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Targeting Suppressor of Variegation 3-9 Homologue 2 (SUV39H2) in Acute Lymphoblastic Leukemia (ALL)[1]

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Abstract

Although recent progress in understanding the biology and optimizing the treatment of acute lymphoblastic leukemia (ALL) has improved cure rates of childhood ALL to nearly 90%, the cure rate in adult ALL remains less than 50%. The poor prognosis in adult ALL has in part been attributed to larger proportion of high-risk leukemia showing drug resistance. Thus, identifying novel therapeutic targets in ALL is needed for further improvements in treatment outcomes of adult ALL. Genetic aberration of chromatin-modifying molecules has been recently reported in subtypes of ALL, and targeting components of chromatin complexes has shown promising efficacy in preclinical studies. Suppressor of variegation 3-9 homologue 2 (SUV39H2), also known as KMT1B, is a SET-domain–containing histone methyltransferase that is upregulated in solid cancers, but its expression is hardly detectable in normal tissues. Here, we show that *SUV39H2* is highly expressed in ALL cells but not in blood cells from healthy donors and also that *SUV39H2* mRNA is expressed at significantly higher levels in bone marrow or blood cells from patients with ALL obtained at diagnosis compared with those obtained at remission ($P = .007$). In four ALL cell lines (Jurkat and CEM derived from T-ALL and RS4;11 and REH derived from B-ALL), *SUV39H2* knockdown resulted in a significant decrease in cell viability (~77%, $P < .001$), likely through induction of apoptosis. On the other hand, SUV39H2 overexpression made cells more resistant to chemotherapy. We conclude that SUV39H2 is a promising therapeutic target and further investigation of this therapeutic approach in ALL is warranted.

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Introduction

Acute lymphoblastic leukemia (ALL) is a hematological malignancy characterized by maturation arrest and overproduction of immature, aberrant lymphoid cells. Based on the immunophenotype of the leukemic cells, ALL is classified as either B-cell or T-cell lymphoblastic leukemia [1,2]. The 5-year event-free survival rate is nearly 90% in childhood ALL but only approximately 40% in adult ALL [3–7]. Notably, the poor prognosis in adult ALL has, in part, been attributed to a higher proportion of high-risk leukemias that demonstrate eventual drug resistance and subsequent relapse. Furthermore, patients of all ages with primary resistant leukemia or whose leukemia relapses after a transient response have few effective therapeutic options and face a dismal prognosis [8–10]. Therefore, a better understanding of the pathogenesis and the basis of chemoresistance is critically important to further improve the prognosis of these high-risk leukemias [11,12].

Several studies have shown that epigenetic dysregulation characterized by changes in DNA methylation and histone modification ultimately influences transcriptional regulation and oncogenic signaling pathways in ALL [13,14]. Notably, whole-genome and whole-exome sequencing in addition to global chromatin profiling

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studies has identified recurrent somatic mutations in genes encoding epigenetic modifiers in ALL, including *MLL-FP*, *CBP*, and *NSD2* [13,14]. Furthermore, aberrant amplification of genes encoding chromatin modifiers such as *EZH2*, *MLL5*, *SUV39H1*, and *BRD4* has also been attributed to resistance to targeted therapy in T-ALL [15]. Indeed, approaches focused on targeting components of chromatin complexes have emerged as promising therapeutic strategies particularly when combined with chemotherapy [14,16]. Taken together, these emerging findings strongly suggest that chromatin modifiers may play an important role in the development and progression of ALL.

Suppressor of variegation 3-9 homologue 2 (SUV39H2), also known as KMT1B, is a SET-domain–containing histone methyltransferase that is expressed only in testis among normal tissues [17]. However, SUV39H2 has been found to be upregulated in lung, cervical, bladder, esophageal, and prostate cancers as well as osteosarcomas and soft tissue sarcomas [18]. SUV39H2, which has been found to be localized in the nucleus, is known to epigenetically regulate gene expression by selectively trimethylating histone H3 on lysine 9 (H3K9), a modification associated with heterochromatin formation and subsequent gene silencing [19,20]. Our recent study has also shown that SUV39H2 methylates histone H2AX on lysine 134 and subsequently enhances the phosphorylation of H2AX on serine 139 [18]. Phosphorylated histone H2AX (γ-H2AX) is associated with activation of DNA-damage repair pathways and enhanced radio and chemoresistance of cancer cells [21–25].

Here, we hypothesized that an SUV39H2-dependent pathway might play a role in survival and proliferation of ALL cells and that targeting the gene would result in antileukemic activity. Our investigation aimed to characterize the expression of SUV39H2 in ALL and examine possible biological roles of this gene in the pathogenesis and progression of the disease.

Results

SUV39H2 Overexpression in ALL Primary Blasts and Cell Lines

To examine the potential of SUV39H2 as a therapeutic target in ALL, we first compared *SUV39H2* mRNA levels in primary cells obtained from 30 patients with ALL as well as 9 B-ALL and 8 T-ALL cell lines with those in normal peripheral blood mononuclear cells (nPBMNCs) obtained from 4 healthy donors. We found that *SUV39H2* expression levels were significantly higher in ALL blasts and cell lines than in the nPBMNCs ($P < .001$; Figure 1*A*). No difference was observed in *SUV39H2* mRNA levels between T-ALL and B-ALL cell lines. We also assessed *SUV39H2* mRNA expression in paired bone marrow or peripheral blood samples obtained from 30 patients with ALL at diagnosis and remission. We found *SUV39H2* expression to be significantly upregulated in cells obtained at the diagnosis compared with those obtained at the remission point ($P = .007$; Figure 1*B*). Furthermore, we analyzed SUV39H2 protein levels in nPBMNCs obtained from four healthy donors, and four T-ALL and three B-ALL cell lines, and found that SUV39H2 was only detectable in the ALL cell lines. Consistently, trimethylated H3K9 (H3K9me3) was detected in ALL cell lines but not in nPBMCs from healthy donors (Figure 1*C*).

The Effect of SUV39H2 Knockdown on Cell Viability in ALL Cell Lines

To assess the biological function of SUV39H2 in ALL cells, we first applied a loss-of-function approach using two T-ALL (Jurkat and CEM) and two B-ALL (REH and RS4;11) cell lines. Knockdown of *SUV39H2* in these cells was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR) (Figure 2*A*) and Western blot (Figure 2*B*) 20 and 30 hours after the transfection of siRNAs, respectively. Cells transfected with *SUV39H2* siRNA showed a significant decrease in cell viability (~77%, $P < .001$) compared with those transfected with control siRNA as assessed by Cell Counting Kit–8 (CCK-8) assay 48 hours after the transfection (Figure 2*C*). These results were also validated in Jurkat cells using a second independent siRNA against *SUV39H2* (Figure S1, *A*–*C*).

The Effect of SUV39H2 Knockdown on Apoptosis in ALL Cell Lines

To further examine whether the decrease in cell viability with *SUV39H2* siRNA in the four ALL cell lines (Jurkat, CEM, REH, and RS4;11) was caused by induction of apoptosis, we prepared protein lysates 30 hours after the transfection and examined the activation of caspase 3 by Western blot analysis. We observed an increase in cleaved caspase 3 in cells transfected with *SUV39H2* siRNA compared with the cells transfected with control siRNA (Figure 3*A*). In addition, Annexin V and propidium iodide (PI) staining performed on the same set of cells at 48 hours after the transfection showed a significantly higher proportion of Annexin V/PI–positive cells in the cells transfected with *SUV39H2* siRNA than cells transfected with control siRNA (69% vs 14%, $P < .05$; Figure 3, *B* and *C*).

The Effect of SUV39H2 Overexpression on Cell Sensitivity to Chemotherapy

To further investigate the biological functions of SUV39H2 in ALL cells, we used 293T cells to generate lentiviral particles designed to express SUV39H2. We confirmed by Western blot analysis the ectopic expression of SUV39H2 and the increase of H3K9m3 in the 293T cells (Figure S2*A*). Jurkat cells were then transduced with the generated lentiviral particles, and SUV39H2 overexpression was confirmed by qRT-PCR (Figure 4*A*) and Western blot analyses (Figure 4*B*). We also confirmed the increase of H3K9m3 in SUV39H2-overexpressing (OE) Jurkat cells by Western blot analysis (Figure 4, *B* and *C*). In addition, we observed a reduction in the expression levels of downstream target genes (*H19 and CD2*) [26] in SUV39H2 OE Jurkat cells compared with Jurkat cells infected with empty vector (Jurkat EV) (Figure S3). Jurkat SUV39H2 OE and Jurkat EV cells showed no significant difference in their growth rate (Figure 4*D*). However, when cells were treated with cytarabine (Ara-C), Jurkat SUV39H2 OE cells were less sensitive to the treatment than Jurkat EV cells (Figure 4*E*). Notably, calculated IC_{50} values of cell viability after 72 hours of cytarabine treatment were 69 nM for Jurkat EV cells and 99 nM for Jurkat SUV39H2 OE cells ($P < .001$). A similar pattern of reduced sensitivity in Jurkat SUV39H2 OE cells was observed when cells were treated with either doxorubicin or dexamethasone (two other drugs used in the treatment of ALL) (Figure S4, *B* and *C*). In addition, Annexin V and PI staining showed significantly less proportion of dead cells in Jurkat SUV39H2 OE cells than in Jurkat EV cells following treatment with 100 nM cytarabine ($P = .002$; Figure 4, *F* and *G*).

Discussion

Emerging evidence has implicated aberrations in chromatin modifiers in the majority of cancer cells. In particular, dysregulation of histone H3 lysine methyltransferases and demethylases has been shown to play a role in leukemogenesis. Increase of H3K9 methylation as well as DNA methylation, partly attributed to inactivation of histone

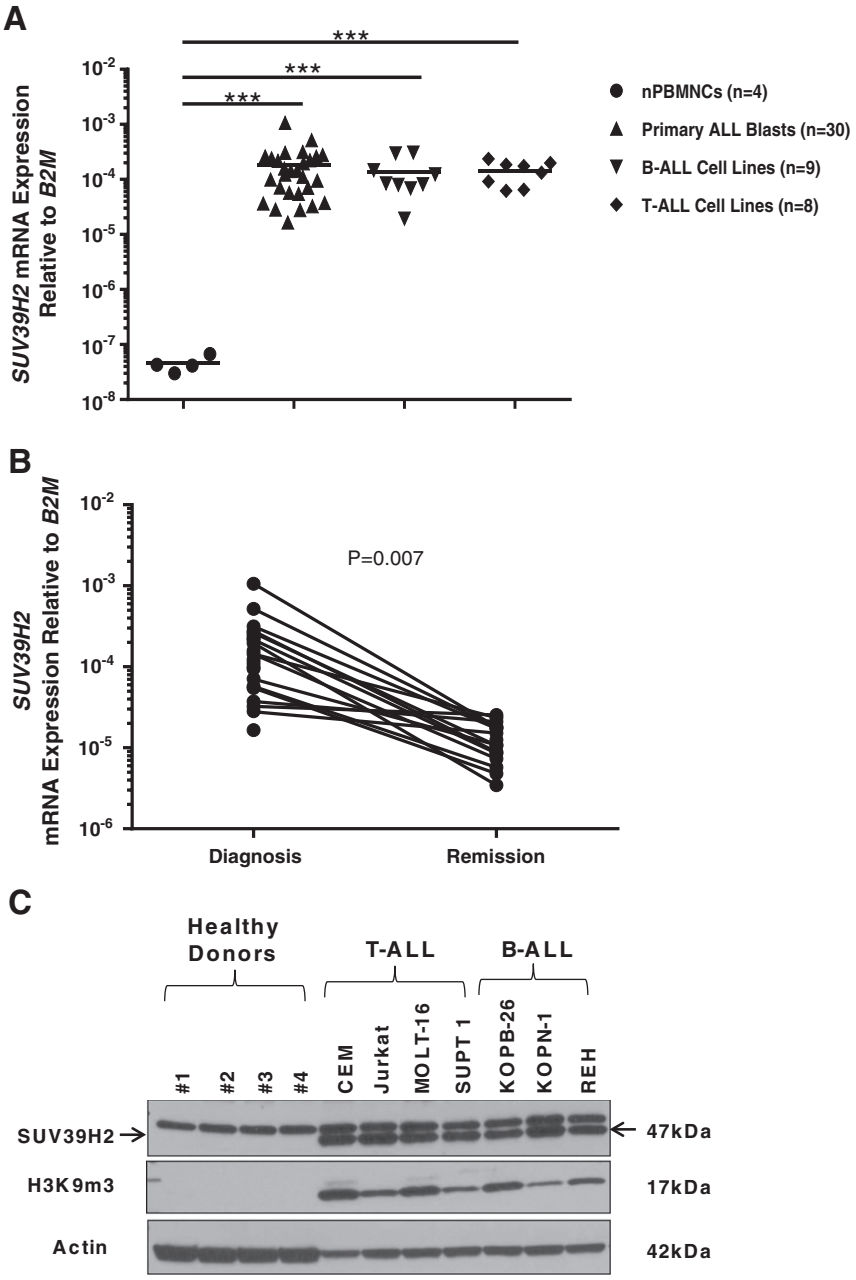

Figure 1. *SUV39H2* mRNA expression is upregulated in ALL. (A) *SUV39H2* mRNA levels in PBMNCs obtained from four healthy donors, primary blasts from 30 patients with ALL, as well as ALL cell lines (B-ALL, $n = 9$ and T-ALL, $n = 8$) as measured by RT-PCR. (B) *SUV39H2* mRNA levels in paired samples obtained at diagnosis and at remission from patients with ALL ($n = 30$) as measured by RT-PCR. (C) Comparison of SUV39H2 and H3K9me3 protein expression in PBMNCs obtained from four healthy donors with that in ALL cell lines (B-ALL, $n = 3$ and T-ALL, $n = 4$) by Western blot analyses. mRNA levels were normalized to *B2M* expression levels. *P* values were calculated using Student's *t* test (***$P < .001$).

demethylases such as KDM4C, has been found to inhibit differentiation of hematopoietic cells [27]. Furthermore, mutations in histone methyltransferase enhancer of zeste homologue 2 (EZH2) has been documented in a wide variety of B- and T-cell lymphoproliferative disorders [28,29]. Whole-genome and subsequent target sequencing studies of early T-cell precursor ALL have also identified high-frequency somatic alterations in histone-modyifying genes such as EZH2, EED, SUZ12, SETD2, and EP300 [30–32]. It is plausible that targeting components of chromatin repressor complexes that are upregulated in cancer cells may be potentially effective in treating lymphoid malignancies, including ALL. Indeed, components of chromatin complexes such as HDAC and EZH2 have been extensively investigated as drug targets in leukemia, and *in vitro* and *in vivo* results confirmed that they are promising targets for novel drug development [16,29,33–36].

SUV39H2 governs methylation of H3K9 in heterochromatic regions as a part of repressor chromatin-remodeling complexes resulting in silencing of gene expression. SUV39H2 was originally identified as an embryonic- and testis-specific histone methyltransferase [19] but was found to be upregulated in lung cancer. It was also

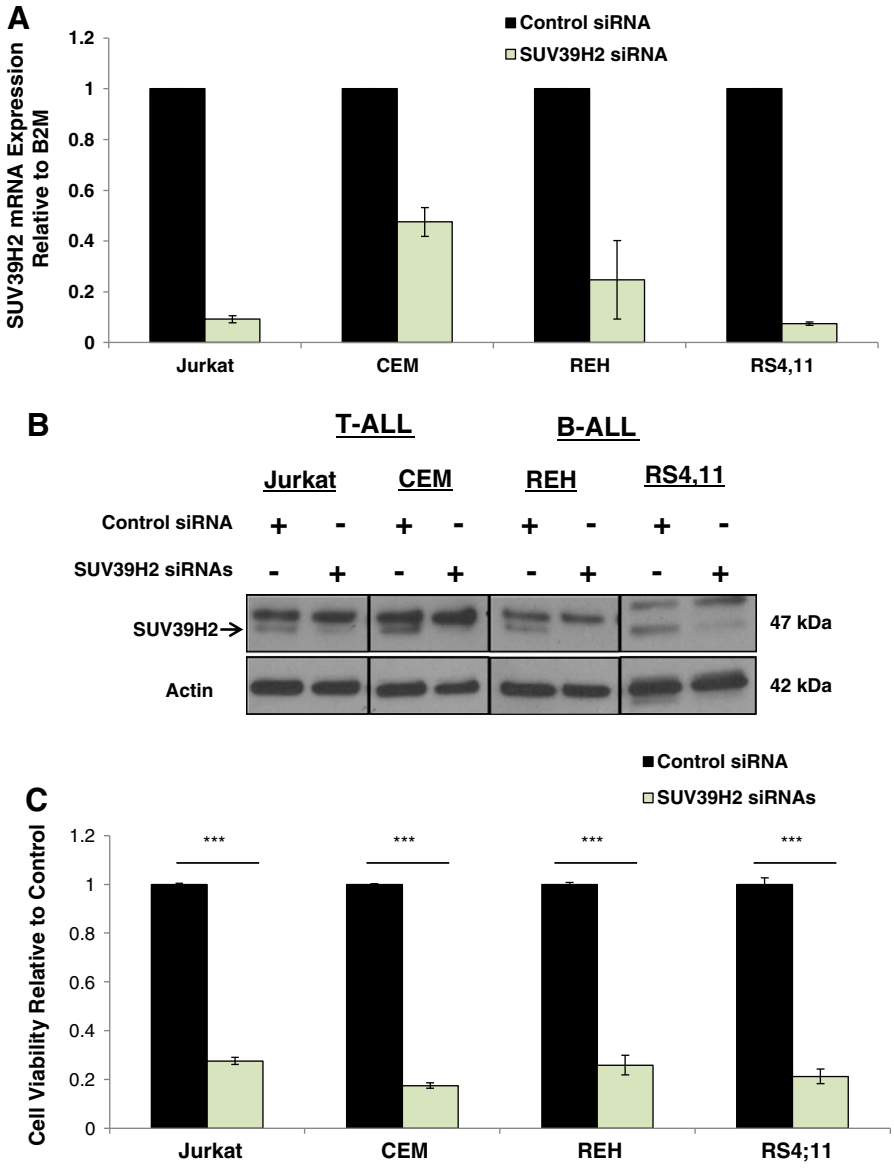

Figure 2. SUV39H2 knockdown decreased cell viability in ALL cell lines. (A) *SUV39H2* mRNA expression, (B) protein expression, and (C) cell viability in Jurkat, CEM, REH, and RS4;11 cells transfected with *SUV39H2* siRNA(#2 and 4) or control siRNA. mRNA levels were normalized to *B2M* expression levels. Data are presented as mean ± SE; *P* values were calculated using Student's *t* test (***$P < .001$).

reported that SUV39H2 expression was hardly detectable in normal tissues except in the testis [18]. Here we have demonstrated that SUV39H2 was upregulated in the majority of ALL cell lines and in leukemia cells from adults with newly diagnosed ALL. We detected much higher expression levels of this gene in leukemia blasts obtained at diagnosis than in cells obtained from marrows or peripheral blood following induction of remission. ALL cells derived from both B-cell and T-cell lineages expressed significantly higher level of SUV39H2 than mononuclear cells obtained from healthy donors. These observations suggest that the upregulation of SUV39H2 in ALL cells is involved in leukemogenesis. Consistent with this hypothesis, we revealed that knockdown of *SUV39H2* in ALL cell lines resulted in a significant decrease in cell viability associated with the enhancement of apoptosis. Although SUV39H2 overexpression in Jurkat cells did not result in increased cell proliferation, interestingly, we found that SUV39H2 overexpression resulted in cytarabine, doxorubicin, and dexamethasone chemoresistance in Jurkat cells. This finding is consistent with our recent report showing that knockdown of *SUV39H2* increased the sensitivity of HeLa cells to doxorubicin by suppression of H2AX methylation and subsequent suppression of production of γ-H2AX [18], a DNA-damage marker that accumulates at sites of double strand breaks and triggers the DNA damage response signaling cascade [22,37]. The potential for synergy using drugs targeting components of the repressor chromatin complexes, which are upregulated in cancer cells, with chemotherapy (for example, HDAC inhibitors in combination with cytarabine) has been demonstrated previously [16]. However, toxicity and selectivity hurdles facing clinical development of some of these combinational approaches suggest that identifying new targets may provide an alternative therapeutic strategy.

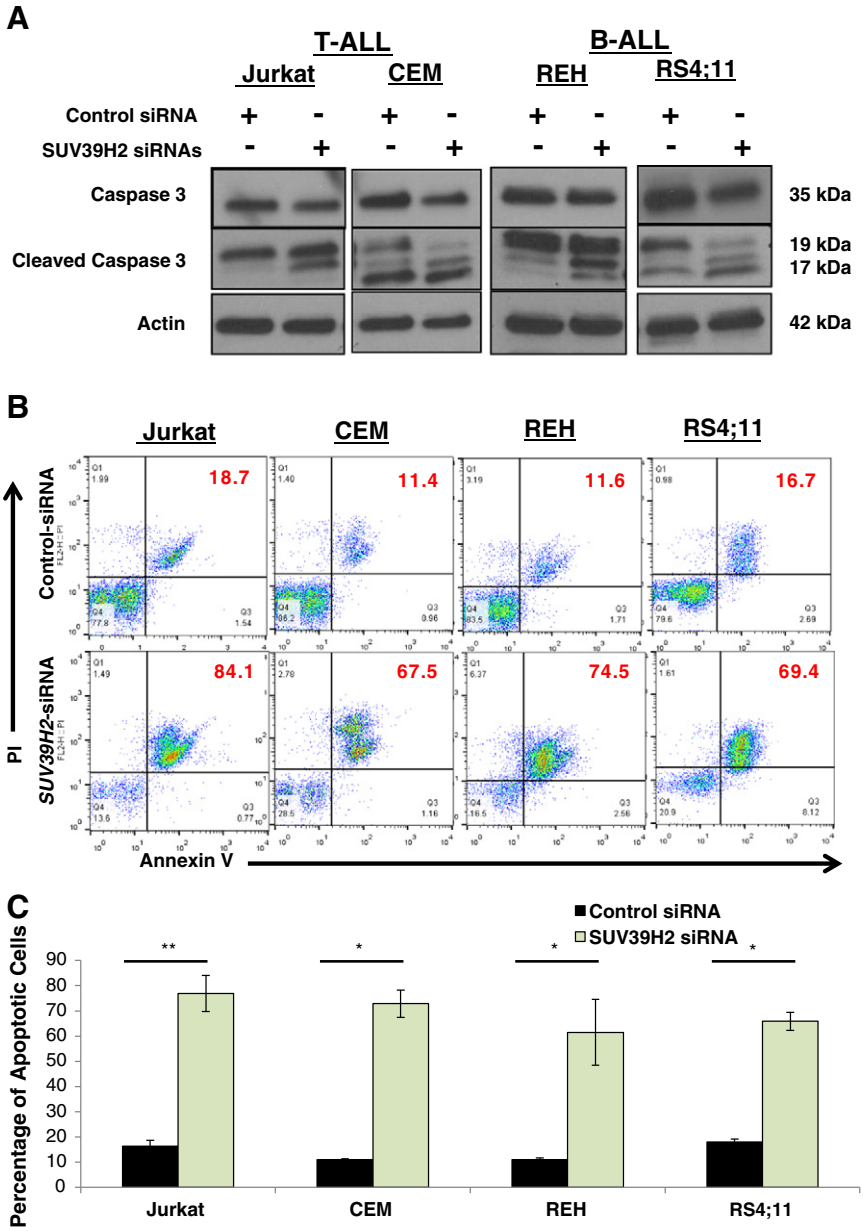

Figure 3. SUV39H2 knockdown induced apoptosis. (A) Assessment of caspase 3 activation by Western blot and (B) representative figure showing Annexin V and PI staining post *SUV39H2* siRNA(#2 and 4) or control siRNA transfection in Jurkat, CEM, REH, and RS4;11 (the numbers represent the percentage of total cells). (C) Graphical quantification of average Annexin V and PI staining results in Jurkat, CEM, REH, and RS4;11 from two independent experiments. Data are presented as mean ± SE; *P* values were calculated using Student's *t* test (*$P < .05$; ** $P < .01$; ***$P < .001$).

Given the upregulation of SUV39H2 in ALL and the dramatic increase of cell death upon its silencing, targeting SUV39H2 in ALL may be a promising therapeutic approach particularly in combination with DNA damage–inducing cytotoxic agents employed for treatment of this disease. Thus, clinical investigation of the potential of targeting SUV39H2 in ALL is warranted.

Materials and Methods

Cell lines for In Vitro Experiments

ALL cell lines were obtained from American Type Culture Collection. Cells are tested and authenticated by DNA profiling for polymorphic short tandem-repeat markers. Cells were passaged in our laboratory for fewer than 6 months after receipt. Cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% FBS (Life Technologies). List of cell lines used in this study is shown in the supplementary material (Table 1).

Patient Samples

RNA analysis was performed on paired pre-treatment and post-remission bone marrow/peripheral blood samples from 30 patients with ALL (age range, 17 to 73 years; median age, 41 years; 10 females and 20 males). Informed consent to use the tissue for investigational studies was obtained from each patient according to University of Chicago institutional guidelines and institutional review board approvals.

Transient Transfection, RNA Interference

siRNA oligonucleotide duplexes were purchased from Sigma-Aldrich for targeting the human *SUV39H2* transcript, and sequences

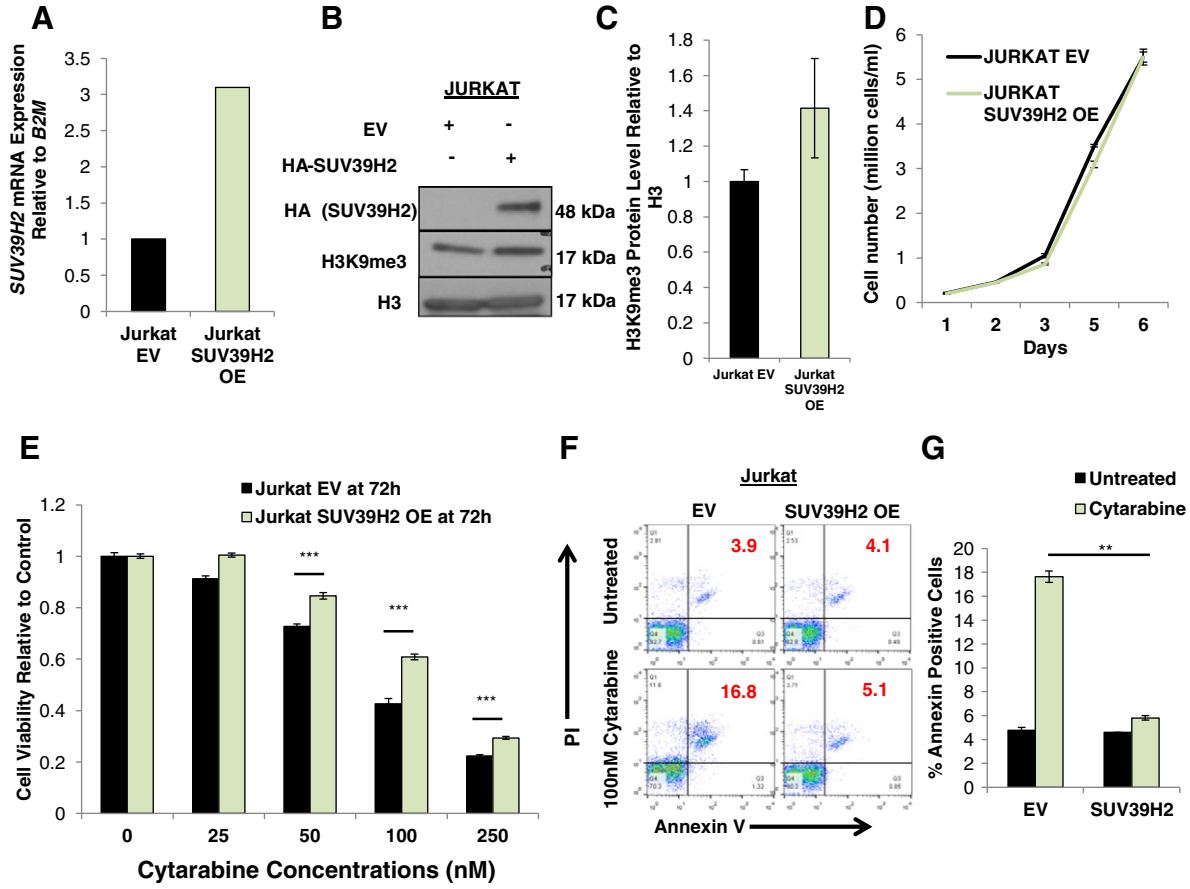

Figure 4. Effect of SUV39H2 overexpression on proliferation and chemosensitivity. (A) *SUV39H2* mRNA expression and (B) protein expression of HA-SUV39H2 and H3K9m3 in Jurkat cells following lentiviral transduction. (C) Densitometry of H3K9me3 protein band obtained from triplicate experiments. (D) Comparison of proliferation between control Jurkat EV and Jurkat SUV39H2 OE. (E) Cell viability in Jurkat EV and Jurkat SUV39H2 OE cells following treatment with increasing concentrations of cytarabine. (F) Representative figure of Annexin V and PI staining and (G) graphical quantification of average Annexin V and PI staining results in Jurkat EV and Jurkat SUV39H2 OE cells after treatment with 100 nM cytarabine. Data are presented as mean ± SE; *P* values were calculated using Student's *t* test (*$P < .05$; ** $P < .01$; ***$P < .001$).

were as follows: *SUV39H2* siRNA#1 (CACAGAUUGCUUCUUUCAA), siRNA#2 (CUUUGGUUGUUCAUGCACA), and siRNA#4 (CUGGAAUCAGCUUAGUCAA). For control, MISSION siRNA universal negative control 1 from Sigma-Aldrich was used.

Transient transfection of cells was performed using 1 nmol of siRNA and 100 μl of Gene Pulser buffer per reaction, and the cells were electroporated with a single electric pulse of 180 V for 12 milliseconds using the Bio-Rad Gene Pulse Xcell (Bio-Rad).

RNA Extraction, RNA Expression Quantification

Total RNA was extracted using Trizol reagent (Life Technologies). *SUV39H2* mRNA expression in ALL cell lines and primary cells was measured by the ViiA 7 system according to the manufacturer's instructions. Each cDNA was synthesized using SuperScript III reagents (Life Technologies) according to the manufacturer's instructions. qRT-PCR was performed using commercially available TaqMan Gene Expression Assay probes or Sigma-Aldrich SYBR Green probes with the ViiA 7 system (Life Technologies). The expression levels were normalized to *B2M* gene.

Western Blot Analysis and Antibodies

Samples were prepared from the cells lysed with CelLytic M cell lysis reagent (Sigma-Aldrich) or NE-PER reagent (Thermo Scientific) containing a complete protease inhibitor cocktail (Roche Applied Science). Total proteins were separated by electrophoresis on 4% to 12% gradient PAGE gels (Bio-Rad) and transferred to polyvinylidine difluoride membrane (GE Healthcare). Proteins of interest were detected by incubating membrane with horseradish peroxidase–conjugated antibodies (Santa Cruz) and visualized with enhanced chemiluminescence (GE Healthcare) or SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific). The following antibodies were used: rabbit polyclonal SUV39H2 antibody (ab71683; Abcam), rabbit polyclonal H3 antibody (ab1791; Abcam), rabbit polyclonal H3K9me3 antibody (ab8898; Abcam), caspase-3 and cleaved caspase-3 (Cell Signaling), anti-hemagglutinin (HA) high affinity (clone 3F10; Roche Life Science), and mouse monoclonal β-actin (AC-15; Sigma-Aldrich).

Viability and Apoptosis Analyses

For viability analysis, CCK-8 assay (Dojindo Molecular Technologies, Inc.,) was performed in a 96-well plate. A total of 5×10^4 cells were plated per well.

For viability and apoptosis analyses, cells were collected, spun down then washed with PBS, and resuspended in 50 μl of binding buffer containing 2 μl of Annexin V-APC (eBioscience) and 5 μl of PI (eBioscience). After 20-minute incubation, fluorescence was

quantified by flow cytometry on a FACSCalibur instrument (BD Biosciences).

Constructs and Viral Transduction

SUV39H2 was cloned in pLVX-Puro Vector (Clontech) containing a puromycin resistance gene. HA tag sequence was added downstream of the *SUV39H2* gene. Sequence of the construct was checked by Sanger sequencing. For the virus production, 293T cells were transfected with a mix containing gag/pol vector (Addgene), pCMV-VSV-G vector (Addgene), and pLVX construct. Medium was changed after 12 hours, and infectious virions were harvested from the culture media at 48 and 72 hours posttransfection. Jurkat cells were then incubated with the harvested culture media for 72 hours before the selection of cells conferring resistance against puromycin (TOKU-E).

Proliferation Assay

Control Jurkat cells (containing an empty vector) and SUV39H2 OE Jurkat cells were suspended in 1 ml of RPMI medium supplemented with 10% FBS at a concentration of 1×10^5 cells/ml. Cells were then counted on days 1, 2, 3, 4, and 5 using Countess automated cell counter (Invitrogen).

Chemosensitivity Experiments

Jurkat cells containing an empty vector (Jurkat EV) or Jurkat cells stably overexpressing SUV39H2 (Jurkat SUV39H2 OE) were treated with increasing doses of cytarabine or doxorubicin or dexamethasone in a 96-well plate, each well containing 5×10^4 cells. At day 3 for cytarabine and doxorubicin, and day 7 for dexamethasone, CCK-8 reagent (Dojindo Molecular Technologies) was added to assess cell viability.

For viability and apoptosis analyses, Jurkat EV or Jurkat SUV39H2 OE cells were treated with 100 nM cytarabine. Cells were collected at 72 hours, and Annexin V and PI staining was performed as described in the viability and apoptosis subsection.

Statistical Analysis

Mechanistic and biological experiments were analyzed with paired and unpaired two-sided *t* tests. *P* values $<.05$ were considered statistically significant. Experiments were performed in duplicate or triplicate; results were presented as mean ± SE.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2015.07.003.

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