-associated gene expression program. The up-regulation of \(CD300LF\) expression in Myb-depleted cells\(^{41}\) as well as in human and mouse \(JMJD1C\)-depleted cells (Table S5 and S6) suggests that it contributes to the \(JMJD1C\) suppression phenotype both in human and mouse transformed cells. Indeed, the mRNA levels of \(Cd300lf\) were partially rescued with overexpression of Myb or Myc in \(jmjd1c\)-depleted cells (Figure 7B).

Down-regulation of Src family kinase \(LYN\) and its regulator \(PAG1\) appear to be primary effects of \(JMJD1C\) knock-down in SEM cells (Figure 7C). Whether \(JMJD1C\) directly regulates these genes remains unknown, as we have failed to obtain reliable chromatin immunoprecipitation data with several \(JMJD1C\) antibodies. In addition, and in agreement with two recent studies\(^{34,35}\), we have not detected any \(JMJD1C\) H3K9 demethylase activity \textit{in vitro} or by over-expression in HEK293 cells, nor did the knock-down generate global accumulation of H3K9 methyl marks in SEM cells (Figure S7). Moreover, a study recently reported a non-histone target of \(JMJD1C\) demethylase activity\(^{61}\). It is therefore possible that \(JMJD1C\) also exerts an indirect function in gene transcription regulation.

The correlation of the \(JMJD1C\) knock-down expression profile in SEM and murine MLL-AF9 cells with several of the expression signatures ties \(JMJD1C\) to MLL-fusion dependent transformation programs\(^{41,51,56}\). \(JMJD1C\) function however is neither exclusive to nor entirely overlapping with MLL-rearranged leukemia: The knock-down profile either did not correlate, or inversely correlated with defined MLL-rearranged
signatures\textsuperscript{46,48} or an MLL-AF4 target gene set in SEM cells\textsuperscript{39} (Figure S4C) and non-MLL-rearranged cell lines are also affected by JMJ1C depletion (Figure 4C-D). We show that in the case of MLL-fusion-driven leukemia, JMJ1C expression levels are required for the maintenance of transformation programs (Figure 7D). Taken together, our findings implicate \textit{JMJ1C} as a crucial gene in leukemia and qualify it as a potential therapeutic target in leukemia subtypes spanning a range of lineages and MLL-rearranged cytogenetic status.
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Authorship Contributions

Conceived and designed the experiments: PS, VAC, JPB, SM, BP, KH. Performed the experiments: PS, VAC, JPB, SM. Analyzed the data: PS, VAC, JPB, SM, FOB, KH. Contributed reagents/materials/analysis tools: PS, VAC, JPB, SM, JW, MBS, FOB. Wrote the paper: PS, VAC, KH. Assisted with writing: JPB, FOB.

Disclosure of Conflicts of Interest

KH is a cofounder of EpiTherapeutics, works as a consultant for the company and has shares and warrants in the company. All other authors do not have any conflicts of interest.
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FIGURE LEGENDS

Figure 1. Pooled shRNA screens with mouse retroviral shRNA epi-library

(A) Schematic map of the pMLS vector. (B) Categories of chromatin-associated factors represented in the mouse retroviral shRNA epi-library. (C) Screening strategy. Mouse primary MLL-AF9 AML cells or c-Kit-enriched mouse BM cells were transduced with the epi-library. Two days after transduction, GFP-positive cells were FACS-sorted and cells were harvested at day 0 and day 14 for genomic DNA. shRNA hairpins were PCR-amplified and submitted for sequencing. (D) Screening result in MLL-AF9 AML cells. Data are presented as ratio of normalized read number at day 14 to normalized read number at day 0 for each shRNA in the library. The result is an average of two replicates. Positive controls: orange, negative (non-targeting): green, shRNAs targeting Jmjd1c: blue. Inset shows correlation of normalized reads per shRNA between two replicates. (E) Screening result in c-Kit-enriched BM cells. Two independent experiments are presented. Control and Jmjd1c shRNAs are marked as in D.

Figure 2. Differential sensitivity of mouse MLL-AF9 AML cells and normal BM cells to Jmjd1c knock-down

(A) Competitive proliferation assay of MLL-AF9 cells transduced with shRNAs targeting Jmjd1c (867 and 868) or with a non-targeting control (Scr). Graph shows percentage of pMLS-GFP transduced cells normalized to the percentage observed at day 0 (2 days after transduction). (B) Competitive proliferation assay of c-Kit-enriched BM cells transduced with shRNAs targeting Jmjd1c (867 and 868) or with a non-targeting control (Scr). Graph shows percentage of pMLS-GFP transduced cells normalized to day 0 (2 days after transduction). (C) Relative expression of Jmjd1c in MLL-AF9 cells transduced with Scr or Jmjd1c-targeting shRNAs at day 2 after transduction. (D) Relative expression
of Jmjd1c in c-Kit+ cells transduced with Scr or Jmjd1c-targeting shRNAs at day 2 after transduction. (E) Relative Jmjd1c mRNA expression in c-Kit+ mouse BM cells and in MLL-AF9 primary mouse leukemic cells. (F) Number of colonies generated by MLL-AF9 cells transduced by pMLS-Scr, -867 or -868. Cells were plated in semi-solid media 2 days after transduction. Error bars indicate standard deviation of the mean (n=3). (G) Number of colonies generated by c-Kit+ cells transduced by pMLS-Scr, -867 or -868. Cells were plated in semi-solid media 2 days after transduction. Error bars indicate standard deviation of the mean (n=3). (H) Survival curves of sublethally irradiated mice transplanted with 10^4 MLL-AF9 cells transduced with pMLS-Scr, pMLS-867 or pMLS-868. GFP+ cells were FACS-sorted and transplanted 2 days after transduction.

Figure 3. The effect of Jmjd1c knock-down on growth and differentiation of LSK cells

(A) Experimental overview. (B) Relative expression of Jmjd1c mRNA in Lin- c-Kit+ Sca-1+ cells transduced with non-targeting control (shScr) or Jmjd1c-targeting shRNA (sh868) at day 2 after transduction. These cells were sorted in parallel with Lin- c-Kit+ Sca-1+ (LSK) cells used in the experiment. (C) Proliferation of shScr- and shJmjd1c-transduced LSK cells in liquid culture. (D) Number of hematopoietic colonies generated by shScr- and shJmjd1c-transduced LSK cells in semi-solid media supplemented with stem cell factor (SCF), interleukin 6 (IL6), interleukin 3 (IL3) and erythropoietin (EPO). (E) Flow cytometry analysis of lineage markers, c-Kit and Sca-1 expression in Jmjd1c-depleted and control cells 14 days after sort. (F) Number of erythroid colonies generated by shScr- and shJmjd1c-transduced LSK cells in semi-solid culture supplemented with erythropoietin (EPO). (G) Erythroid colony morphology generated by shScr- or shJmjd1c-transduced cells. (H) Flow cytometry analysis of Ter119 and CD71 expression in cultures generated in (F).
Figure 4. Effect of *JMJD1C* depletion on human leukemic cells

(A) Relative *JMJD1C* mRNA levels in a panel of human leukemic cell lines normalized to mRNA levels in SEM cells. (B) Box plots of mean *JMJD1C* mRNA levels in MLL-rearranged and non-MLL-rearranged cell lines from (A). (C) Relative cell number of the indicated cell lines transduced with shScr, sh*MJD1C*#1 or sh*MJD1C*#2 at day 8 after GFP+ sort. Cells were FACS sorted 4 days after transduction. Error bars indicate standard deviation (n=3 technical replicates). See also Figure S2. (D) Number of colonies generated in semi-solid media by GFP+ cells transduced with shScr, sh*MJD1C*#1 or sh*MJD1C*#2. Error bars indicate standard deviation (n=3 technical replicates).

Figure 5. *JMJD1C* depletion triggers apoptosis

(A) Relative percentage of annexin V-positive MLL-AF9 cells at 3, 4 and 6 days after transduction with non-targeting control (shScr) or shRNAs targeting *jmjd1c* (sh867 and sh868). Average of 3 independent experiments, error bars indicate standard deviation. (B) Relative percentage of annexin V-positive SEM cells over a time-course starting from IPTG induction at day 0. Average of 3 independent experiments, error bars indicate standard deviation. (C) Western blot showing *JMJD1C*, PARP and Caspase 3 levels in control (pLKO Scr) and *JMJD1C*-depleted SEM cells (sh*1C*#i60 and sh*1C*#i61). Vinculin was used as a loading control. (D) Number of SEM cells over a time-course of *JMJD1C* knock-down induction by IPTG. Fresh IPTG was added at days 0, 2 and 4. (E) Cell cycle analysis of *JMJD1C*-depleted SEM cells (sh*i60) compared to control cells (Scr). Error bars indicate standard deviation (n=3 for days 0-2 and n=2 for day 3). (F) Flow cytometry analysis of c-Kit and Mac1 expression in MLL-AF9 cells with shScr or sh*mjd1c*. (G) Relative mRNA levels of the indicated genes in cells transduced with shScr or sh*mjd1c*. (H) Representative images of May-Grünewald-Giemsa stained MLL-AF9 cells transduced with shScr or sh*mjd1c*.
Figure 6. Gene expression changes upon JMJD1C knock-down

(A and B) Classification of genes with significant change in expression in SEM cells (A) and MLL-AF9 cells (B) into Gene Ontologies describing cellular function (top) and molecular activity (bottom). (C and D) Enrichment of indicated gene sets in JMJD1C knock-down versus control SEM (C) or MLL-AF9 (D) cells as revealed by Gene set enrichment analysis (GSEA). NES: Normalised Enrichment Score, q-value: False discovery rate. See also Table S2 and Figure S4C.

Figure 7. JMJD1C contributes to MLL-rearranged leukemia maintenance by affecting MYB, MYC and HOXA9-MEIS1 gene expression programs

(A) MLL-AF9 cells were co-transduced with pMLS-YFP carrying shScr or shjmjd1c (B66) and empty vector or pMSCV-GFP vector expressing mouse Myb or Myc cDNA. Normalized ratios of GFP+YFP+ cell percentages between shjmjd1c and shScr samples are plotted over a 10-day time course starting from day 2 after transduction, average of 3 independent experiments, error bars indicate standard deviation. (B) Relative mRNA levels of the indicated genes in MLL-AF9 cells from one of the experiments in panel A. (C) Relative mRNA levels of the indicated genes in SEM cells over a 48h JMJD1C inducible knock-down time-course with 12h intervals. (D) Model for MLL-rearranged implementation of transformation programs. JMJD1C, MYC, MYB, HOXA9 and MEIS1 are bound and their transcription is maintained by MLL-AF4 and -AF9. In the presence of sufficient levels of JMJD1C, cells remain transformed. Upon reduction of JMJD1C levels however, transformation and stem cell programs are hindered through de-regulation of MYC, MYB and HOXA9-MEIS1 target-gene expression, leading to cells displaying an apoptotic phenotype.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
shRNA screening identifies JMJD1C as being required for leukemia maintenance

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