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KDM2b/JHDM1b, an H3K36me2-specific demethylase, is required for initiation and maintenance of acute myeloid leukemia

Jin He[1,2], Anh Tram Nguyen[1,2], and Yi Zhang[1,2 *]

[1]Howard Hughes Medical Institute, [2]Department of Biochemistry and Biophysics, Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7295

*To whom correspondence should be addressed
Phone: 919-843-8225
Fax: 919-966-4330
e-mail: yi_zhang@med.unc.edu

Running Title: KDM2b/JHDM1b and leukemia

Abstract

The histone H3 lysine 36 dimethyl (H3K36me2)-specific demethylase KDM2b/JHDM1b, which is highly expressed in various human leukemias, was previously found to be important in regulating cell proliferation and cellular senescence. However, its functions in leukemia development and maintenance are unclear. Here we demonstrate that ectopic expression of *Kdm2b/Jhdm1b* is sufficient to transform hematopoietic progenitors. Conversely, depletion of *Kdm2b/Jhdm1b* in hematopoietic progenitors significantly impairs *Hoxa9/Meis1* induced leukemic transformation. In leukemic stem cells, knockdown of *Kdm2b/Jhdm1b* impairs their self-renewing capability *in vitro* and *in vivo*. The functions of *Kdm2b/Jhdm1b* are mediated by its silencing of $p15^{Ink4b}$ expression through active demethylation of H3K36me2. Thus, our study suggests that *Kdm2b/Jhdm1b* functions as an oncogene and plays a critical role in leukemia development and maintenance.

Introduction

Previous studies have demonstrated that human leukemic cells from the same patient are composed of heterogeneous cell populations with varying proliferation capacities and differentiation status. In the proposed leukemia stem cell (LSCs) model, a fraction of LSCs resides at the apex of leukemia cellular hierarchy. Similar to hematopoietic stem cells (HSCs) in normal blood development, LSCs can give rise to the entire cellular hierarchy and sustain leukemia expansion through an unlimited self-renewal capability [1]. This model is supported by studies in which LSC-enriched cell populations, such as the CD34+CD38- leukemic cells in human acute myeloid leukemia (AML), transplanted into SCID mice are able to fully recapitulate the process of leukemia development [2, 3].

LSCs can be derived from different cellular compartments according to the leukemia type and disease stage. In a *Jun-B* inactivation induced chronic myeloid leukemia (CML) murine model, the CML-like disease can only develop from *Jun-B* inactivated HSCs but not progenitor cells, indicating that LSCs may derive from HSCs [4]. However, in the accelerated and myeloid blast crisis phases of human CML, only leukemic granulocyte-macrophage progenitors (GMP) can be expanded and display an aberrant self-renewing capacity *in vitro* in the methylcellulose re-plating assay [5]. In addition, the fact that certain murine AMLs can be induced by retroviral transduction of oncogenes, such as *Mll* fusion genes, into the GMP population indicates that LSCs can originate from committed progenitor cells directly [6, 7]. These studies suggest that the "stemness" program of LSCs could be activated by various oncogenic stimuli in different cellular contexts. However, the molecular mechanisms underlying LSC self-renewal is not very well understood [8].

The leukemic stem cell model implies that epigenetic regulation at certain critical gene loci might be important in determining the phenotypic difference between self-renewing LSCs and their non-self-renewing progeny [8]. One example that supports this notion comes from the demonstration that the *Ink4a-Arf-Ink4b* locus, which encodes three tumor suppressors including $p16^{Ink4a}$, $p15^{Ink4b}$ and ARF, is controlled by the Polycomb repressive complex 1 (PRC1) in both normal HSCs and LSCs [9, 10]. Biochemical analysis demonstrated that the PRC1 complex contains

an ubiquitin E3 ligase activity and catalyzes the monoubiquitylation of histone H2A at lysine 119, which may serve as an epigenetic mark for the recruitment of other transcriptional repressors to the *Ink4a-Arf-Ink4b* locus [11, 12]. Consistently, deletion of BMI-1, a component of the PRC1 complex, in LSCs leads to de-repression of *Ink4a-Arf* expression and loss of their self-renewal capacity [10]. In addition, Cleary's group also found that some epigenetic modifiers, such as chromobox 5 (CBX5) and high mobility group box 3 (HMGB3), are up-regulated in LSCs and coordinate each other to maintain the LSC program [13]. However, it is unclear whether the functions of CBX5 and HMGB3 in LSC maintenance are mediated through the *Ink4/Arf* or other gene loci.

In an effort to identify other epigenetic regulators important for LSC maintenance, we analyzed the expression level of all known epigenetic factors in human leukemias using the available databases. Interestingly, we found that KDM2b/JHDM1b, a JmjC-domain containing protein, is highly expressed in human leukemia samples. *Kdm2b/Jhdm1b* was first identified as a hotspot for proviral insertion in murine tumors generated by random MMLV mutagenesis [14]. However, it was shown paradoxically to function as both an oncogene and a tumor suppressor depending on the screen and analytic methods [14, 15]. In our previous studies, we have demonstrated that KDM2b/JHDM1b is an H3K36me2-specific demethylase important for maintaining proliferation of murine embryonic fibroblasts (MEFs) as depletion of *Kdm2b/Jhdm1b* causes premature cellular senescence and defective cellular proliferation [16], supporting an oncogenic function for *Kdm2b/Jhdm1b*.

The observation that *Kdm2b/Jhdm1b* is up-regulated in human leukemias prompted us to investigate its role in leukemogenesis. Here we demonstrate that enforced expression of *Kdm2b/Jhdm1b* facilitates proliferation of hematopoietic progenitor cells and induces leukemic transformation. This leukemic property depends on its H3K36me2-demethylase activity and its down-stream target $p15^{Ink4b}$. In addition, we show that *Kdm2b/Jhdm1b* is necessary for the development and maintenance of leukemia in a mouse AML model. Our study thus establishes KDM2b/JHDM1b as a critical epigenetic factor for leukemogenesis and raises the possibility that KDM2b/JHDM1b might serve as a potential therapeutic target for the treatment of leukemia.

Methods

Lentiviral vector construction and virus production

Stable knockdown was achieved using a lentiviral system obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program. The mouse U6 promoter was cloned from mouse genomic DNA and inserted into the NotI site of pTY-EF1α-nLacZ. For the LV-U6 shRNA-Pgk-Pac construct, the Pgk-Pac cassette is inserted between NotI/EcoRI sites to replace the EF1α-nLacZ cassette. The hairpin RNA targeting Kdm2b/Jhdm1b (5'-GCTCCAACTCAGTTACTGT-3') and the control shRNA (5'-GTTCAGATGTGCGGCGAGT-3') were cloned into the BbsI/HindIII sites under the control of U6 promoter [17]. To generate Hoxa9-Meis1-GFP expression vector, the Hoxa9, Meis1 and GFP cDNAs were PCR amplified, ligated to a P2A linker (5'- CAACTGCTGAATTTTGACCTTCTTAAGTTGGCGGG AGACGTCGAGTCCAACCCTGGGCCC-3') and cloned into the SpeI/EcoRI sites under EF1α promoter. To generate wild-type and mutant Jhdm1b-H211A rescue constructs, the siRNA target site of Jhdm1b was mutated to 5'-ATTGCGTTGAGTTACTGT-3' by PCR mutagenesis. The siRNA-resistant wild-type, mutant Kdm2b/Jhdm1b and truncated LacZ cDNAs were PCR amplified and cloned into the SpeI/EcoRI sites of the Pac-2a cassette. To generate lentiviral viruses, the transducing vectors pTY, pHP and pHEF1α-VSVG were co-transfected into 293T cells. The supernatant was harvested at 24, 36 and 48 hours after transfection, filtered through 0.45 μm membrane, and concentrated using Spin-column. Concentrated viruses were snap-frozen and saved at -80°C for later use.

Hematopoietic progenitor isolation, culture, and viral transduction

Hematopoietic progenitor cells are isolated from either E14.5 fetal liver or femurs of 4-6 week Ly5.2 C57BL/6 mice. The red blood cells in the fetal liver and bone marrows are lysed by ammonium chloride solution (Stemcell Technologies 07800) and filtered with 70 μm nylon filter. The isolated single cell suspension is labeled with APC conjugated anti-c-kit antibody in staining buffer (1xPBS, 2% FBS) at 4°C for 30 min. c-kit+ hematopoietic progenitor cells are sorted using a BD FACS Aria II flow cytometry. The sorted cells are cultured in DMEM medium supplemented with 15% (v/v) fetal bovine serum, 1% (v/v) penicillin/streptomycin, 1x essential amino acid, 1x sodium pyruvate and 1x GlutaMax, 16 ng/ml murine recombinant

interleukin 3, 20 ng/ml murine recombinant interleukine-6 and 100 ng/ml stem cell factor. For transduction of hematopoietic progenitor cells, $5x10^5$ cells were divided into 10 wells of 96-well plate and transduced with 10 µl concentrated virus (MOI ~10) plus polybrene (8 ng/ml). For each round of transduction, the plates were centrifuged at 2000rpm for 2 hours at 20°C. 2-3 rounds of transduction were performed for each experiment.

Serial methylcellulose re-plating assay and bone marrow transplantation

Approximately $3x10^4$ sorted GFP+ cells were mixed with 3 ml methylcellulose (MethoCult® GF M3534) medium supplemented with GM-CSF (10 ng/ml) and evenly distributed into three 25 cm^2 plates. After 10-14 days, the colony numbers were counted under a microscope. The colonies were picked up, and cells were pooled and re-plated (10^4cells /plate) onto secondary methylcellulose plates. 3-4 rounds of re-plating were performed for each experiment.

For bone marrow transplantation, recipient Ly5.1 C57BL/6 mice were subjected to total body irradiation at a dose of 950rad using a cesium irradiator. $2.5x10^5$ donor cells and $2.5\ x10^5$ radiation protector cells isolated from bone marrow of Ly5.1 C57BL/6 mice were mixed in 1xPBS and transplanted into the recipient mice through retro-orbital injection. For the transplantation of Kdm2b/Jhdm1b over-expressed hematopoietic progenitor cells, we transplanted $2.5x10^5$ transduced cells and $5.0x10^5$ Ly5.1+Ly5.2+ protecting cells into irradiated C57BL/6 Ly5.1+Ly5.2+ recipient mice. The mice were fed with Trimethoprim/ Sulfamethoxazole (TMP/SMZ) supplemented water for 4 weeks after transplantation.

FACS analysis and cell sorting

For FACS analysis and cell sorting, cells were stained with antibodies in staining buffer (1xPBS, 2% FBS) and incubated at 4°C for 30 min. The samples were washed once with staining buffer before subjected to FACS analysis using BD FACSAriaII cell sorter. The antibodies used in this study include: anti-Mac-1(ebiosciences), anti-Gr-1(ebioscience), anti-c-kit (ebioscience), anti-B220 (ebioscience), anti-CD3 (ebioscience), anti-Ly5.1(ebioscience) and anti-Ly5.2 (ebioscience).

Histological analysis

Mouse tissues were fixed in 4% paraformaldehyde, dehydrated in 70% ethanol, and paraffin embedded. Hematoxylin and Eosin staining was performed as previously described [18]. For May-Grunwald/Giemsa staining, bone marrow and peripheral blood smears are stained with Jenner's solution (EMS, #26250-1A) for 6 minutes and Giemsa solution (EMS, #26250-02) for 15 minutes. The slides were washed with distilled water before viewed under a microscope.

RT-qPCR and ChIP assays

RNA was extracted and purified from cells using Qiashredder (Qiagen) and RNeasy (Qiagen) spin columns. Total RNA (1 µg) was subjected to reverse transcription using random primers (Promega) and the Superscript II reverse transcriptase (Invitrogen). cDNA levels were assayed by real-time PCR using SYBR GreenER (Invitrogen) and analyzed on the ABI 7300 Real Time PCR system with DSD software version 1.3.1. The expression of individual genes is normalized to expression level of GAPDH. Chip assays using M2 anti-Flag antibody (Sigma) and anti-H3K36me2 antibody [13, 19]were carried out as reported previously [16] with the following modifications: 20 µl of M2 agarose (Sigma) was used in the immunoprecipitation and chromatin-bound beads were washed three times each with TSEI, TSEII and TESIII followed by two washes in 10 mM Tris, pH 7.5, 1mM EDTA. Histone modification ChIPs were carried out as previously reported [16]. ChIPed DNA was analyzed by qPCR and data are presented as percentage of input as determined using Applied Biosystems's SDS software Absolute Quantification protocol. Primers for qPCR and ChIP assays are listed in Supplementary Table 1 and Table 2, respectively.

Bisulfite sequencing analysis

Genomic DNA was isolated and subjected to bisulfite conversion using the EZ DNA methylation-gold Kit (Zymo Research). The high CpG regions of $p15^{Ink4b}$ promoter and first exon reported to be methylated in certain cancers [20, 21] (318–644 bp, GenBank accession number U66084) was PCR amplified and cloned in pST-Blue vector (Novagen). DNA sequences of the PCR primers are: forward: 5'-TAAGTTTTAGATTAGGAAATTTAAAGTTTT G-3'; reverse: 5'-TAAAATTACTACAACCTAATCTC-3'. The clones were sequenced by UNC-CH genome analysis facility.

Western Blot analysis

Total proteins were extracted using RIPA buffer and separated by electrophoresis using 6-8% gel. Flag monoclonal M2 antibody (Sigma) was used at the dilution of 1:10000 for western blot analysis.

Results

Expression of Kdm2b/Jhdm1b is up-regulated in leukemic stem cells

We have previously demonstrated that the histone H3 lysine 36 dimethyl (H3K36me2)-specific demethylase KDM2b/JHDM1b plays an important role in promoting cell proliferation and preventing cellular senescence in murine embryonic fibroblasts (MEFs) [16]. Interestingly, an analysis of the Oncomine database (www.oncomine.org) indicated that the expression of *Kdm2b/Jhdm1b* is up-regulated in samples of human acute myeloid leukemia (AML), T-cell acute lymphoid leukemia (T-ALL), and B-cell acute lymphoid leukemia (B-ALL) compared to normal controls (Fig. 1A). These findings raised the possibility that *Kdm2b/Jhdm1b* may have a role in leukemogenesis and/or maintenance of leukemic stem cells (LSCs). To address this possibility, we analyzed *Kdm2b/Jhdm1b* expression in a murine AML model and found that it is expressed at a higher level in the *Hoxa9/Meis1*-induced leukemia cells when compared to normal bone marrow (Fig. 1B) [22]. To further analyze the expression of *Kdm2b/Jhdm1b* in different leukemic cell populations, we sorted the *Hoxa9/Meis1* induced AML cells into two populations based on their c-kit expression level (c-kit ^{high} vs. c-kit ^{low}) (Fig. 1C). Methylcellulose colony formation assays revealed that the c-kit ^{high} cell population has a higher capability to form colonies compared to the c-kit ^{low} cell population (Fig. 1D), indicating that the c-kit ^{high} cell population is enriched for LSCs while the c-kit ^{low} cell population is depleted of LSCs. RT-qPCR analysis demonstrates that the expression level of *Kdm2b/Jhdm1b* is about 3-fold higher in the LSC-enriched c-kit ^{high} population compared to the LSC-depleted c-kit ^{low} population (Fig. 1E). Collectively, these analyses indicate that high *Kdm2b/Jhdm2b* expression correlates with leukemic stem cell potential and might play an important role in leukemia development and maintenance.

Enforced expression of Kdm2b/Jhdm1b is sufficient for bone marrow transformation

To examine whether over-expression of *Kdm2b/Jhdm1b* is sufficient to immortalize hematopoietic progenitor cells (HPCs) *in vitro*, c-kit positive HPCs were isolated from mouse bone marrow and transduced with lentivirus expressing *Kdm2b/Jhdm1b*. As controls, transductions with lentiviruses expressing GFP or *Hoxa9/Meis1* were also performed in parallel. The transduction efficiency of HPCs was estimated about 80-90% based on the percentage of

GFP positive cells analyzed at 48 hours after transduction (Fig. S1A). After verifying over-expression of *Kdm2b/Jhdm1b* in the transduced cells (Fig. S1B), its effect on colony formation was evaluated by methylcellulose assay. Compared to GFP expressing cells, whose colony formation capacity decreases with each round of re-plating, enforced expression of *Kdm2b/Jhdm1b* confers methylcellulose re-plating capability although the colony numbers are fewer than that obtained from cells transduced with *Hoxa9/Meis1* (Fig. 2A). Importantly, cells derived from enforced *Kdm2b/Jhdm1b* expression can sustain in liquid culture medium supplemented with IL-3, IL-6 and SCF. In contrast, the control GFP expressing cells were not able to proliferate under the same conditions (Fig. 2B). These results suggest that enforced expression of *Kdm2b/Jhdm1b* alone is sufficient to immortalize c-kit positive cells *in vitro*, consistent with a potential role of *Kdm2b/Jhdm1b* in leukemogenesis.

To determine whether enforced expression of *Kdm2b/Jhdm1b* in HPCs is sufficient to induce leukemia *in vivo*, we transplanted the transduced $Ly5.2^+$ HPCs with normal $Ly5.1^+Ly5.2^+$ radioprotective bone marrow cells into lethally irradiated mice. The proliferation rate of transplanted cells was monitored by measuring the percentage of $Ly5.2^+$ cells in the peripheral blood. Compared to control *LacZ* transduced HPCs, the percentage of $Ly5.2^+$ cells was greater in mice transplanted with *Kdm2b/Jhdm1b* over-expressing HPCs (Fig. 2C). To determine whether the transplanted cells were transformed, we isolated the $Ly5.2^+$ $c\text{-}kit^+$ cells from the bone marrow of recipient mice 4 weeks after transplantation and performed serial methylcellulose re-plating assay. Compared to the *LacZ* transduced cells, which failed to form colonies after second round of plating, the *Kdm2b/Jhdm1b* over-expressing bone marrow cells have increased colony numbers at each round of re-plating (Fig. 2D). FACS analysis of these transformed cells indicates that they express high levels of c-kit and relative low levels of Mac-1/Gr-1 (Fig. 2E). The recipient mice in the same group gradually display splenomegaly (Fig. 2F) and the typical leukoblasts are also observed in bone marrow smears (Fig. 2G). Consequently, mice transplanted with *Kdm2b/Jhdm1b* over-expressing HPCs died within 4-6 months after transplantation. In contrast, all of the mice in the control group survived in the same period (Fig. 2H). Collectively, these results suggest that enforced expression of *Kdm2b/Jhdm1b* is sufficient to transform hematopoietic progenitors *in vivo*.

Kdm2b/Jhmd1b is required for Hoxa9/Meis1 induced leukemic transformation in vitro

To examine whether *Kdm2b/Jhdm1b* is necessary for AML development, we used the well-established murine AML model involving lentiviral transduction of *Hoxa9/Meis1* oncogenes into c-Kit+ hematopoietic progenitor cells followed by methylcellulose re-plating assays (Fig.3A) [10, 22]. To facilitate isolation and tracing of the genetically modified cells in the study, transduced cells are marked by GFP. HOXA9, MEIS1 and GFP proteins are linked by the foot-and-mouth disease virus peptide 2A, which allows expression of multiple proteins in a single expression vector [16]. To examine the role of *Kdm2b/Jhdm1b* in *Hoxa9/Meis1* induced leukemic transformation, a shRNA against all three isoforms of *Kdm2b/Jhdm1b* is delivered into the cells using the same vector. After FACS sorting (Fig. S2A), *Kdm2b/Jhdm1b* mRNA levels in transduced GFP+ hematopoietic progenitor cells were analyzed by RT-qPCR. Results shown in Fig. S2B indicate that *Kdm2b/Jhdm1b* mRNA level decreased to 15% of the control level upon knockdown. Methylcellulose re-plating assays demonstrate that knockdown of *Kdm2b/Jhdm1b* greatly affected the transformation capacity of *Hoxa9/Meis1* (Fig. 3B). Although colonies can still be derived from the *Kdm2b/Jhdm1b* knockdown cells in the third round of re-plating, these cells failed to proliferate when cultured in liquid media in the presence of IL-3 (Fig. 3C). Flow cytometry analysis demonstrates that compared to control knockdown cells, *Kdm2b/Jhdm1b* knockdown cells has a higher percentage of cells in G1 phase and a lower percentage of cells in S phase (Fig. 3D), indicating a G1 to S transition defect. Collectively, the above results suggest that *Kdm2b/Jhdm1b* is necessary for *Hoxa9/Meis1* induced leukemic transformation as well as proliferation *in vitro*.

Kdm2b/Jhmd1b is required for Hoxa9/Meis1 induced AML development in vivo

To examine whether *Kdm2b/Jhdm1b* is required for leukemia development in animals, we co-transplanted $2.5x10^5$ various genetically modified GFP+ cells and $2.5x10^5$ normal radioprotective bone marrow cells into lethally irradiated syngenic mice (Fig. 4A). At different time points post-transplantation, donor-derived cells in peripheral blood were analyzed by FACS analysis using GFP and lineage markers. Results demonstrate that cells transduced with *Hoxa9/Meis1/GFP* (HMG) and control shRNA (CKD-HMG) can repopulate the recipient by 1 week with 40-45% of GFP+ cells in the Gr-1 and Mac-1 positive myeloid population (Figs. 4B, C). In addition, *Hoxa9/Meis1* transformed cells proliferated faster than the radioprotective cells

since the majority of the Mac-1 and Gr-1 positive cells are GFP+ at 4 weeks post-transplantation (Figs. 4B, C). However, the *Kdm2b/Jhdm1b* knockdown cells (J1bKD-HMG) failed to repopulate *in vivo* as indicated by a persistently low percentage of GFP+ cells after transplantation (Fig. 4B, C). Cells transduced with GFP control decreased gradually due to the limited self-renewing capacity of normal hematopoietic progenitor cells (Fig. 4B, C). Consistent with a GFP+ repopulating capability, the CKD-HMG transplanted mice developed typical AML. The mice displayed splenomegaly (Fig. 4D) with leukemic cell infiltration in multiple organs including liver, kidney and lung (Fig. 4E). In addition, typical leukoblasts are found in the peripheral blood and bone marrow smears (Fig. 4F, Fig. S3A). FACS analysis indicated that the leukemic cells express both c-kit and myeloid lineage markers such as Mac-1 and Gr-1 (Fig. S3B). The mice also developed other leukemia symptoms such as anemia, bleeding and succumb to death within 6-10 weeks after transplantation (Fig. 4G). In contrast, similar to mice transplanted with control GFP cells, the mice transplanted with J1bKD-HMG transduced cells appear healthy and survived over 3 months longer. Collectively, these results suggest that maintaining the expression of *Kdm2b/Jhdm1b* is important for *Hoxa9/Meis1* induced leukemia development in mice.

The enzymatic activity of Kdm2b/Jhdm1b is required for self-renewal of LSCs

After establishing a role for *Kdm2b/Jhdm1b* in transformation and development of Hoxa9/Meis1 induced AML, we asked whether KDM2b/JHDM1b and its associated histone demethylase activity are required for self-renewal of LSCs. To this end, GFP+ leukemic cells were isolated from bone marrow of primary AML mice and cultured in methylcellulose to select for LSCs. Cells capable of forming colonies were transduced with lentiviral vectors expressing control shRNA or *Kdm2b/Jhdm1b* shRNA. To determine whether the phenotypes caused by Kdm2b/Jhdm1b knockdown are related to its H3K36me2 demethylase activity, a mock gene (truncated lacZ), an shRNA-resistant wild-type or JmjC domain mutant form of *Kdm2b/Jhdm1b* coupled with *Kdm2b/Jhdm1b* shRNA is delivered into the cells by the same lentiviral vector (Fig. 5A and Fig. S4A). The transduced cells were subjected to serial methylcellulose plating assay as well as leukemia transplantation assay. Methylcellulose plating assays demonstrate that not only the size but also the number of colonies reduced dramatically upon *Kdm2b/Jhdm1b* KD, suggesting that *Kdm2b/Jhdm1b* expression level in cells is critical for both proliferation of

leukemic cells and self-renewal of LSCs. This defect in colony formation can be rescued by expression of an shRNA-resistant wild-type but not a JmjC-domain mutant KDM2b/JHDM1b protein or LacZ in cells (Fig. 5B, C). The differential effect of the wild-type and JmjC-domain mutant is not due to differences in their expression level as similar expression of *Kdm2b/Jhdm1b* is verified by both RT-qPCR and Western blot analysis (Fig. S4B, C). Consistent with the colony formation assay, leukemia transplantation demonstrates that mice transplanted with control KD or wild-type *Kdm2b/Jhdm1b* rescued leukemic cells died within 3-6 weeks after transplantation. In contrast, all mice transplanted with *Kdm2b/Jhdm1b* KD leukemic cells, 7 out of 10 mice transplanted with JmjC-domain mutant rescued KD leukemic cells, and 9 out of 10 mice transplanted with mock rescued KD leukemic cells survive for at least 3 months after transplantation (Fig. 5D). These results suggest not only that the knockdown is specific, but also that the enzymatic activity of KDM2b/JHDM1b is responsible for maintaining the leukemic state.

Kdm2b/Jhdm1b directly regulates p15^{Ink4b} expression in leukemic cells

To understand how *Kdm2b/Jhdm1b* regulates leukemic cell proliferation and LSC self-renewal, we focused on proliferation-related genes. Given that genes with immediate response to *Kdm2b/Jhdm1b* manipulation are more likely to be its direct targets, we examined the expression of a panel of proliferation related genes 72 hours after viral transduction using the cells generated in Fig. 5A. Consistent with our finding in MEFs [14, 15], *p15^{Ink4b}* is up-regulated significantly upon *Kdm2b/Jhdm1b* KD. Importantly, *p15^{Ink4b}* can be re-silenced by introducing an shRNA-resistant wild-type *Kdm2b/Jhdm1b*, but not a catalytic mutant or irrelevant *LacZ* (Fig. 6A). This result suggests that the H3K36me2 demethylase activity of *Kdm2b/Jhdm1b* is involved in p15^{Ink4b} repression. In addition to p15^{Ink4b}, p16^{Ink4a} and p19^{Arf} are also modestly increased in response to *Kdm2b/Jhdm1b* knockdown (Fig. 6A). However, since *p15^{Ink4b}* had the most significant changes, we analyzed this effect further.

To determine whether *p15^{Ink4b}* is a direct target of *Kdm2b/Jdhm1b*, chromatin immunoprecipitation (ChIP) assay was carried out using chromatin prepared from leukemic cells harboring wild-type or catalytic mutant rescue *Kdm2b/Jhdm1b* transgenes (Fig. 5A). Both wild-type and mutant KDM2b/JHDM1b-Flag were found to be enriched from the promoter region

(amplicon 1) to the first intron (amplicon 3) when compared to the LacZ-Flag control (Fig. 6B, C), indicating that *p15*^{Ink4b} is a direct KDM2b/JHDM1b target. Consistent with its H3K36me2-specific demethylase activity, knockdown of *Kdm2b/Jhdm1b* results in an increased H3K36me2 levels across the three regions when compared to control knockdown (Fig. 6B, D). Importantly, re-introduction of an shRNA-resistant wild-type, but not the catalytic mutant, *Kdm2b/Jhdm1b* results in a decreased level of H3K36me2 comparable to that of control knockdown (Fig. 6D). The change in H3K36me2 level at *p15*^{Ink4b} locus appears to be gene specific as no significant increase in the global H3K36me2 level is observed upon *Kdm2b/Jhdm1b* knockdown (Fig. S5A).

To further substantiate *p15*^{Ink4b} as a direct target, we measured the *p15*^{Ink4b} expression by RT-qPCR in mock and *Kdm2b/Jhdm1b* over-expressing HPCs that were cultured over 2 weeks. The result demonstrates that over-expression of *Kdm2b/Jhdm1b* resulted in repression of *p15*^{Ink4b} (Fig. S5B). Further ChIP analysis using the same cells demonstrates that KDM2b/JHDM1b-Flag is enriched and the H3K36me2 level is reduced at the *p15*^{Ink4b} locus in *Kdm2b/Jhdm1b* over-expressing cells (Fig. S5C). Since previous studies have suggested a link between DNA methylation and *p15*^{Ink4b} silencing in leukemia [20, 21], we asked whether *p15*^{Ink4b} silencing upon Kdm2b/Jhdm1b over-expression uses a similar mechanism. To this end, we analyzed the DNA methylation levels of *p15*^{Ink4b} promoter and first exon in normal HPCs, *Kdm2b/Jhdm1b* immortalized HPCs, and *Hoxa9/Meis1* transformed cells by bisulfite sequencing. Results shown in Fig. S5D demonstrate no significant differences in DNA methylation levels, indicating that changes in histone modifications, but not DNA methylation, are responsible for down-regulation of *p15*^{Ink4b} in the *Kdm2b/Jhdm1b* over-expressing and *Hoxa9/Meis1* transformed cells. These results suggest that silencing of *p15*^{Ink4b} in leukemia may use multiple mechanisms under different conditions. Collectively, these data suggest that *p15*^{Ink4b} is a KDM2b/JHDM1b direct target and that KDM2b/JHDM1b down-regulates *p15*^{Ink4b} expression by maintaining low levels of H3K36me2.

p15^{Ink4b} *is a critical target mediating the function of Kdm2b/Jhdm1b in Hoxa9/Meis1 induced AML*

After demonstrating that *p15*^{Ink4b} is a direct target of KDM2b/JHDM1b, we asked whether *p15*^{Ink4b} is a major effector mediating the function of *Kdm2b/Jhdm1b* in leukemogenesis. To this

end, c-kit+ hematopoietic progenitor cells were isolated from bone marrow of *p15*^{Ink4b} knock-out mice. After transduction with lentiviral vectors expressing control GFP, CKD-HMG control shRNA, and J1bKD-HMG, GFP+ cells were sorted and subjected to serial methylcellulose re-plating assay and bone marrow transplantation assays as described above. Results shown in Fig. 7A demonstrate that *Hoxa9/Meis1* can induce transformation of *p15*^{Ink4b}-null hematopoietic progenitor cells regardless of whether *Kdm2b/Jhdm1b* is knocked down or not. This is in direct contrast with the results obtained when wild-type hematopoietic progenitor cells are used (Fig. 3B) indicating that transformation by *Hoxa9/Meis1* is largely dependent on *Kdm2b/Jhdm1b*-mediated repression of *p15*^{Ink4b}. Consistent with this *in vitro* result, mice transplanted with J1bKD-HMG *p15*^{Ink4b} -null cells exhibited the same splenomegaly phenotype as that of mice transplanted with CKD-HMG cells, indicating that the transformed cells can proliferate in animals even if *Kdm2b/Jhdm1b* is depleted (Fig. 7B). In addition, FACS analysis demonstrates that GFP+ cells can repopulate and dominate the myeloid lineage cell population in mice 4 weeks after transplantation regardless of whether *Kdm2b/Jhdm1b* is depleted (Fig. 7C). Finally, all mice transplanted with J1bKD-HMG *p15*^{Ink4b} -null cells died of leukemia within 3 months of transplantation although there is a 2-3 weeks delay compared to mice transplanted with CKD-HMG cells (Fig. 7D), indicating that other *Kdm2b/Jhdm1b* targets beside p15^{Ink4b} may also contribute to *Kdm2b/Jhdm1b*'s effect in leukemogenesis. Nevertheless, the above results suggest that *p15*^{Ink4b} is a major target that mediates *Kdm2b/Jhdm1b*'s function in leukemogenesis.

Discussion

The *Kdm2b/Jhdm1b* gene was first identified as a hotspot for proviral insertion in murine tumors generated by random MMLV insertion [16]. Our previous studies have demonstrated that depletion of *Kdm2b/Jhdm1b* in MEFs results in defective cellular proliferation and premature senescence, implicating that *Kdm2b/Jhdm1b* has proto-oncogene properties [23]. In the current study, we provide three lines of evidence which further support that *Kdm2b/Jdhm1b* functions as an oncogene and plays critical roles in both leukemogenesis and LSC self-renewal. First, over-expression of *Kdm2b/Jhdm1b* in normal hematopoietic progenitor cells can increase their methylcellulose re-plating capability and proliferation *in vitro* and *in vivo* (Fig. 2), suggesting that an increased level of *Kdm2b/Jdhm1b* can confer a cell growth advantage and induce leukemic transformation. In addition, the colonies with over-expressed *Kdm2b/Jhdm1b* maintain a high level of progenitor marker c-kit and express low levels of myeloid markers Mac-1/Gr-1after serial methylcellulose re-plating, indicating that over-expression of *Kdm2b/Jhdm1b* is able to restrict cells in the progenitor-like status and suppress differentiation (Fig 2E). Second, depletion of *Kdm2b/Jhdm1b* impairs *Hoxa9/Meis1*-induced leukemogenesis (Figs. 3, 4). This defect can be largely rescued in $p15^{Ink4b}$-null hematopoietic progenitor cells, indicating a block of cellular proliferation due to de-repression of $p15^{Ink4b}$ as a primary cause. Third, depletion of *Kdm2b/Jdhm1b* in LSCs impairs the capability of LSCs to self-renew, which is manifested by both small size and low number of colonies in the methylcellulose re-plating assay. This further suggests that high levels of *Kdm2b/Jdhm1b* might not only promote the proliferation of LSCs, but also inhibit their differentiation. In addition, the oncogenic function of *Kdm2b/Jhdm1b* is supported by the finding that *Kdm2b/Jdhm1b* is highly expressed in various human leukemias, *Hoxa9/Meis1*-induced murine AML cells, as well as the LSC-enriched cell population. However, it is currently not clear how *Kdm2b/Jhdm1b* expression is up-regulated during leukemogenesis and maintained in LSCs but not in non-self-renewing leukemic progeny cells. Further chromatin immunoprecipitation assays using oncoprotein-specific antibodies to map their binding sites in the *Kdm2b/Jhdm1b* regulatory regions will help elucidate the relationship between oncogenic stimuli and *Kdm2b/Jhdm1b* expression in the initiation and maintenance of leukemia.

The *Ink4a-Arf-Ink4b* locus plays an important role in regulating cellular proliferation, premature

senescence, and apoptosis [24]. Mutations and deletions in this locus are frequently found in hematopoietic malignancies [9, 10, 25]. Generally, expression of this locus is suppressed in normal adult stem cells and cancer stem cells by various epigenetic regulators. Depletion of *Bmi-1*, a component in the Polycomb repressive complex 1 (PRC1), can activate the expression of *Ink4a-Arf* and result in self-renewal defects in hematopoietic stem cells, neural stem cells, as well as leukemia stem cells [26]. Although $p15^{Ink4b}$ and $p16^{Ink4a}$ have a similar mechanism in regulating cell cycle progression and work together in preventing tumorigenesis [19], the transcriptional regulation of $p15^{Ink4b}$ and its role in cancer stem cells is less characterized. In this study, we demonstrate that *Kdm2b/Jhdm1b* regulates LSC self-renewal mainly through modulating the expression levels of $p15^{Ink4b}$. We show that the expression of $p15^{Ink4b}$ is up-regulated immediately upon *Kdm2b/Jhdm1b* knockdown. We also demonstrate that deletion of $p15^{Ink4b}$ can largely rescue the phenotypes caused by *Kdm2b/Jhdm1b* knockdown (Fig. 7). These results suggest that although the *Ink4a-Arf-Ink4b* locus is regulated by multiple epigenetic factors, $p15^{Ink4b}$ and $p16^{Ink4a}$ might have differential regulatory mechanisms. Accordingly, the existence of multiple regulatory mechanisms may allow tight control of the expression of the *Ink4a-Arf-Ink4b* locus in different conditions. Since the *Ink4a-Arf-Ink4b* locus also plays an important role in self-renewal of normal adult stem cells, we speculate that *Kdm2b/Jdhm1b* likely has a similar function as that of *Bmi-1 in* normal HSCs. Therefore, for therapeutic purposes, it is important to find out whether there is a sensitivity difference between normal HSCs and LSCs in response to *Kdm2b/Jdhm1b* depletion. Further studies using *Kdm2b/Jdhm1b* knockout mice will help answer this question.

Kdm2b/Jdhm1b is a paralog of the first JmjC domain-containing histone demethylase Jhdm1a, which specifically targets H3K36me2 for demethylation [16]. We have previously demonstrated that *Kdm2b/Jhdm1b* also targets H3K36me2 for demethylation [27]. In this study, we found that the LSCs defects caused by *Kdm2b/Jhdm1b* depletion can be rescued by wild-type, but not a catalytic mutant, *Kdm2b/Jdhm1b*, implying that its histone demethylase activity is critical in mediating its function in LSCs (Fig. 5). Consistent with the observed defect in LSC self-renewal, we found that the tumor suppressor $p15^{Ink4b}$ is de-repressed upon *Kdm2b/Jhdm1b* knockdown, which is also rescued by wild-type, but not a catalytic mutant *Kdm2b/Jhdm1b*. Collectively, these data suggests that H3K36me2 levels is directly linked to $p15^{Ink4b}$ expression (Fig. 5). ChIP

analysis confirmed that KDM2b/JHDM1b binds to and is responsible for maintaining a low H3K36me2 level at the $p15^{Ink4b}$ locus, resulting in $p15^{Ink4b}$ down-regulation. Our data indicate that the level of H3K36me2 correlates with transcriptional activity, which is consistent with previous studies demonstrating the association of the H3K36 methyltransferase Set2 with the elongating RNA polymerase II [27, 28] and that H3K36 methylation is coupled with transcription elongation[28]. Given that KDM2b/JHDM1b can actively remove the methyl groups from H3K36, it is not surprising that *Kdm2b/Jhdm1b* plays an important role in silencing $p15^{Ink4b}$. Since KDM2b/JHDM1b has been shown to be part of a Polycomb group complex[29, 30] , it is likely that KDM2b/JHDM1b-mediated H3K36 demethylation is coupled with transcription repression by Polycomb group proteins. Further studies should clarify the exact molecular mechanism underlying $p15^{Ink4b}$ repression in LSCs.

In summary, we propose that KDM2b/JHDM1b is a critical epigenetic factor involved in leukemogenesis and LSC self-renewal (Fig.7E). In response to oncogenic signals, expression of *Kdm2b/Jhdm1b* is up-regulated during leukemogenesis and is maintained at a high level in LSCs. Binding of KDM2b/JHDM1b to the $p15^{Ink4b}$ locus results in demethylation of H3K36me2 and concomitant repression of $p15^{Ink4b}$ by associated Polycomb group of proteins. Conversely, depletion of *Kdm2b/Jhdm1b* causes an increase in H3K36me2 and loss of Polycomb group proteins at the $p15^{Ink4b}$ locus, which leads to de-repression of $p15^{Ink4b}$ and blocks the proliferation of leukemia cells and LSCs. Based on this model, developing small molecule inhibitors for KDM2b/JHDM1b enzymatic activity might be a potential strategy for leukemia treatment.

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Author Contributions

J.H. designed and performed the experiments and contributed data for figure 1, 2, 3, 4, 5, 6 and 7; A.T.N. contributed data for figure 4; Y.Z. oversaw the projects; J.H. and Y.Z. wrote the paper.

Conflict of Interest Disclosure

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Expression of *Kdm2b/Jhdm1b* is up-regulated in leukemic stem cells

(**A**) *Kdm2b/Jhdm1b* is over-expressed at RNA level in human AML, T-ALL, and B-ALL when compared to their normal controls. Data is derived from Oncomine (www.oncomine.com).

(**B**) *Kdm2b/Jhdm1b* is over-expressed in Hoxa9/Meis1 induced AML samples when compared to that of normal bone marrow. Relative mRNA levels are measured by RT-qPCR and normalized to *Gapdh* level. The level in normal bone marrows is arbitrarily set to 1.

(**C**) Separation of *Hoxa9/Meis1* transformed murine AML cells into c-kit^{high} and c-kit^{low} populations by FACS sorting. These two cell populations are used for methylcellulose colony formation assays.

(**D**) c-kit^{high} cell population has higher colony formation capability when compared to the c-kit^{low} population.

(**E**) RT-qPCR analysis demonstrates that c-kit^{high} cell population expresses higher level of *Kdm2b/Jhdm1b* compared to c-kit^{low} cell population. Relative mRNA levels are measured by RT-qPCR and normalized to *Gapdh* level. The level of c-kit^{low} population is arbitrarily set to 1.

Figure 2. Enforced expression of *Kdm2b/Jhdm1b* is sufficient for bone marrow transformation

(**A**) Enforced expression of *Kdm2b/Jhdm1b* is capable of immortalizing bone marrow cells *in vitro*. Shown are colony numbers of c-kit^{+} hematopoietic progenitor cells transduced with lentiviral vectors expressing *GFP*, *Kdm2b/Jhdm1b*, or *Hoxa9-Meis1* at each round of methylcellulose re-plating.

(**B**) Growth curve of c-kit^{+} hematopoietic progenitors transduced with lentiviral vectors expressing *GFP*, *Kdm2b/Jhdm1b*, or *Hoxa9-Meis1* in suspension culture.

(**C**) FACS analysis of the genetically modified Ly5.2^{+} donor cells, *LacZ* and *Jhdm1b* over-expression (*Jhdm1b* OE) in the peripheral blood of recipients. The results demonstrate that the percentage of *Kdm2b/Jhdm1b* over-expressing Ly5.2^{+} cells increases gradually after transplantation.

(**D**) Methylcellulose re-plating assay demonstrates that *Kdm2b/Jhdm1b* over-expressing bone marrow cells isolated from recipient mice can form increased numbers of colonies. In contrast, mock transplanted bone marrow cells fail to grow continuously in the methylcellulose re-plating assay. Colony numbers for each round of re-plating are shown.

(**E**) Flow cytometry analysis demonstrates that *Kdm2b/Jhdm1b* over-expressed colonies express high level of c-kit and low levels of myeloid lineage markers Mac-1/Gr-1.

(**F**) Splenomegaly is observed in mice transplanted with *Kdm2b/Jhdm1b* over-expressing bone marrow cells. Shown is a representative picture of spleens harvested from mice 6 weeks after transplantation of bone marrow cells transduced with lentiviral vectors expressing *LacZ* or *Kdm2b/Jhdm1b*. Bar size represents 1.0 cm.

(**G**) May-Grunwald/Giemsa staining showing typical leukoblasts in the bone marrow of recipient mice transplanted with *Kdm2b/Jhdm1b* over-expressing bone marrow cells. Bar size represents 10 μm.

(**H**) Survival curve shows mice transplanted with mock cells survived at least 210 days after transplantation, while the majority of mice transplanted with *Kdm2b/Jhdm1b* over-expressing cells died within 120-180 days after transplantation.

Figure 3. *Kdm2b/Jhdm1b* is required for *Hoxa9-Meis1* induced leukemic transformation *in vitro*

(**A**) Flow chart of experimental procedure. To examine the role of *Kdm2b/Jhdm1b* in *Hoxa9-Meis1* induced leukemic transformation *in vitro*, c-kit$^+$ hematopoietic progenitors were isolated from E14.5 fetal liver and transduced with lentiviral vectors expressing various combinations of proteins and shRNAs. Transduced cells were then plated on methylcellulose to evaluate the effect of *Kdm2b/Jhdm1b* knockdown on colony formation and re-plating capacity.

(**B**) Knockdown of *Kdm2b/Jhdm1b* in *Hoxa9-Meis1* induced leukemic cells impairs their methylcellulose colony re-plating capacity. Colony numbers for each round of re-plating are shown.

(**C**) Growth curves indicate knockdown of *Jhdm1b* in *Hoxa9/Meis1/GFP* (J1bKD-HMG) transformed leukemic cells impairs cell proliferation compared to *Hoxa9/MeisI/GFP*

transformed cells with control knockdown (CKD-HMG). Transformed cell colonies were picked after 3rd round of methylcellulose re-plating and cultured in liquid medium.

(**D**) Knockdown of *Kdm2b/Jhdm1b* results in a block at G1 to S-phase transition. Flow cytometry analysis of cell cycle status shows that *Kdm2b/Jhdm1b* knockdown (J1bKD-HMG) results in a higher percentage of cells in the G1 phase compared to that of control knockdown (CKD-HMG).

Figure 4. *Kdm2b/Jhdm1* is required for *Hoxa9-Meis1* induced AML development *in vivo*

(**A**) Flow chart of experimental procedure for bone marrow transplantation assays. To examine the role of *Kdm2b/Jhdm1b* in *Hoxa9-Meis1* induced acute myeloid leukemia development *in vivo*, c-kit+ progenitors were isolated from E14.5 fetal liver of Ly5.2 C57BL/6 embryos. After lentiviral transduction with vectors expressing various combinations of proteins and shRNAs, the genetically modified cells were mixed with normal irradiation protector cells, and were transplanted into lethally irradiated Ly5.1 C57BL/6 mice.

(**B**) FACS analysis of the accumulation kinetics of the genetically modified donor cells (GFP, CKD-HMG and J1bKD-HMG) in the peripheral blood of recipients. The results indicate that *Kdm2b/Jhdm1b* knockdown cells (J1bKD-HMG) failed to repopulate in the Mac-1+ myeloid lineage while the control knockdown cells (CKD-HMG) can.

(**C**) The percentage of genetically modified cells, marked by GFP, contribute to the myeloid lineage (Gr-1+ and Mac-1+) of peripheral blood at various time points after transplantation. The results show that *Kdm2b/Jhdm1b* knockdown inhibits re-populating by *Hoxa9-Meis1* transduced cells in recipient mice. The percentage of GFP+ cells in a particular lineage is calculated by dividing GFP and lineage marker double positive cells with the total lineage marker positive cells. All error bars represent S.D. (n=10)

(**D**) Splenomegaly of mice transplanted with lentiviral transduced bone marrow cells expressing CKD-HMG. Shown is a representative picture of spleens harvested from mice 6 weeks post-transplantation of bone marrow cells transduced with lentiviral vectors expressing GFP, J1bKD-HMG, or CKD-HMG. Bar size represents 1.0 cm.

(**E**) Hematoxylin/Eosin staining shows leukemic infiltration of multiple organs (spleen, kidney, and lung) in recipient mice transplanted with *Hoxa9-Meis1* induced leukemic cells (CKD-

HMG), while there is no obvious leukemic cell infiltration in recipient mice transplanted with bone marrow cells transduced with lentiviral vectors expressing GFP or J1bKD-HMG cells.

(**F**) May-Grunwald/Giemsa staining shows typical leukemic cells in the peripheral blood of recipient mice transplanted with CKD-HMG cells. Bar size represents 50 μm.

(**G**) Survival curve shows mice transplanted with GFP and J1bKD-HMG cells survived at least 70 days after transplantation, while the mice transplanted with CKD-HMG cells all died within 70 days after transplantation.

Figure 5. The enzymatic activity of KDM2b/JHDM1b is required for the self-renewal of leukemic stem cells

(**A**) Flow chart of experimental procedure for analyzing the role of *Kdm2b/Jhdm1b* in LSC self-renewal. Leukemic cells were isolated from primary AML mice and selected for LSCs through re-plating on methylcellulose. Cells derived from the colonies were transduced with various lentiviral vectors, followed by methylcellulose colony formation assay *in vitro* and secondary transplantation assays *in vivo*.

(**B**) Photographs of the methylcellulose colony formation assay plates show that *Kdm2b/Jhdm1b* knockdown results in decease of both size and number of colonies. This phenotype can be rescued by wild-type, but not a catalytic mutant, *Kdm2b/Jhdm1b* or *LacZ.*

(**C**) Quantification of the colony numbers derived from the methylcellulose colony re-plating assays.

(**D**) Survival curve shows prolonged survival of mice receiving transplantation of primary *Hoxa9-Meis1* leukemia cells with knockdown of Jhdm1b. This phenotype can be reversed by wild-type, but not catalytic mutant *Kdm2b/Jhdm1b* or *LacZ.*

Figure 6. KDM2b/JHDM1b directly regulates *p15^{Ink4b}* expression in leukemic cells

(A) RT-qPCR analysis demonstrates that *p15^{Ink4b}* is significantly up-regulated in response to *Kdm2b/Jhdm1b* knockdown. Relative mRNA levels are measured by RT-qPCR and normalized to *Gapdh* level.

(B) Schematic representation of the *p15^{Ink4b}* locus in mouse indicating the genomic structure (exons are represented by black boxes), as well as the location of the three amplicons analyzed by ChIP assays.

(C) ChIP experiments using chromatin prepared from leukemic cells expressing LacZ-Flag, wild-type KDM2b/JHDM1b-Flag and mutant KDM2b/JHDM1b-Flag were carried out using anti-Flag antibody. KDM2b/JHDM1b-Flag binding was assayed by qPCR at the three genomic regions depicted in panel **B**.

(D) ChIP experiments using chromatin prepared from leukemic cells with control KD, *Kdm2b/Jhdm1b* KD, *Kdm2b/Jhdm1b* KD reconstituted with wild-type *Kdm2b/Jhdm1b* or mutant *Kdm2b/Jhdm1b* were carried out using anti-H3K36me2 antibody. H3K36me2 level was assayed by qPCR at the three genomic regions depicted in panel **B**.

Figure 7. *p15*^{Ink4b} is a critical target mediating the function of KDM2b/JHDM1b in *Hoxa9-Meis1* induced AML

(**A**) Loss of *p15*^{Ink4b} abrogates the effect of *Kdm2b/Jhdm1b* knockdown on *Hoxa9-Meis1* induced leukemic transformation. Serial methylcellulose re-plating assay shows that similar numbers of colonies were obtained from *p15*^{Ink4b}-null hematopoietic progenitor cells transduced with *Hoxa9-Meis1* regardless of whether *Kdm2b/Jhdm1b* was knocked down or not.

(**B**) Splenomegaly is observed in mice transplanted with both control knockdown (CKD/HMG) and *Kdm2b/Jhdm1b* knockdown (J1bKD-HMG) *Hoxa9-Mesi1* transduced *p15*^{Ink4b} -null hematopoietic progenitors.

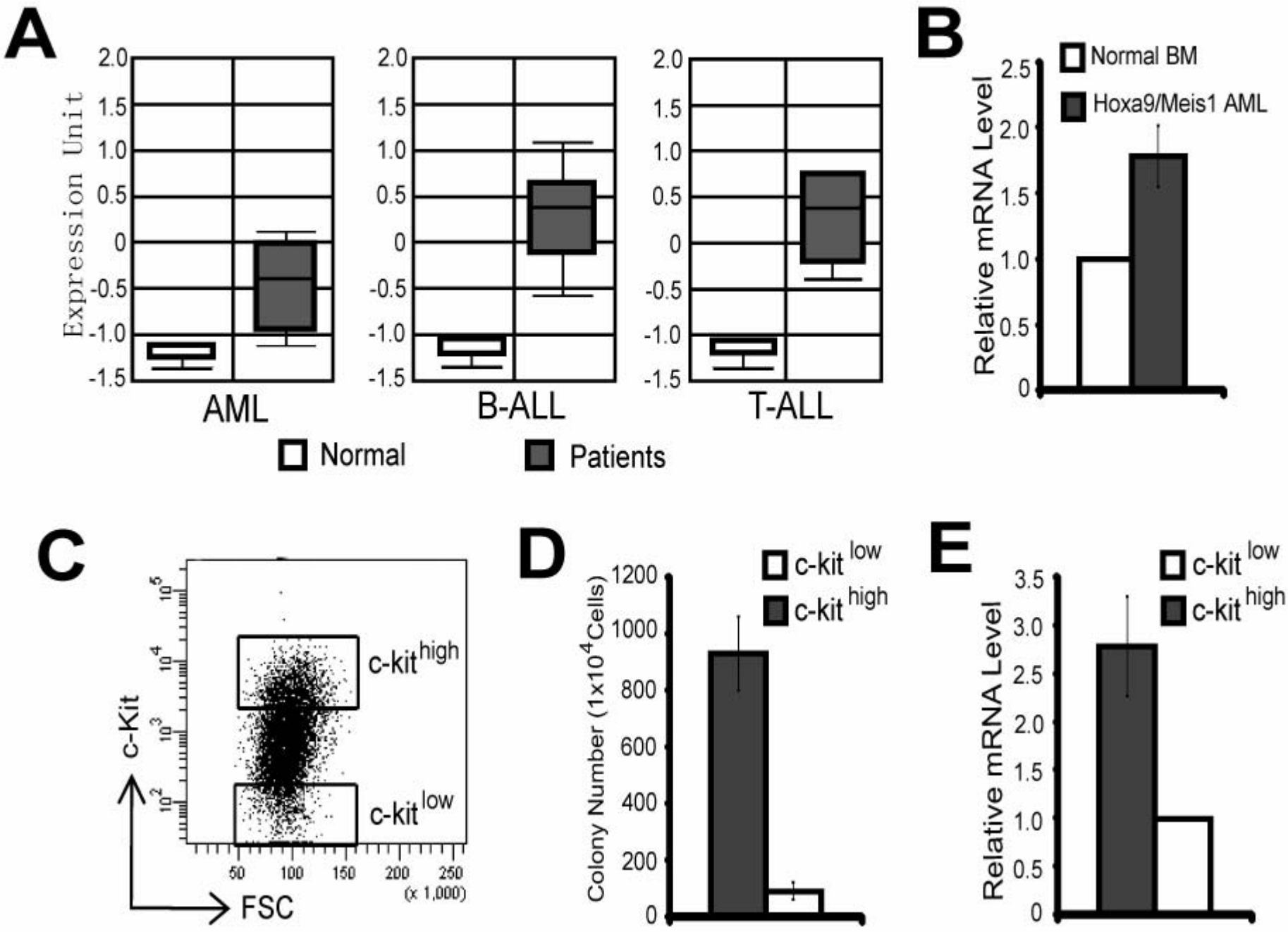
(**C**) Flow cytometry analysis demonstrates that both J1bKD-HMG and CKD/HMG transduced *p15*^{Ink4b}-null hematopoietic progenitor donor cells can repopulate and dominate peripheral blood of recipient mice 4 weeks after transplantation.

(**D**) Survival curve shows that recipient mice transplanted with *p15*^{Ink4b}-null hematopoietic progenitor cells transduced with either CKD-HMG or J1bKD-HMG die within 90 days after transplantation.

(**E**) Proposed model for epigenetic regulation of the Ink4b locus in LSCs. In LSCs, *p15*^{Ink4b} is suppressed by multiple epigenetic modifiers including KDM2b/JHDM1b and Polycomb group proteins. In this model, *Kdm2b/Jhdm1b* is up-regulated by oncogenic stimuli and maintains at a high level in LSCs. Demethylation of H3K36 by KDM2b/JHDM1b and concomitant H2A ubiquitylation by the associated Polycomb group of proteins results in silencing of *p15*^{Ink4b}. Conversely, depletion of *Kdm2b/Jhdm1b* causes an increase in the

H3K36me2 level concomitant with loss of Polycomb group proteins leading to de-repression of *p15*^{Ink4b}, resulting in defects in leukemic cell proliferation and LSC self-renewal.

Figure 1


A
Expression Unit
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AML
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Normal
Patients
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Normal BM
Hoxa9/Meis1 AML
Relative mRNA Level
2.5
2.0
1.5
1.0
0.5
0
C
c-Kit
FSC
c-kit high
c-kit low
50
100
150
200
250
(× 1,000)
D
Colony Number (1x10^4Cells)
1200
1000
800
600
400
200
0
c-kit low
c-kit high
E
Relative mRNA Level
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
c-kit low
c-kit high

Figure 2

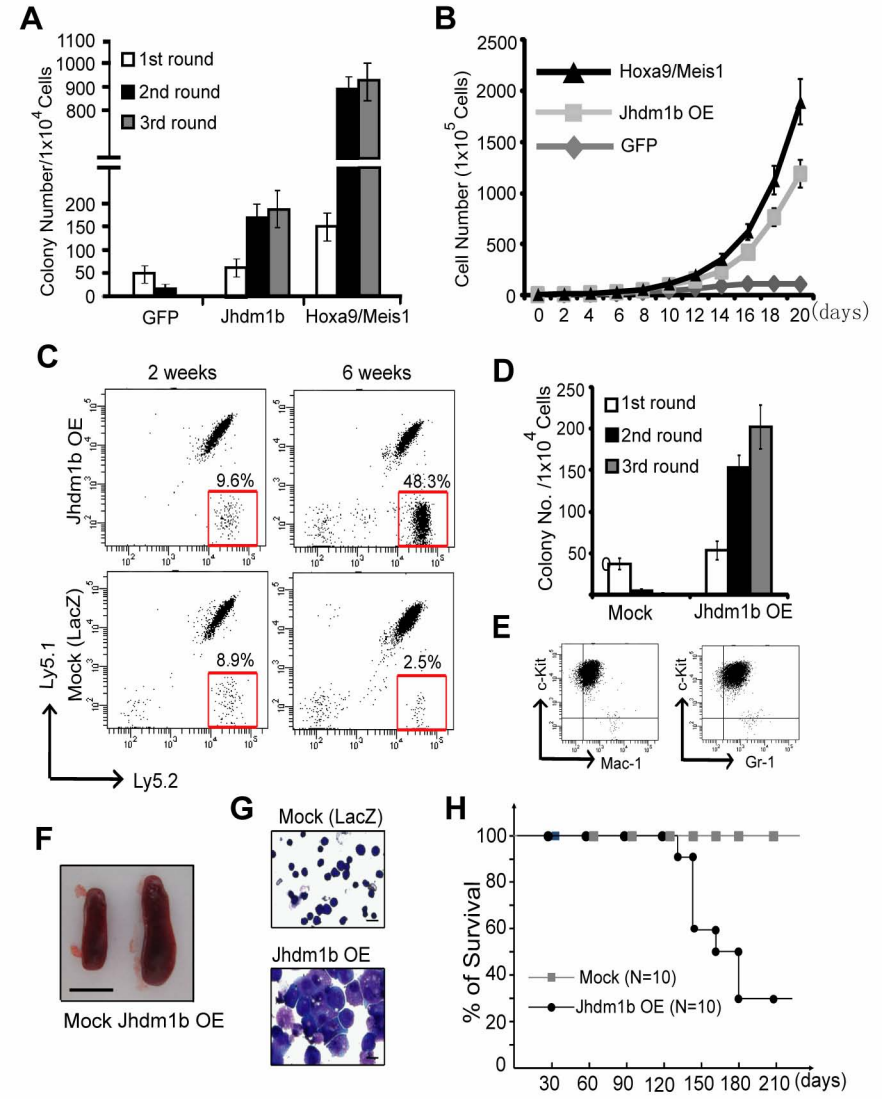

A
Colony Number/1x10^4 Cells
1st round
2nd round
3rd round
GFP
Jhdm1b
Hoxa9/Meis1
B
Cell Number (1x10^5 Cells)
Hoxa9/Meis1
Jhdm1b OE
GFP
(days)
C
2 weeks
6 weeks
Jhdm1b OE
Mock (LacZ)
9.6%
48.3%
8.9%
2.5%
Ly5.1
Ly5.2
D
Colony No. /1x10^4 Cells
1st round
2nd round
3rd round
Mock
Jhdm1b OE
E
c-Kit
Mac-1
Gr-1
F
Mock Jhdm1b OE
G
Mock (LacZ)
Jhdm1b OE
H
% of Survival
Mock (N=10)
Jhdm1b OE (N=10)
(days)

Figure 3

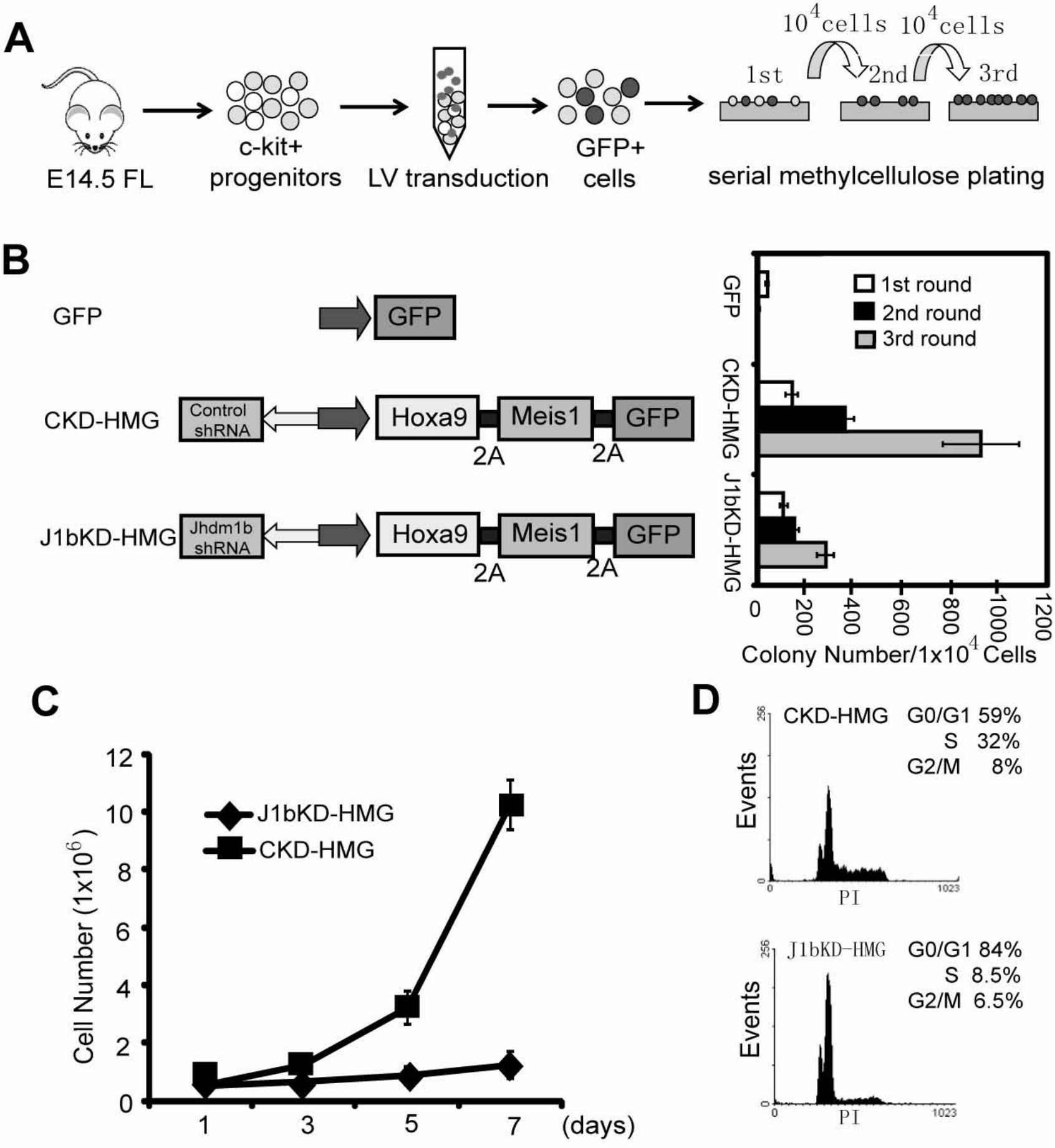

A
10^4cells 10^4cells
1st
2nd
3rd
E14.5 FL
c-kit+
progenitors
LV transduction
GFP+
cells
serial methylcellulose plating
B
GFP
GFP
CKD-HMG
Control
shRNA
Hoxa9
Meis1
GFP
2A
2A
J1bKD-HMG
Jhdm1b
shRNA
Hoxa9
Meis1
GFP
2A
2A
1st round
2nd round
3rd round
GFP
CKD-HMG
J1bKD-HMG
0
200
400
600
800
1000
1200
Colony Number/1x10^4 Cells
C
Cell Number (1x10^6)
J1bKD-HMG
CKD-HMG
0
2
4
6
8
10
12
1
3
5
7
(days)
D
CKD-HMG
G0/G1 59%
S 32%
G2/M 8%
Events
PI
J1bKD-HMG
G0/G1 84%
S 8.5%
G2/M 6.5%
Events
PI

Figure 4

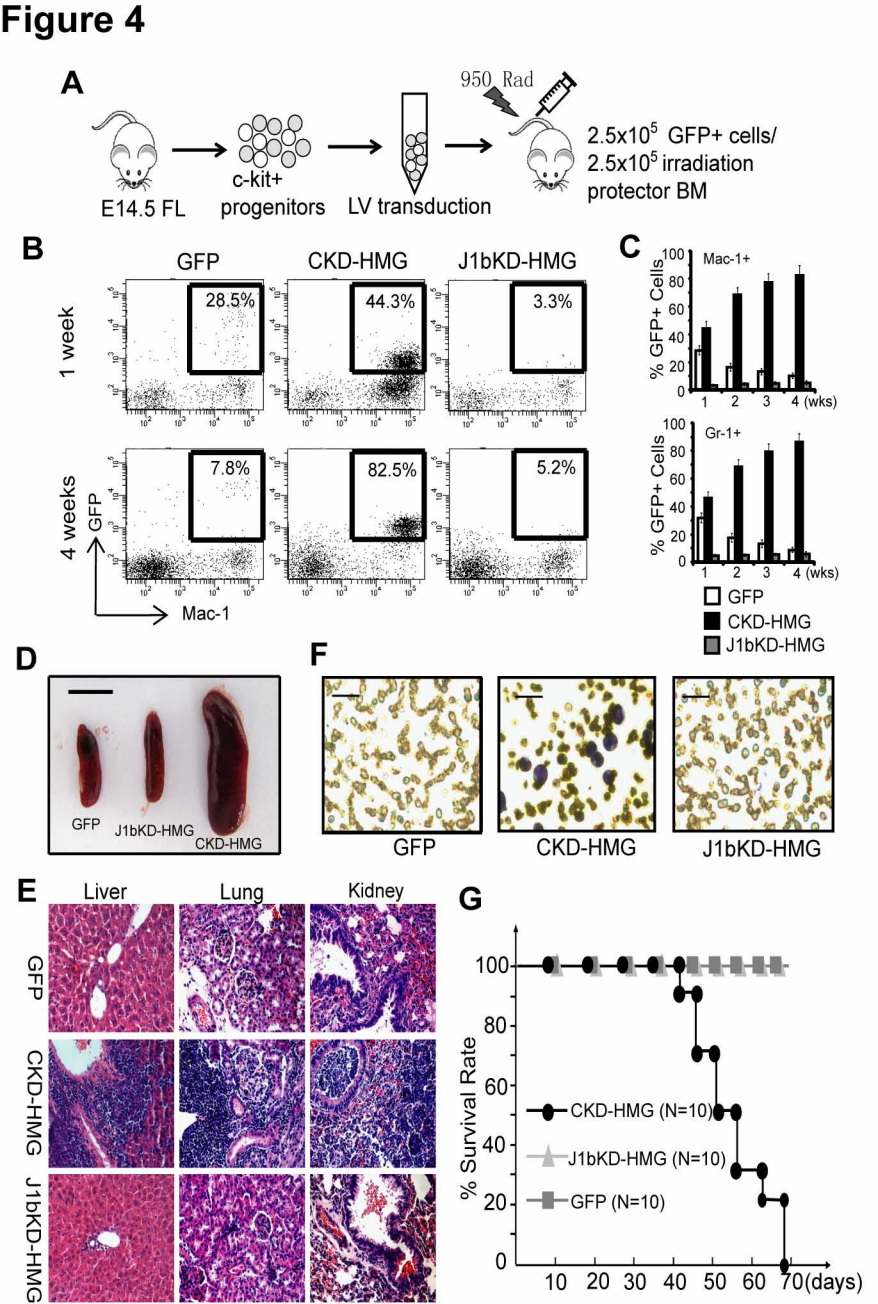

A
950 Rad
E14.5 FL
c-kit+ progenitors
LV transduction
2.5x10^5 GFP+ cells/ 2.5x10^5 irradiation protector BM
B
GFP
CKD-HMG
J1bKD-HMG
1 week
4 weeks
28.5%
44.3%
3.3%
7.8%
82.5%
5.2%
GFP
Mac-1
C
Mac-1+
Gr-1+
% GFP+ Cells
1 2 3 4 (wks)
GFP
CKD-HMG
J1bKD-HMG
D
GFP
J1bKD-HMG
CKD-HMG
F
GFP
CKD-HMG
J1bKD-HMG
E
Liver
Lung
Kidney
GFP
CKD-HMG
J1bKD-HMG
G
% Survival Rate
CKD-HMG (N=10)
J1bKD-HMG (N=10)
GFP (N=10)
10 20 30 40 50 60 70(days)

Figure 5

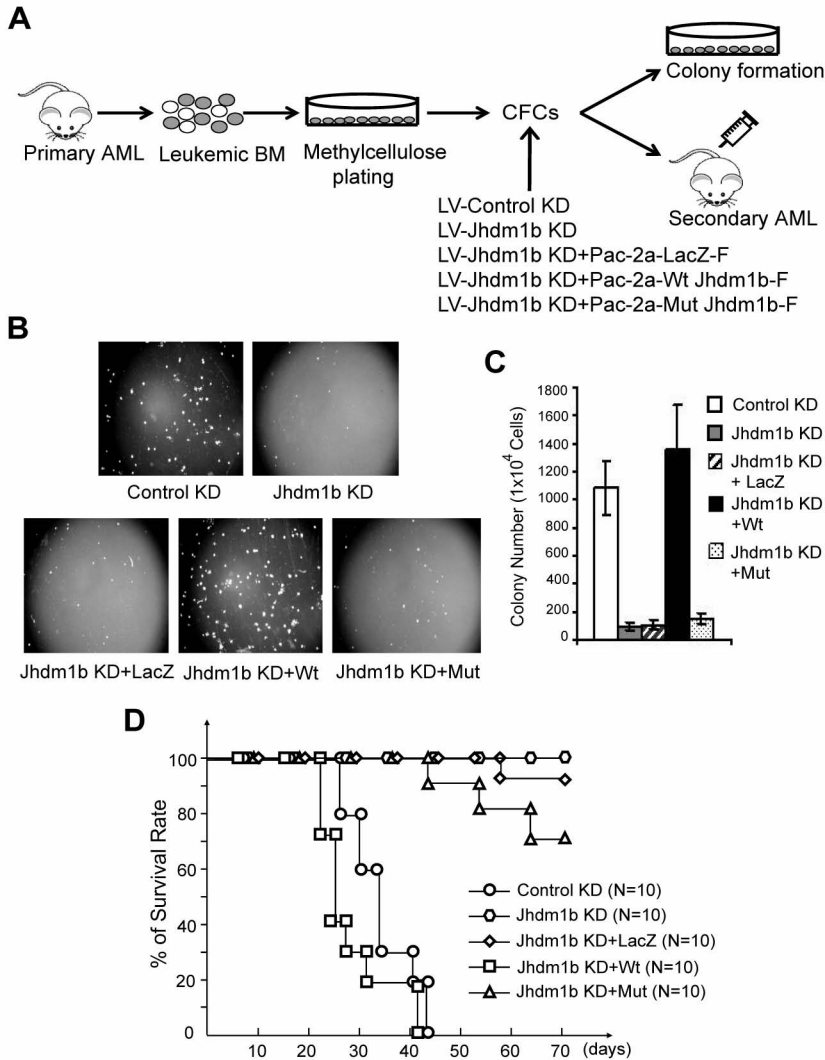

A
Primary AML
Leukemic BM
Methylcellulose plating
CFCs
Colony formation
Secondary AML
LV-Control KD
LV-Jhdm1b KD
LV-Jhdm1b KD+Pac-2a-LacZ-F
LV-Jhdm1b KD+Pac-2a-Wt Jhdm1b-F
LV-Jhdm1b KD+Pac-2a-Mut Jhdm1b-F
B
Control KD
Jhdm1b KD
Jhdm1b KD+LacZ
Jhdm1b KD+Wt
Jhdm1b KD+Mut
C
Colony Number (1x10^4 Cells)
1800
1600
1400
1200
1000
800
600
400
200
0
Control KD
Jhdm1b KD
Jhdm1b KD + LacZ
Jhdm1b KD +Wt
Jhdm1b KD +Mut
D
% of Survival Rate
100
80
60
40
20
0
10
20
30
40
50
60
70
(days)
Control KD (N=10)
Jhdm1b KD (N=10)
Jhdm1b KD+LacZ (N=10)
Jhdm1b KD+Wt (N=10)
Jhdm1b KD+Mut (N=10)

Figure 6

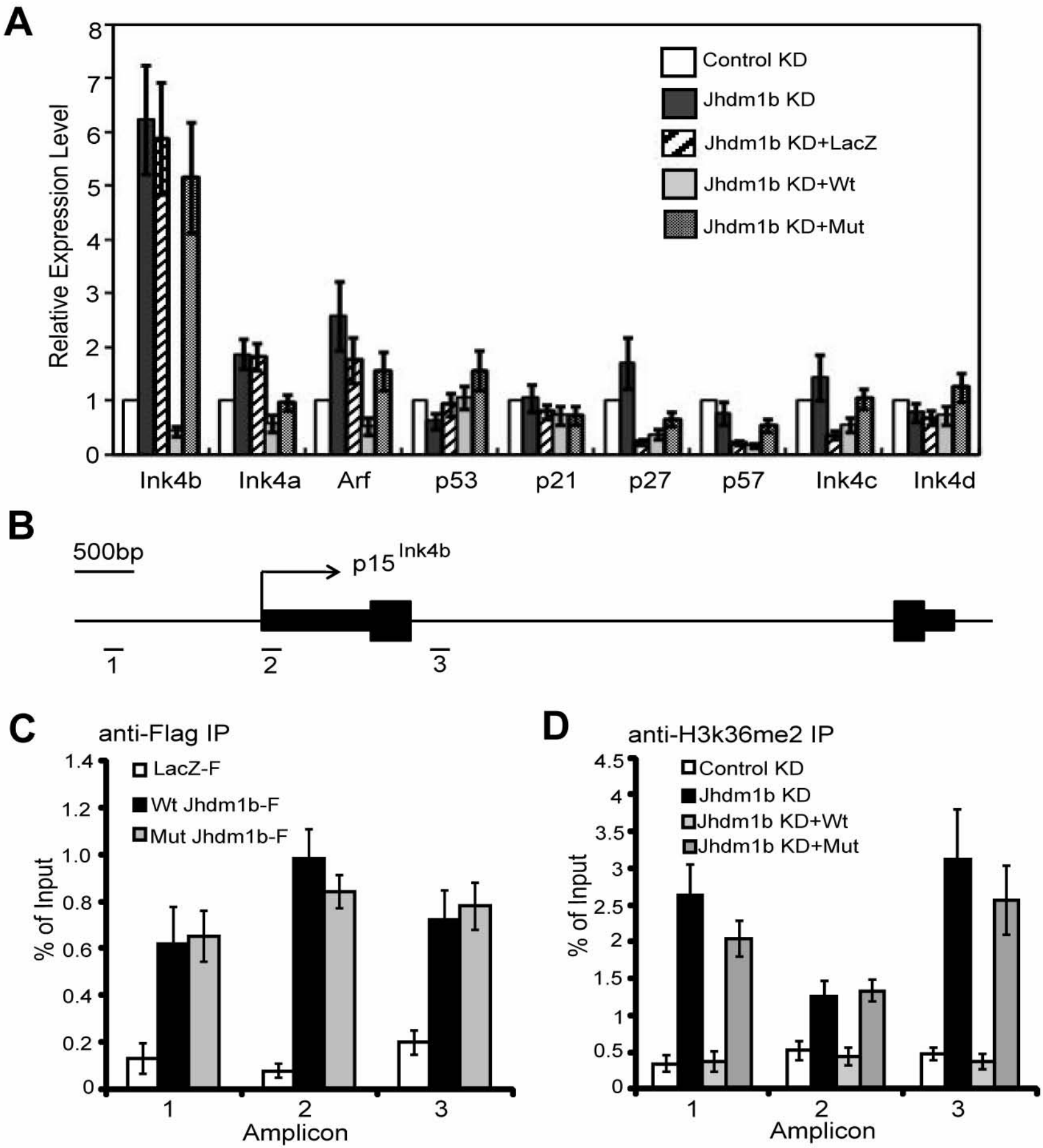
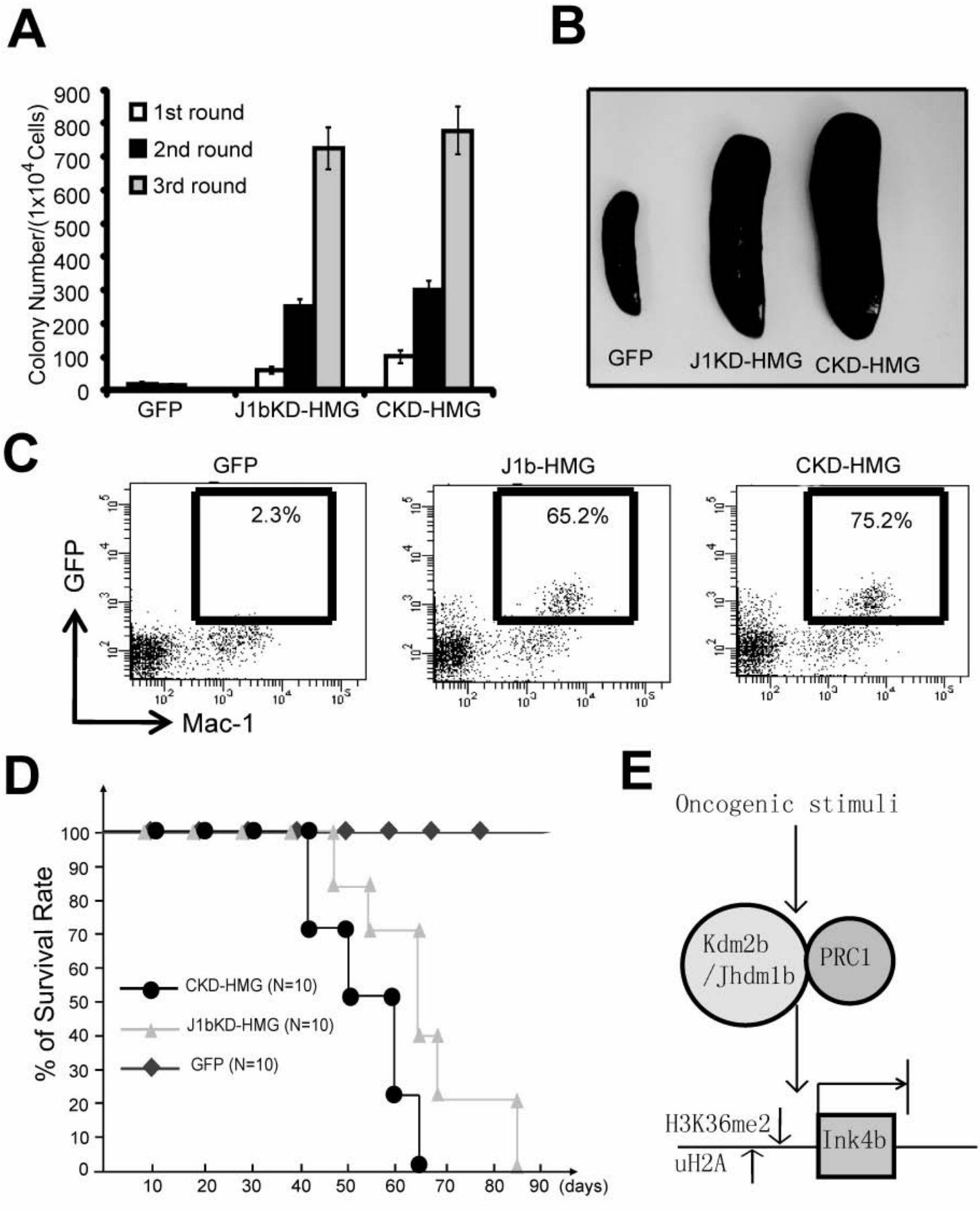

A
Relative Expression Level
Control KD
Jhdm1b KD
Jhdm1b KD+LacZ
Jhdm1b KD+Wt
Jhdm1b KD+Mut
Ink4b
Ink4a
Arf
p53
p21
p27
p57
Ink4c
Ink4d
B
500bp
p15 Ink4b
1
2
3
C
anti-Flag IP
LacZ-F
Wt Jhdm1b-F
Mut Jhdm1b-F
% of Input
Amplicon
D
anti-H3k36me2 IP
Control KD
Jhdm1b KD
Jhdm1b KD+Wt
Jhdm1b KD+Mut
% of Input
Amplicon

Figure 7


A
Colony Number/(1x10^4Cells)
900
800
700
600
500
400
300
200
100
0
1st round
2nd round
3rd round
GFP
J1bKD-HMG
CKD-HMG
B
GFP
J1KD-HMG
CKD-HMG
C
GFP
J1b-HMG
CKD-HMG
2.3%
65.2%
75.2%
GFP
Mac-1
D
% of Survival Rate
100
90
80
70
60
50
40
30
20
10
0
CKD-HMG (N=10)
J1bKD-HMG (N=10)
GFP (N=10)
10
20
30
40
50
60
70
80
90
(days)
E
Oncogenic stimuli
Kdm2b
/Jhdm1b
PRC1
H3K36me2
uH2A
Ink4b