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Epigenetic silencing of *SOCS5* potentiates JAK-STAT signaling and progression of T-cell acute lymphoblastic leukemia

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Abstract

Activating mutations in cytokine receptors and transcriptional regulators govern aberrant signal transduction in T-cell lineage acute lymphoblastic leukemia (T-ALL). However, the roles played by suppressors of cytokine signaling remain incompletely understood. We examined the regulatory roles of suppressor of cytokine signaling 5 (SOCS5) in T-ALL cellular signaling networks and leukemia progression. We found that *SOCS5* was differentially expressed in primary T-ALL and its expression levels were lowered in *HOXA*-deregulated leukemia harboring *KMT2A* gene rearrangements. Here, we report that *SOCS5* expression is epigenetically regulated by DNA methyltransferase-3A-mediated DNA methylation and methyl CpG binding protein-2-mediated histone deacetylation. We show that *SOCS5* negatively regulates T-ALL cell growth and cell cycle progression but has no effect on apoptotic cell death. Mechanistically, *SOCS5* silencing induces activation of JAK-STAT signaling, and negatively regulates interleukin-7 and interleukin-4 receptors. Using a human T-ALL murine xenograft model, we show that genetic inactivation of *SOCS5* accelerates leukemia engraftment and progression, and leukemia burden. We postulate that *SOCS5* is epigenetically deregulated in T-ALL and serves as an important regulator of T-ALL cell proliferation and leukemic progression. Our results link aberrant

Abbreviations: 5-AzaC, 5-azacitidine; DNMT, DNA methyltransferase; ETP, early T-cell precursor; HDAC, histone deacetylase; IL-7R, interleukin-7 receptor; *KMT2A*-R, *KMT2A* gene rearrangements; MBD, methyl-CpG-binding domain protein; MeCP2, methyl CpG binding protein-2; NSG, NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ; qRT-PCR, quantitative real-time PCR; SOCS, suppressor of cytokine signaling; T-ALL, T-cell lineage acute lymphoblastic leukemia; Th, T-helper; TSA, Trichostatin A.

downregulation of *SOCS5* expression to the enhanced activation of the JAK-STAT and cytokine receptor-signaling cascade in T-ALL.

KEYWORDS
DNA methylation, histone deacetylation, JAK-STAT, signal transduction, T-ALL

1 | INTRODUCTION

T-cell lineage acute lymphoblastic leukemia is an aggressive hematopoietic malignancy accounting for 15% of pediatric ALLs.[1,2] Over the past few decades, the cure rate in T-ALL has significantly increased; however, survival is poor in patients who suffer treatment failure or early relapse.[2,3] Further improvements in survival for T-ALL will require improved understanding of the mechanism governing leukemogenesis to develop novel treatment approaches. Although much progress has been made in understanding the stage-specific transformation of T-cell progenitors in leukemic transformation, the mechanisms of epigenetic dysregulation remain less well understood.[4] Genes involved in T-cell receptor signaling and differentiation, and tumor suppressor genes are commonly differentially methylated genes in T-ALL.[5,6] Hypermethylation of CpG islands located in the promoter and/or 1st exon/intron region was proposed as an alternative mechanism for tumor suppressor gene inactivation.[7-9]

The JAK-STAT signaling pathway plays an important role in hematopoietic cell growth, differentiation, and survival.[10] Similar to other leukemias, dysregulation in JAK-STAT signaling networks were found in a subset of T-ALL.[1,10,11] Studies of JAK-STAT activating mutations, including *IL7R*, *JAK1*, *JAK2*, *JAK3*, and *TYK2* have been undertaken,[11-18] but the potential roles of negative regulators of signal transduction, including SOCS, remain largely unexplored in the pathogenesis of T-ALL.

The SOCS family of cytokine-inducible negative regulators of JAK-STAT and other signaling pathways includes 8 structurally related family members, SOCS1-7 and CIS, all of which contain a central Src-homology 2 domain and a conserved C-terminal domain termed the SOCS box.[19,20] There is growing evidence implicating SOCS family members in a range of inflammatory diseases and tumors, including hepatocellular carcinoma, colorectal, cervical, and breast cancer.[20-23] Downregulation of *SOCS* genes was reported in solid tumors with an unfavorable prognosis and hematological malignancies, including AML, and myeloproliferative disorders.[21,22,24-27]

SOCS5 is expressed in a variety of adult tissues, particularly in primary B and T cells located in the spleen, lymph nodes, thymus, and bone marrow.[20,28] Consistent with its expression in lymphoid organs, *SOCS5* has been implicated in Th cell differentiation, particularly in the balance between Th1 and Th2 cells, with *SOCS5* preferentially expressed in Th1 cells.[28,29] Growing evidence suggests *SOCS5* is tumor suppressor gene, negatively regulating the epidermal growth factor receptor and JAK-STAT signaling pathways.[24,30-32] However, little is currently known about the mechanisms by which *SOCS5* regulates signal transduction in leukemic cells.

Given the roles of *SOCS5* in normal T cell development, we hypothesized that SOCS5 is a critical mediator of JAK-STAT signaling and T-ALL progression. Here, we report that *SOCS5* is epigenetically regulated by DNA methylation and histone deacetylation. We provide evidence that *SOCS5* negatively regulates the activation of the JAK-STAT signaling pathway and cytokine receptors in T-ALL. We show that *SOCS5* silencing significantly increases T-ALL proliferation in vitro and leukemia engraftment in a murine model of human leukemia. In summary, we have identified a novel regulator underlying aberrant JAK-STAT activation in T-ALL.

2 | MATERIALS AND METHODS

2.1 | Reagents

All reagents were purchased from Thermo Fisher Scientific (Carlsbad, CA, USA) unless specified otherwise.

2.2 | Cells and patient samples

Human T-ALL cell lines (MOLT4, ALL-SIL, Jurkat, CCRF-CEM, KoptK1, and PF382) were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, and 100 U/mL penicillin G-streptomycin in a 5% CO_2 incubator at 37°C. The 293-FT and Phoenix cells were maintained following manufacturer instructions. Murine hematopoietic BaF3 cell line was cultured in RPMI-1640, 10% FBS, 10 ng/mL mouse IL-3 (PeproTech, Rocky Hill, NJ, USA), 2 mmol/L L-glutamine, and 100 U/mL penicillin G-streptomycin. Human bone marrow $CD34^+$ cells were purchased from Stemcell Technologies (Cambridge, MA, USA). Peripheral blood mononuclear cells were isolated from buffy coats of normal donors (United Blood Services, Albuquerque, NM, USA) by centrifugation in a Ficoll-Paque (GE Healthcare, Pittsburgh, PA, USA) density gradient. Normal T cells were extracted using a human Pan T-cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA). Cryopreserved primary samples were obtained from patients enrolled in Children's Oncology Group T-ALL study AALL0434.[33] All patients or their parent(s)/guardian(s) provided written, informed consent for future research in accordance with the Declaration of Helsinki and local institutional guidelines. The primary cells were cultured as described previously.[34]

2.3 | In vivo leukemia cell transplantation

NSG mice (8-10 weeks old) were obtained from the University of New Mexico Comprehensive Cancer Center Animal Models Shared

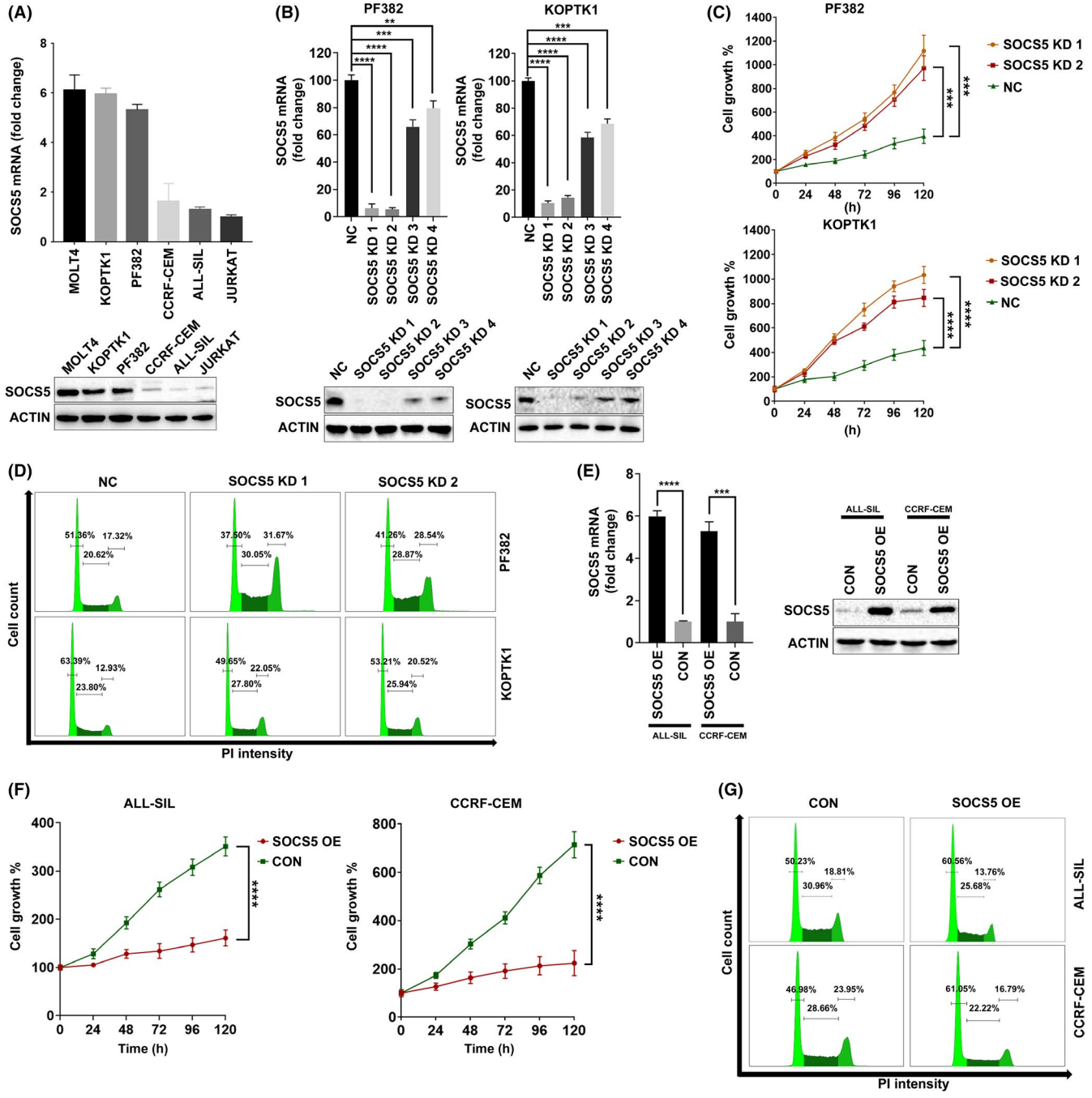


FIGURE 1 Suppressor of cytokine signaling 5 (*SOCS5*) negatively regulates T-cell lineage acute lymphoblastic leukemia (T-ALL) proliferation and cell cycle progression. A, *SOCS5* mRNA and protein levels in T-ALL cell lines (n = 6). For quantitative real-time (qRT)-PCR analyses, data are means ± SD for 3 independent experiments. B, PF382 and KOPTK1 cells were lentivirally transduced with 4 *SOCS5* shRNA (SOCS5 KD1, SOCS5 KD2, SOCS5 KD3, and SOCS5 KD4) and scrambled control (NC), and the decrease in *SOCS5* transcript and protein levels was confirmed by qRT-PCR and immunoblotting (**P < .005, *** P < .0005, **** P < .0001). E, Overexpression of *SOCS5* mRNA and protein in ALL-SIL and CCRF-CEM cells transduced with SOCS5 expressing plasmid (SOCS5 OE) compared to negative control plasmid (CON) was confirmed by qRT-PCR and immunoblotting (***P < .0005, **** P < .0001). C,F, Growth curves of the transduced T-ALL cell lines were determined by MTS assay. Data are means ± SD for 2 independent experiments carried out in triplicate (repeated measure ANOVA with Tukey's multiple comparisons test (***P < .0005, **** P < .0001). D,G, Cell cycle distribution was carried out 48 h post transduction by propidium iodide staining followed by flow cytometry analyses. Data are representative for 1 of 3 independent experiments

Resource (Albuquerque, NM, USA) and housed in a specific pathogen-free, AAALAC-accredited facility as described previously.[35] Animals were injected through a tail vein with 1 × 10^6 cells per mouse (T-ALL cells transduced with SOCS5-shRNA or with negative control scrambled shRNA). For survival experiments, animals (10 mice per group) were immediately killed when they showed signs

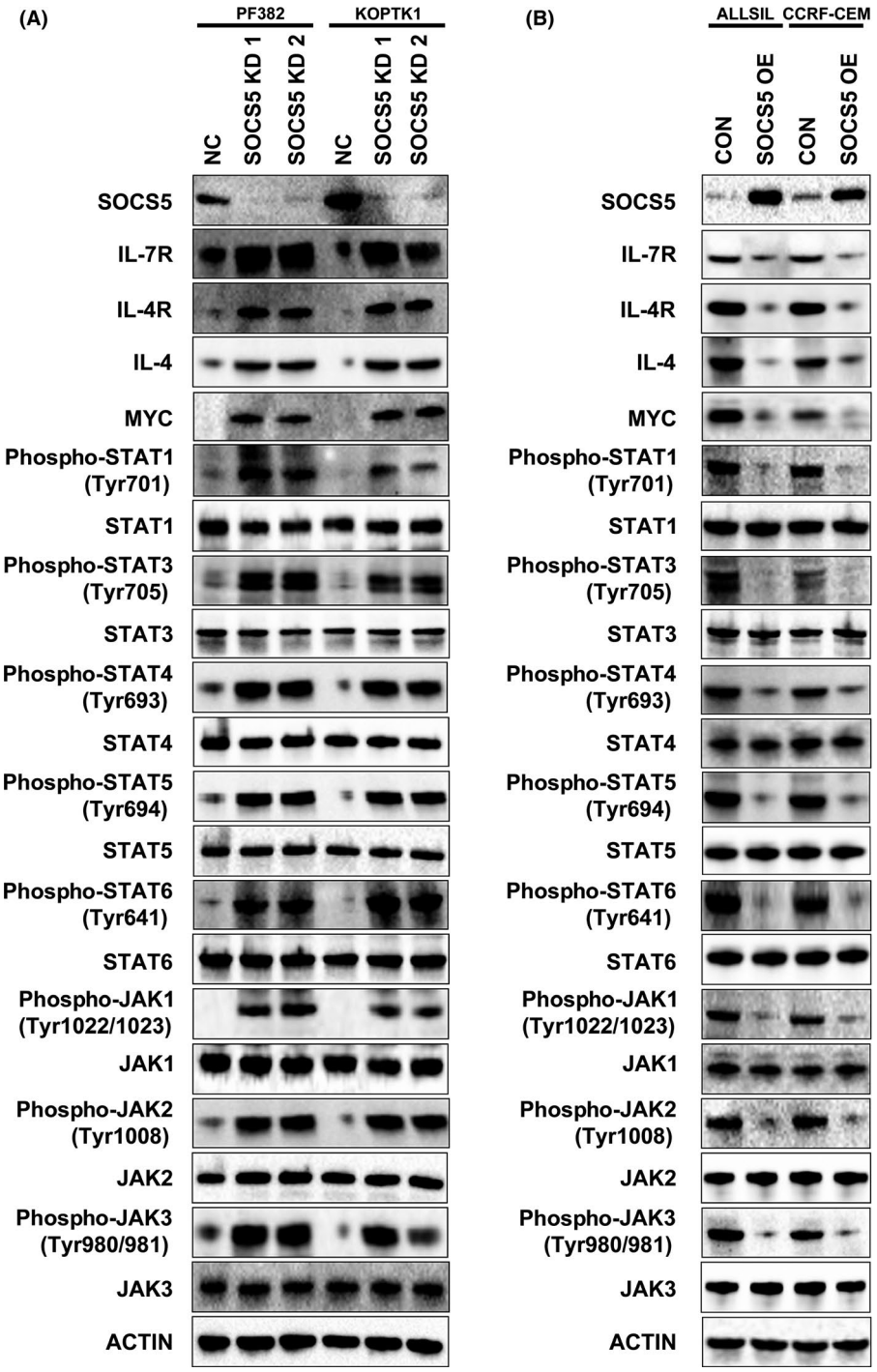


FIGURE 2 Suppressor of cytokine signaling 5 (SOCS5) negatively regulates cytokine receptors and the JAK-STAT signaling pathway in T-cell lineage acute lymphoblastic leukemia cell lines. A, PF382 and KOPTK1 cells were transduced with *SOCS5* shRNA (SOCS5 KD1 and SOCS5 KD2) and scrambled negative control (NC). B, ALL-SIL and CCRF-CEM cells were transduced with *SOCS5* expressing plasmid (SOCS5 OE) or control plasmid (CON). A,B, Cells were lysed and subjected to immunoblotting for the expression of interleukin-7 receptor (IL-7R), IL-4R, IL-4, and MYC, levels of phosphorylated and total STAT proteins, and activation and total levels of JAK. Western blots were undertaken 3 times and the representative blots are shown

of being moribund or had weight loss that exceeded 10%-15% of their total weight. For leukemia burden analyses (4 mice per group), all mice were killed 25 days post-engraftment. Leukemic cells were extracted from the bone marrow of femurs, spleen, liver, and brain by centrifugation in a Percoll (GE Healthcare) density gradient. The cells were stained with fluorescent labelled anti-human APC-CD45$^+$ and anti-mouse BV-421-CD45$^+$ Ab (BD Biosciences, San Jose, CA, USA) and analyzed by flow on the LSRFortessa flow cytometer. Kaluza Analysis Software (Indianapolis, IN, USA) was used for data analysis.

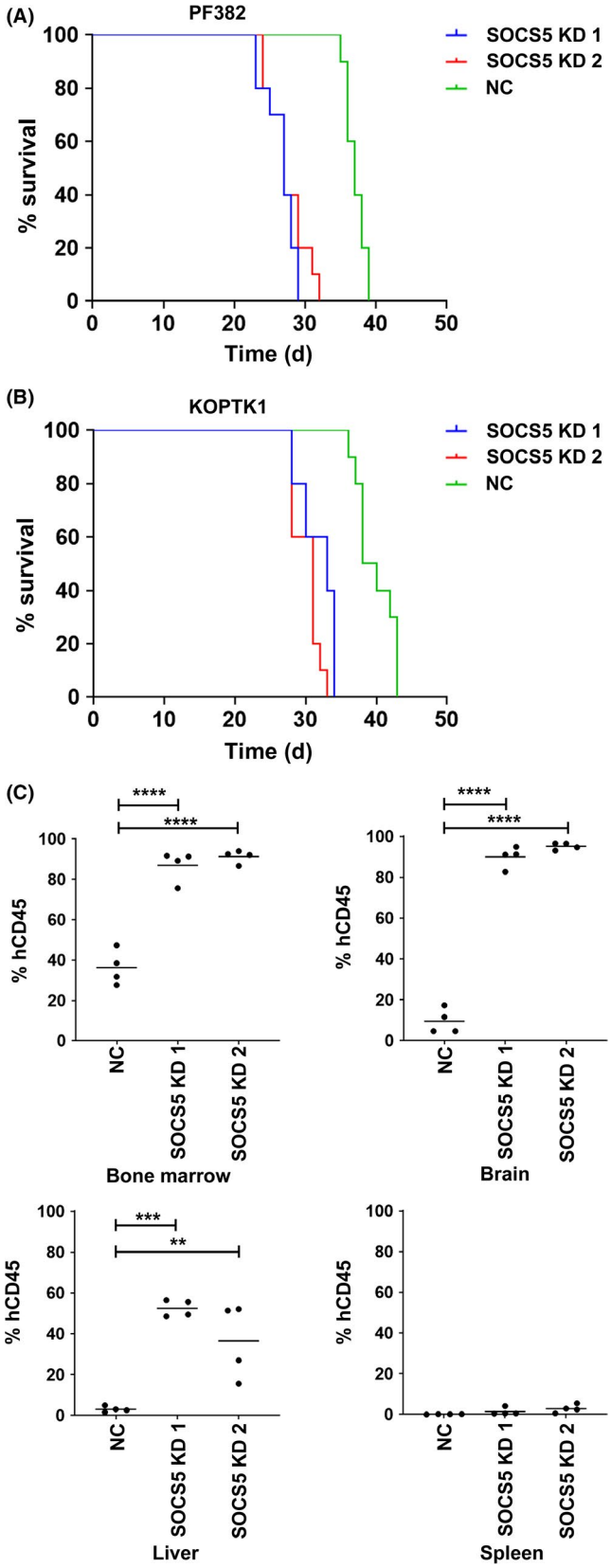


FIGURE 3 Silencing of suppressor of cytokine signaling 5 (*SOCS5*) accelerates leukemia progression in murine xenograft model of human T-cell lineage acute lymphoblastic leukemia. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice were engrafted with 1 × 10^6 cells (PF382 or KoptK1) transduced with 2 shRNAs targeting SOCS5 (SOCS5 KD1 and SOCS5 KD2) and scrambled control (NC), respectively. A,B, Kaplan-Meier plot of animal survival in each transplanted group (n = 10 mice per group). For both, PF382 SOCS5 KD1 and SOCS5 KD2 group median survival was 27 d compared to the NC group, in which median survival was 37 d (log-rank Mantel-Cox test: SOCS5 KD1 vs NC, $P < .0001$; SOCS5 KD2 vs NC, $P < .0001$). For KoptK1 SOCS5 KD1 and SOCS5 KD2 groups, median survival was 33 and 31 d, respectively, compared to the SOCS5 NC group with median survival of 40 d (log-rank Mantel-Cox test: SOCS5 KD1 vs NC, $P < .0001$; SOCS5 KD2 vs NC, $P < .0001$). C, For leukemia burden analyses, all mice (4 mice per group) were killed 25 d post-inoculation with transduced PF382 cells and the levels of human and murine CD45$^+$ cells were assessed by flow cytometry (one-way ANOVA with Dunnett's multiple comparison test: $^{**}P < .005$; $^{***}P < .0005$, $^{****}P < .0001$)

2.4 | Microarray and RNA sequencing datasets

All of the microarray datasets were from publicly available data resources, including GSE70536[33] and GSE13159.[36] RNA sequencing data was available through the TARGET website, https://ocg.cancer.gov/programs/target. Data were analyzed as described previously.[1,33,37,38]

2.5 | Methylation-specific PCR and bisulfite sequencing

Genomic DNA was subjected to bisulfite treatment using the DNA Methylation-Direct Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The bisulfite-treated DNA (150 ng) was amplified using 1× ZymoTaq PreMix and 10 μmol/L primers specific to the methylated or unmethylated DNA sequence. The PCR products were separated on a 2% agarose gel, stained with GelGreen Nucleic Acid Staining Solution (Biotium, Fremont, CA, USA) and visualized using the Bio-Rad ChemiDoc XRS equipped with Image Lab 5.0 software (Herkules, CA, USA). Primers and the detailed PCR conditions are summarized in Table S1. For sequencing, bisulfite-treated genomic DNA was amplified using primers listed in Table S2. The PCR products were cloned into the Topo TA cloning kit (Thermo Fisher Scientific). Ten randomly picked clones were sequenced (Eurofins Genomics, Luisville, KY, USA) and aligned using Quantification Tool for Methylation Analysis.[39]

2.6 | Chromatin immunoprecipitation

Chromatin immunoprecipitation was carried out on T-ALL cells as described.[40] DNA was immunoprecipitated with anti-HDAC1 (sc-7872X,) anti-HDAC2 (sc-7872X), anti-HDAC3 (sc-11417X), anti-MeCP2 (sc-137070), nonspecific IgG Abs (Santa Cruz Biotechnology, Dallas, TX, USA) or MBD3 (D1B8F) (Cell Signaling Technology, Danvers, MA, USA) and amplified by qRT-PCR (Table S3) using SYBR Green PCR Master Mix on a StepOnePlus Real-Time PCR System under standard conditions. Results were quantified by SYBR Green Real-Time PCR analysis. The fold enrichment of immunoprecipitated samples was normalized on INPUT and expressed relative to the mock-treated control (IgG). Results were visualized after separating PCR products on 3% agarose gel stained with GelGreen Nucleic Acid Staining.

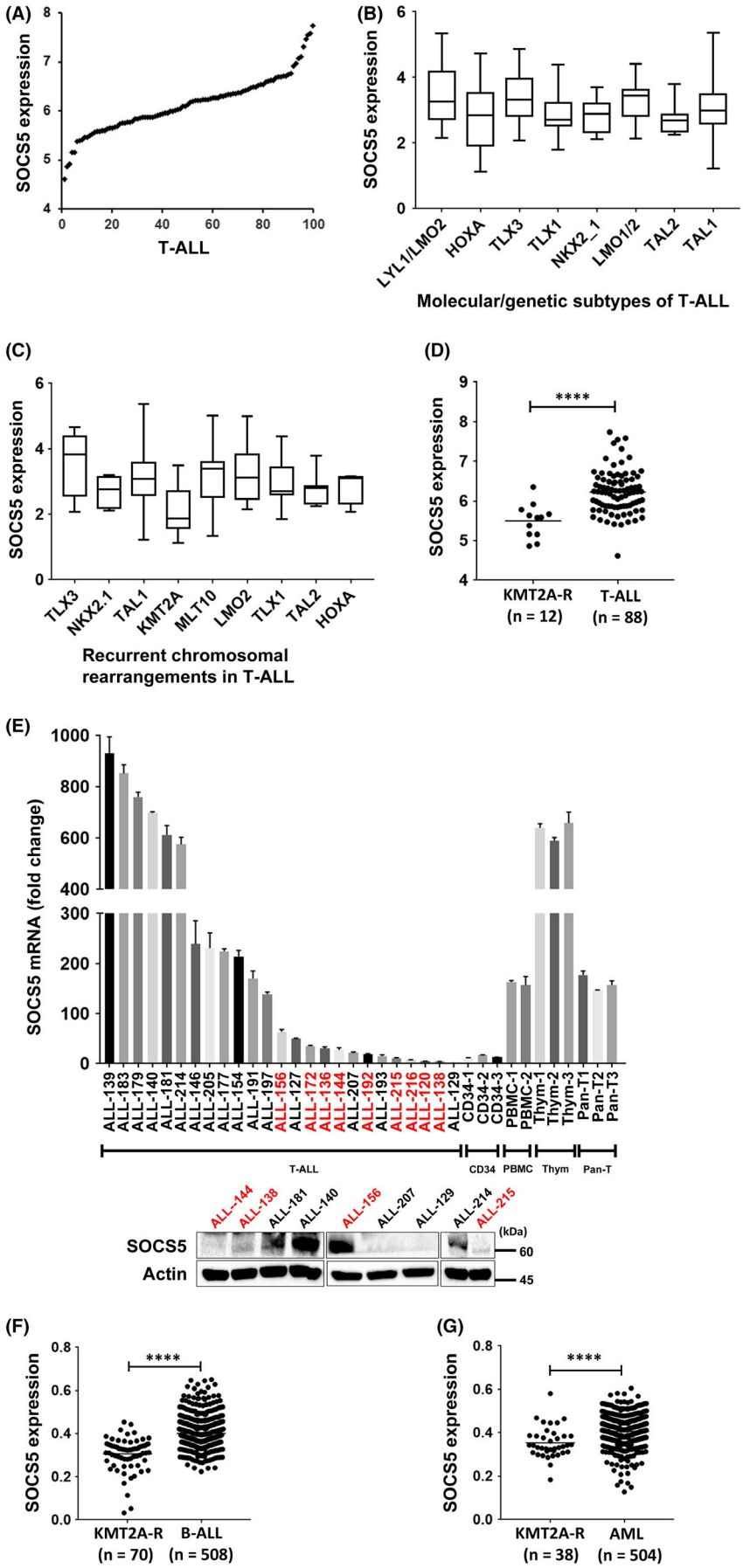

(A)
SOCS5 expression
T-ALL
(B)
SOCS5 expression
LYL1/LMO2
HOXA
TLX3
TLX1
NKX2_1
LMO1/2
TAL2
TAL1
Molecular/genetic subtypes of T-ALL
(C)
SOCS5 expression
TLX3
NKX2.1
TAL1
KMT2A
MLT10
LMO2
TLX1
TAL2
HOXA
Recurrent chromosomal rearrangements in T-ALL
(D)
SOCS5 expression
****
KMT2A-R (n = 12)
T-ALL (n = 88)
(E)
SOCS5 mRNA (fold change)
T-ALL
CD34
PBMC
Thym
Pan-T
SOCS5
Actin
(kDa)
60
45
(F)
SOCS5 expression
****
KMT2A-R (n = 70)
B-ALL (n = 508)
(G)
SOCS5 expression
****
KMT2A-R (n = 38)
AML (n = 504)

FIGURE 4 Suppressor of cytokine signaling 5 (*SOCS5*) expression is lowered in acute leukemias with *KMT2A* gene rearrangements. A, Microarray data analysis of *SOCS5* expression in pediatric and young adult T-cell lineage acute lymphoblastic leukemia (T-ALL) patients (n = 100) treated in the COG AALL0434 study (GSE70636).[33] Multiple probe sets were tested. The representative probe set for *SOCS5* is shown (209647_s_at). B, *SOCS5* expression across distinct molecular/genetic subtypes of T-ALL identified in a cohort of 264 pediatric and young adult T-ALL patients by RNA sequencing (Kruskal-Wallis with Dunn's multiple comparison test, $P < .005$).[1] C, *SOCS5* expression in 173 T-ALL cases classified by recurrent chromosomal rearrangements (*TLX3*, n = 14; *NKX2.1*, n = 10; *TAL1*, n = 77; *KMT2A*, n = 12; *MLLT10*, n = 12; *LMO2*, n = 10; *TLX1*, n = 17; *TAL2*, n = 7; *HOXA*, n = 4) (Kruskal-Wallis with Dunn's multiple comparison test, $P < .005$).[1] D, SOCS5 expression in T-ALL with *KMT2A* gene rearrangements (*KMT2A*-R) in an independent published cohort of 100 T-ALL cases tested by microarray (unpaired Mann-Whitney *U* test, **** $P < .0001$).[33] E, *SOCS5* mRNA levels in primary T-ALL samples (n = 25), normal bone marrow $CD34^+$ cells (n = 3), normal PBMC (n = 2), pan T-cells (Pan-T) (n = 3), and normal thymocytes (Thym, n = 3) by quantitative real-time PCR. Immunoblotting for SOCS5 protein levels in primary T-ALL samples (n = 9), for which cellular material was available. Red text indicates the samples harboring *KMT2A*-R. F,G, Analysis of *SOCS5* mRNA levels in B-cell (B-)ALL and AML with *KMT2A*-R compared to the remaining cases in a previously reported microarray dataset[36] (unpaired Mann-Whitney *U* test, ****$P < .0001$)

2.7 | Statistical analysis

The statistical analyses were undertaken using GraphPad Prism 7.02 software (GraphPad, La Jolla, CA, USA). The results were considered statistically significant when $P < .05$. Additional supplementary methods appear in Data S1.

3 | RESULTS

3.1 | Inactivation of *SOCS5* promotes T-ALL cell proliferation

We first analyzed a panel of 6 T-ALL cell lines and found that the *SOCS5* gene and protein were differentially expressed (Figure 1A). To investigate the biological roles of *SOCS5* in T-ALL, we undertook shRNA-mediated knockdown of *SOCS5* in PF382 and KOPTK1 cells that had higher levels of SOCS5 mRNA and protein. Depletion of SOCS5 mRNA and protein levels was confirmed by qRT-PCR and immunoblotting, respectively (Figure 1B). Downregulation of *SOCS5* expression promoted proliferation of T-ALL cells as shown by an increase in cell number, increased cell cycle progression in S and G_2/M phases, and decreased G_1 phase (Figure 1C,D). We next investigated the effects of lentivirus-induced *SOCS5* expression (Figure 1E) on the proliferation of ALL-SIL and CCRF-CEM cells. Forced *SOCS5* expression suppressed T-ALL cell growth by inhibiting cell proliferation and reducing the cell cycle in S and G_2/M phases, and increased G_1 phase (Figure 1F,G). Interestingly, the knockdown and overexpression of *SOCS5* had no effect on apoptotic cell death (Figure S1). These results indicate that *SOCS5* negatively regulates proliferation of T-ALL cells in vitro.

3.2 | *SOCS5* negatively regulates *MYC* and cytokine receptor expression in T-ALL

With evidence that *SOCS5* regulates T-ALL proliferation, we studied the mechanism by which *SOCS5* controls T-ALL cell growth and signal transduction. We first examined the expression of IL-7R and IL-4R, which are involved in T-ALL progression. Genetic inactivation of *SOCS5* upregulated IL-4R and IL-7R levels in the tested cells (Figure 2A). In a converse experiment, overexpression of SOCS5 led to receptor downregulation, indicating that *SOCS5* negatively regulates the expression of IL-4R and IL-7R in T-ALL (Figure 2B). We also assessed whether the depletion of *SOCS5* affects the expression of IL-4. *SOCS5* silencing increased the levels of IL-4, whereas *SOCS5* upregulation reduced its expression (Figure 2). Thus, downregulation of *SOCS5* promotes the expression of critical T-ALL cytokine receptors to enhance T-ALL proliferation. Because *MYC* is a transcriptional target of JAK-STAT signaling, we examined whether *SOCS5* affects MYC expression. *SOCS5* silencing induced MYC in the tested cells (Figure 2A). Conversely, forced *SOCS5* expression reduced MYC protein levels, indicating that *SOCS5* could have putative tumor suppressor activity in T-ALL (Figure 2B).

3.3 | *SOCS5* downregulation induces activation of the JAK-STAT signaling pathway

To test whether *SOCS5* negatively regulates signal transduction in T-ALL, we examined the JAK-STAT pathway activation in *SOCS5*-depleted cells compared to the negative control. Silencing of *SOCS5* expression markedly enhanced the phosphorylation of STAT1, STAT3, STAT4, STAT5, and STAT6 but had no effect on the total STAT levels (Figure 2A). Accordingly, forced *SOCS5* expression reduced the activation of the tested STAT proteins, consistent with no changes in their total levels (Figure 2B). We next investigated whether *SOCS5* preferentially inhibits JAK1, JAK2, JAK3, and TYK2 in T-ALL cells. *SOCS5* depletion accelerated JAK1, JAK2, and JAK3 phosphorylation but had modest to no effect on TYK2 (Figures 2A and S2). Conversely, overexpressed SOCS5 inhibited activation of all JAK proteins except for TYK2 in the tested cells (Figures 2B and S2). As expected, *SOCS5* had no effect on total JAK protein levels. Thus, *SOCS5* negatively regulates JAK-STAT signaling in T-ALL.

3.4 | *SOCS5* negatively regulates T-ALL progression in vivo

Because *SOCS5* regulates T-ALL cell growth in vitro, we next investigated the effects of *SOCS5* expression on T-ALL progression in NSG mice injected with SOCS5-depleted or control cell lines. *SOCS5* depletion significantly reduced survival of the engrafted NSG mice (SOCS5 KD1, n = 10; SOCS5 KD2, n = 10) when compared to the

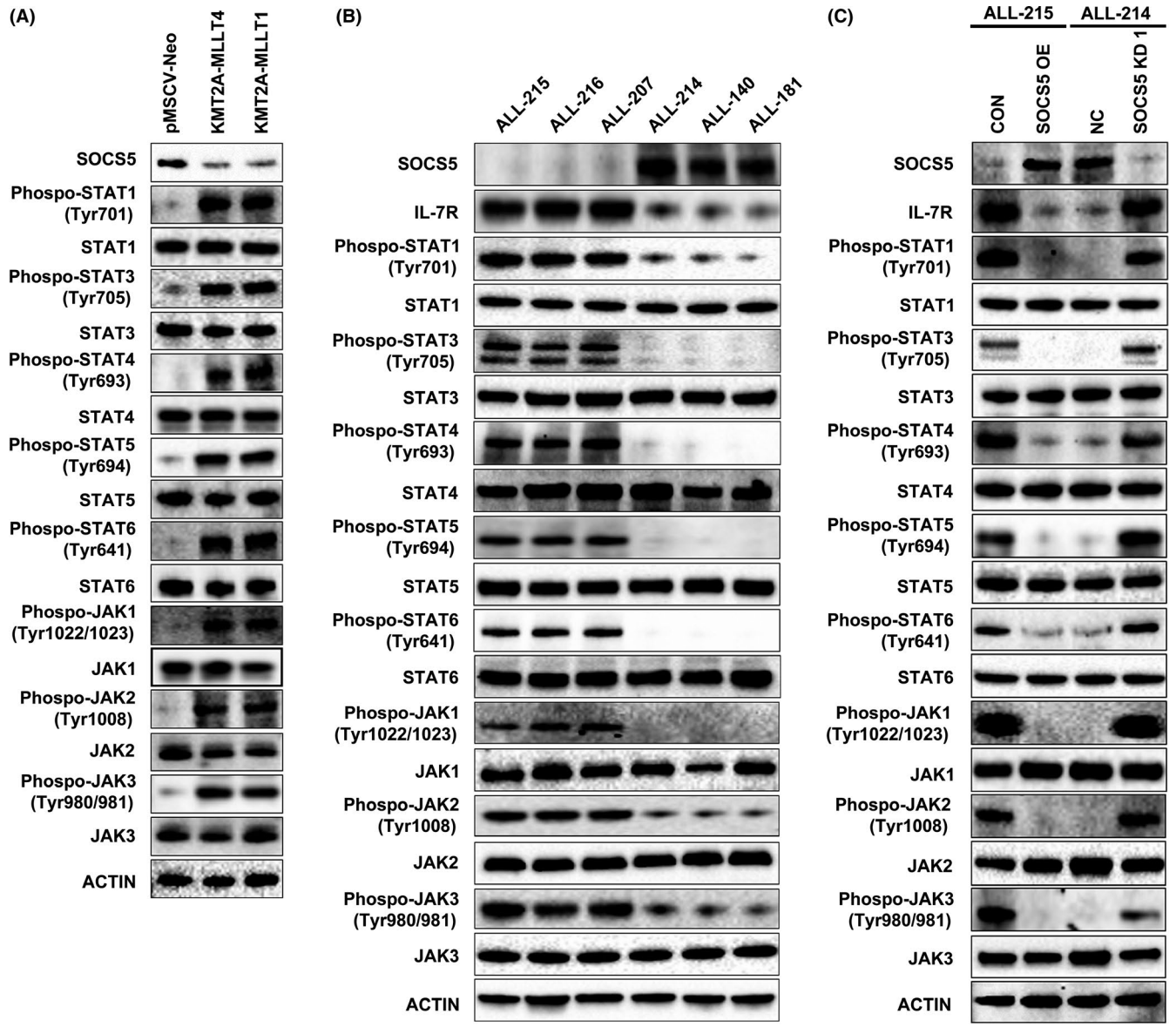


FIGURE 5 Suppressor of cytokine signaling 5 (SOCS5) negatively regulates interleukin-7 receptor (IL7R) and JAK-STAT signaling in BaF3 cells expressing KMT2A-R and primary T-cell lineage acute lymphoblastic leukemia (T-ALL) cells. A, Immunoblotting of BaF3 cells transduced with *KMT2A-MLLT1* or *KMT2A-MLLT4* and negative control (pMSCV-Neo) for SOCS5 and activated or total levels of JAK-STAT proteins. B, Immunoblot analyses for IL-7R and JAK-STAT activation in primary T-ALL, including 3 samples with higher and 3 samples with low/undetectable SOCS5 protein levels. C, Primary T-ALL cells harboring *KMT2A* gene rearrangements (ALL-215) were transduced with *SOCS5*-expressing plasmid (SOCS5 OE) or negative control plasmid (CON). Primary T-ALL sample (ALL-214) was transduced with *SOCS5* shRNA (SOCS5 KD1) only (due to insufficient cellular material) and scrambled negative control (NC). The cell lysates were immunoblotted for the expression of SOCS5 and IL-7R and the levels of phosphorylated and total JAK-STAT proteins. Representative blots are shown

control group (NC, n = 10) (Figure 3A,B). Moreover, tissue analyses from mice killed 25 days post-injection revealed profound evidence of leukemia progression in mice injected with the *SOCS5*-depleted cells (SOCS5 KD1, n = 4; SOCS5 KD2, n = 4) compared to the control group (NC, n = 4). *SOCS5* silencing significantly increased leukemia burden in bone marrow, brain, and liver of the tested mice (Figure 3C). PF382 cells did not infiltrate the spleen in all experimental groups, which is consistent with previously published data for PF382 cells.[41] These results show that *SOCS5* downregulation promotes T-ALL cell proliferation in vivo, suggesting its suppressive role in leukemia engraftment and progression.

3.5 | *SOCS5* expression is reduced in leukemic patients harboring *KMT2A* gene rearrangements

To determine *SOCS5* expression in primary T-ALL, we assessed microarray data for 100 T-ALL samples obtained from children, adolescents, and young adults enrolled in COG AALL0434 (GSE70536),[33]

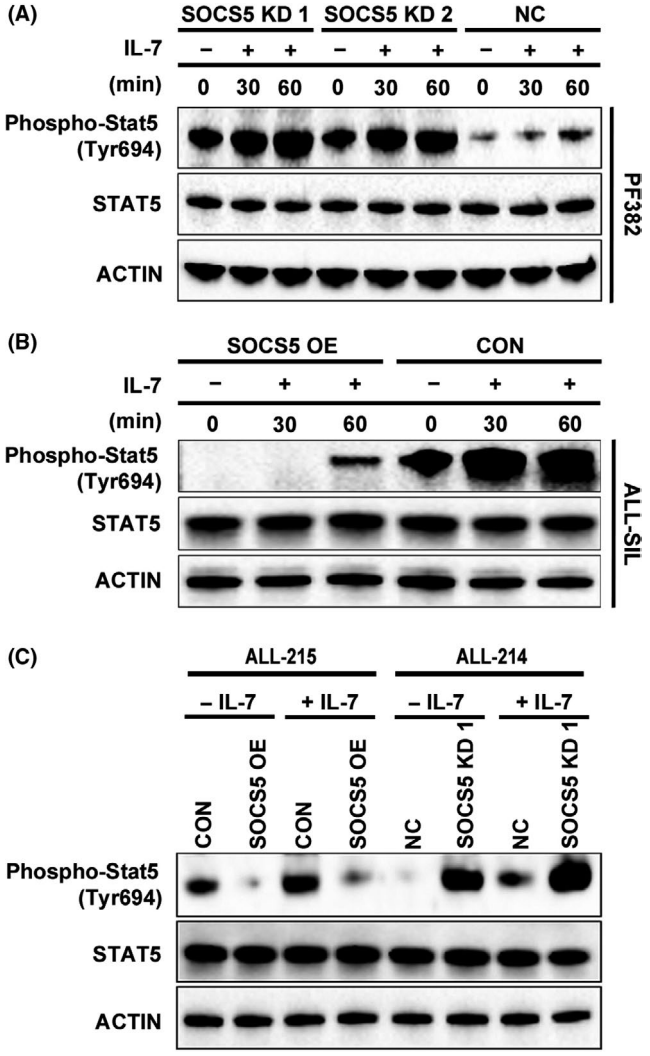


FIGURE 6 Suppressor of cytokine signaling 5 (SOCS5) downregulation enhances interleukin-7 (IL-7)-induced STAT5 activation. PF382 cell line and primary T-cell lineage acute lymphoblastic leukemia T-ALL (ALL-214) cells were transduced with SOCS5 shRNA (SOCS5 KD1 and SOCS5 KD2) and scrambled control (NC). ALL-SIL cell line and primary T-ALL cells (ALL-215) were transduced with *SOCS5*-expressing plasmid (SOCS5 OE) or negative control plasmid (CON). The transduced cell lines (A,B) and primary T-ALL samples (C) (ALL-215 and ALL-214) were serum and cytokine starved (120 min) followed by stimulation with IL-7 (25 ng/mL). The cell lysates were tested for the levels of phosphorylated and total STAT5. Representative blots are shown

and identified different levels of SOCS5 mRNA expression in the tested dataset (Figure 4A). To further identify molecular characteristics associated with distinct *SOCS5* expression levels, we analyzed genomic data available for 264 T-ALL patients reported by Liu et al.[1] *SOCS5* was differentially expressed between the molecular subtypes of T-ALL ($P < .005$) including *HOXA*- and *TLX1*-deregulated cases with the lowest, and *TLX3*-deregulated cases with the highest levels of *SOCS5* mRNA, respectively (Figure 4B). While *SOCS5* mRNA levels were higher in immature *LYL1/LMO2*-deregulated leukemia, there was no evidence of an association between *SOCS5* expression with early T-cell phenotype or differentiation arrest at distinct stages of T cell development (data not shown). Because *HOXA*-deregulated T-ALL is enriched in alterations involving *KMT2A* or *MLLT10* genes,[33,42,43] we sought to determine whether *SOCS5* mRNA levels were associated with recurrent chromosomal alterations found in T-ALL. We found that *SOCS5* expression was downregulated in T-ALL patients harboring *KMT2A* gene rearrangements (*KMT2A*-R, $P < .005$; Figure 4C). The results were consistent with our microarray dataset of 100 T-ALLs, in which *KMT2A*-R samples (n = 12) had lower levels of *SOCS5* mRNA compared to the remaining samples (Figure 4D). To further validate our findings, we tested a limited number of primary T-ALL samples (n = 25; Table S4) and confirmed that both *SOCS5* mRNA and protein are differentially expressed in T-ALL, and that samples harboring *KMT2A*-R have lower levels of *SOCS5* expression (Figure 4E). *SOCS5* mRNA was robustly expressed in PBMC, pan T-cells, and thymocytes from healthy individuals, but its levels were lower in bone marrow $CD34^+$ cells (Figure 4E). These results show that SOCS5 is deregulated in T-ALL. Interestingly, *SOCS5* downregulation was also identified in B-ALL and AML primary samples having *KMT2A*-R (Figure 4F,G), indicating that *SOCS5* inactivation could represent a feature of deregulated signaling networks in *KMT2A*-R leukemias. In order to test for the effects of *KMT2A* rearrangements on SOCS5 expression, BaF3 cells were transduced with *KMT2A-MLLT4* and *KMT2A-MLLT1* constructs, representing the most prevalent *KMT2A*-R in T-ALL (Figure S3, Table S5).[1,33,44] Forced expression of *KMT2A-MLLT4* and *KMT2A-MLLT1* resulted in the decrease in SOCS5 protein levels and the increased phosphorylation of JAK-STAT proteins in BaF3 cells (Figure 5A). We next examined IL-7R expression levels and JAK-STAT pathway activation in a small set of primary T-ALL samples with higher (n = 3) and lower/undetectable (n = 3) SOCS5 protein levels. We identified elevated expression of IL-7R and increased activation of JAK-STAT proteins in primary T-ALL samples with lower/undetectable SOCS5 levels (both ALL-215 and ALL-216 harbor *KMT2A*-R) compared to samples that had higher levels of SOCS5 protein (Figure 5B). We next used lentiviral transduction to induce or silence *SOCS5* expression in 2 primary T-ALL samples, ALL-214 and ALL-215 (Table S4). Forced expression of *SOCS5* in ALL-215 primary cells lowered the expression of IL-7R and decreased the activation of STAT1, STAT3, STAT4, STAT5, and STAT6 as well as JAK1, JAK2, and JAK3 proteins (Figure 5C). Conversely, genetic silencing of *SOCS5* in ALL-214 cells increased the expression of IL-7R and the activation of the JAK-STAT signaling pathway (Figure 5C). Together, these results indicate that SOCS5 downregulation is associated with *KMT2A* gene rearrangements and its lower levels enhance JAK-STAT and IL-7R signaling in T-ALL.

3.6 | *SOCS5* downregulation potentiates IL-7-induced STAT5 activation

To determine whether SOCS5 inactivation reinforces the JAK-STAT signaling in response to IL-7 stimulation, we utilized primary T-ALL cells (ALL-214 and ALL-215) and cell lines (PF382 and ALL-SIL), which were stimulated with IL-7 (25 ng/mL; Figure 6). Under basal conditions, SOCS5-expressing PF382 cells showed the increase in

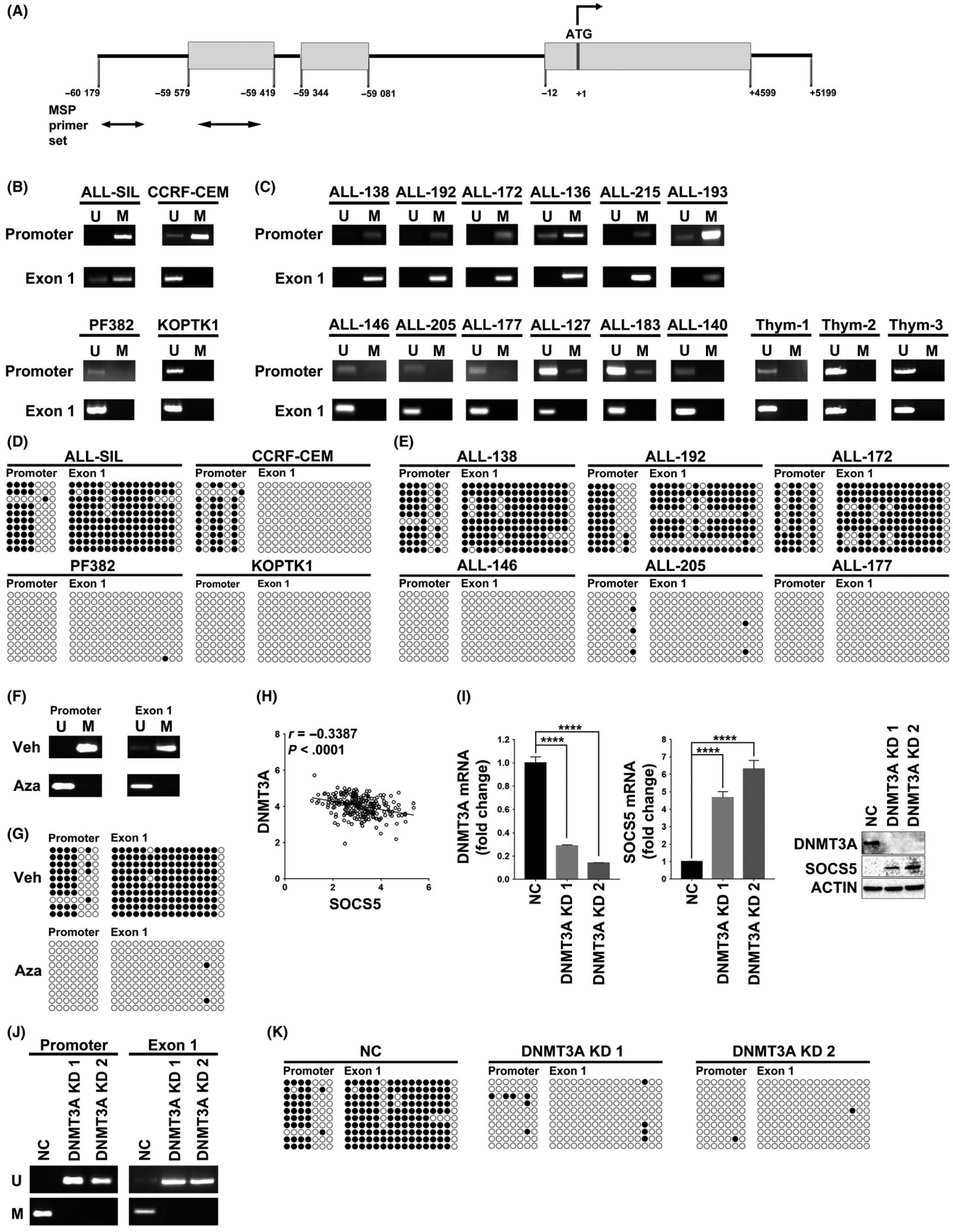

(A)
ATG
−60 179
−59 579
−59 419
−59 344
−59 081
−12
+1
+4599
+5199
MSP primer set
(B)
ALL-SIL
CCRF-CEM
PF382
KOPTK1
U M
Promoter
Exon 1
(C)
ALL-138
ALL-192
ALL-172
ALL-136
ALL-215
ALL-193
ALL-146
ALL-205
ALL-177
ALL-127
ALL-183
ALL-140
Thym-1
Thym-2
Thym-3
(D)
ALL-SIL
CCRF-CEM
PF382
KOPTK1
Promoter
Exon 1
(E)
ALL-138
ALL-192
ALL-172
ALL-146
ALL-205
ALL-177
(F)
Promoter
Exon 1
U M
Veh
Aza
(G)
Promoter
Exon 1
Veh
Aza
(H)
r = −0.3387
P < .0001
DNMT3A
SOCS5
(I)
DNMT3A mRNA (fold change)
SOCS5 mRNA (fold change)
****
NC
DNMT3A KD 1
DNMT3A KD 2
DNMT3A
SOCS5
ACTIN
(J)
Promoter
Exon 1
NC
DNMT3A KD 1
DNMT3A KD 2
U
M
(K)
NC
DNMT3A KD 1
DNMT3A KD 2
Promoter
Exon 1

FIGURE 7 DNA methylation regulates suppressor of cytokine signaling 5 (*SOCS5*) expression. A, Schematic diagram of the *SOCS5* gene. Gray boxes represent exons; the translation start site is at +1, and the arrows indicate direction of translation. The bottom arrows indicate primers used for methylation specific PCR (MS-PCR). DNA methylation of *SOCS5* promoter/1st exon in (B) T-cell lineage acute lymphoblastic leukemia (T-ALL) cell lines (n = 4) and (C) primary T-ALL samples (n = 12) and normal thymocytes (n = 3) were tested by methylation specific (MS)-PCR. M, methylated; U, unmethylated. D,E, Bisulfite sequencing of *SOCS5* promoter/1st exon region in (D) T-ALL cell lines (n = 4) and (E) primary T-ALL samples (n = 6) for which sufficient DNA was available. Unmethylated CpG site in the amplified region is shown as an open, white circle and methylated CpG as a closed, black circle. F,G, DNA methylation (F) and bisulfite sequencing (G) of *SOCS5* promoter/1st exon in ALL-SIL cells treated with demethylating agent 5-azacitidine (Aza) (24 h, 10 μmol/L) or vehicle control (Veh). H, Expression correlation between *SOCS5* and DNA methyltransferase-3A (*DNMT3A*) in a previously published RNASeq dataset for 264 T-ALL patients from the COG study (NCT00408005)[1] (*r*, Pearson correlation; $P < .0001$). I, ALL-SIL cells were infected with scrambled control (NC) or lentivirus expressing shRNA targeting *DNMT3A* (DNMT3A KD1 and DNMT3A KD2). Knockdown of *DNMT3A* and *SOCS5* gene expression was examined by quantitative real-time PCR and immunoblotting. SOCS5 in negative control cells (NC) was normalized to 1. Data are means ± SD for 3 independent experiments (****$P < .0001$; two-tailed Student's test). J,K, DNA methylation and bisulfite sequencing analyses of the SOCS5 promoter/1st exon in DNMT3A-depleted ALL-SIL cells compared to NC

STAT5 phosphorylation after 60 minutes of cytokine stimulation (Figure 6A). Interestingly, genetic inactivation of *SOCS5* led to increased STAT5 activation in serum and cytokine-free environment compared to negative control cells, and increased reactivity of the tested cells to cytokine stimulation. In a converse experiment, low SOCS5 expressing ALL-SIL cells were hypersensitive to IL-7 stimulation, leading to high levels of phosphorylated STAT5. The activation of STAT5 was significantly reduced in the cells with forced SOCS5 expression (Figure 6B). To further validate our findings, similar experiments were carried out in primary T-ALL samples. ALL-215 cells harboring *KMT2A*-R had higher basal levels of STAT5 phosphorylation and showed enhanced STAT5 activation following IL-7 stimulation compared to T-ALL cells obtained from an ALL-214 sample (Figure 6C). Forced *SOCS5* expression inhibited STAT5 activation in primary ALL-215 cells. In contrast, SOCS5 depletion in ALL-214 cells led to a significant increase in STAT5 activation. Together, these results indicate that SOCS5 silencing leads to hyperactivation of IL-7-induced STAT5 signaling.

3.7 | *SOCS5* gene is differentially methylated in T-ALL

Because we did not identify any mutations in the *SOCS5* gene in primary T-ALL samples (Figure 4E; not shown), we hypothesized that *SOCS5* expression is regulated by DNA methylation in T-ALL. We analyzed the genomic sequence of *SOCS5* (Figure 7A) spanning from −60 179 to −59 344 bps and identified densely clustered CpG islands around the promoter and the 1st exon. We performed a methylation-specific PCR using unmethylated or methylated DNA specific primers (Table S1, Figure S4) to investigate the methylation of the *SOCS5* promoter (−60 162 to −59 938 bps) and 1st exon (−59 594 to −59 469 bps) regions in T-ALL cell lines (n = 4), primary T-ALL samples (n = 12; Table S4), and normal thymocytes (n = 3). *SOCS5* promoter/1st exon were hypermethylated in the cell lines showing lower *SOCS5* expression levels (ALL-SIL and CCRF-CEM) compared to PF382 and KOPTK1 that had higher levels of *SOCS5* mRNA, in which the tested regions were hypomethylated (Figures 1A and 7B). Primary T-ALL samples with lower levels of *SOCS5* expression showed methylation in promoter and/or 1st exon regions (Figures 4E and 7C). By contrast, T-ALL samples expressing higher levels of *SOCS5* mRNA showed absent or partial methylation in the tested regions (Figures 4E and 7C). As expected, normal thymocytes had unmethylated promoter/1st exon regions of the *SOCS5* gene (Figures 4E and 7C). To validate our findings, we undertook bisulfite sequencing of the promoter (−60 098 to −59 934 bps) and 1st exon (−59 598 to −59 422 bps) regions of *SOCS5*. We confirmed the presence of methylated CpG sites (Figure 7D, filled circles) in ALL-SIL and CCRF-CEM cells (lower *SOCS5* expression) and unmethylated CpG sites (Figure 7D, open circles) in PF383 and KOPTK1 cell lines (higher *SOCS5* expression). Bisulfite sequencing of 6 primary T-ALL samples for which sufficient DNA was available confirmed CpG island methylation in samples with lower levels of *SOCS5* expression (ALL-138, ALL-192, and ALL-172) compared to unmethylated CpG islands in ALL-146, ALL-205, and ALL-177, in which *SOCS5* was robustly expressed (Figures 4E and 7E). In addition, treatment of T-ALL cells with the DNA demethylating agent 5-AzaC led to a decrease in DNA methylation of the *SOCS5* promoter/1st exon region and increased expression of *SOCS5* mRNA and protein levels (Figures 7F,G and S5). To determine whether *SOCS5* is a target for DNA methyltransferase, we analyzed a previously published RNASeq dataset for 264 T-ALL.[1] Among all DNMTs, only *DNMT3A* expression was inversely correlated with *SOCS5* mRNA levels in primary T-ALL samples (Figure 7H). The role of DNA methylation in regulation of *SOCS5* expression was further examined by shRNA-mediated knockdown of *DNMT3A* methyltransferase in ALL-SIL cells. *DNMT3A* silencing led to increased expression of *SOCS5* mRNA and protein levels, corresponding to decreased methylation in promoter and/or 1st exon regions of the *SOCS5* gene (Figure 7I-K). Together, these results indicate that DNA methylation regulates *SOCS5* expression in T-ALL.

3.8 | Histone deacetylation regulates *SOCS5* expression

Methylated DNA can be bound by MBDs, which recruit multiprotein co-repressor complexes carrying HDACs to facilitate transcriptional repression.[45-47] We first tested whether histone deacetylation contributes to *SOCS5* silencing by treating T-ALL cell lines with TSA, a potent inhibitor of class I and II HDACs, and found that TSA

treatment induced *SOCS5* mRNA and protein expression (Figure S6). To identify specific HDACs involved in the epigenetic regulation of *SOCS5* expression, we carried out ChIP using Abs directed against HDAC1, HDAC2, and HDAC3 followed by qRT-PCR analyses. HDAC1 and HDAC2, but not HDAC3, occupied CpG islands at promoter/1st exon regions of the *SOCS5* gene in ALL-SIL cells and primary T-ALL samples (n = 2; ALL-215 and ALL-216) (Figure 8A). Because HDACs lack DNA binding domains, specific MBDs are required to mediate deacetylation of histone tails. To confirm whether HDAC1 and HDAC2 are recruited to the *SOCS5* locus, we carried out ChIP against MeCP2, which is known to recruit SIN3 complex,[47,48] and against MBD3, which is a core subunit of the NuRD complex.[46] The SIN3 and NuRD co-repressor complexes are commonly associated with HDAC1 and HDAC2.[46,47] We found a significant enrichment of MeCP2 at the promoter and 1st exon region of *SOCS5* in the tested cells, suggesting that silencing of the *SOCS5* expression is regulated by the recruitment of the MeCP2 methyl binding protein and SIN3 co-repressor complex (Figure 8B). To further investigate whether MeCP2 binding depends on the *SOCS5* promoter/1st exon methylation, we treated ALL-SIL cells with 10 μmol/L demethylating agent, 5-AzaC. DNA demethylation abrogated MeCP2 binding to the *SOCS5* gene (Figure 8C). Thus, MeCP2 regulation of *SOCS5* expression is methylation-dependent. In addition, treatment with 5-AzaC induced acetylation of histone 3 tails at the *SOCS5* locus, which is indicative of chromatin decondensation (Figure 8D). Our findings show that histone deacetylation regulates *SOCS5* expression in T-ALL through the recruitment of the MeCP2-SIN3 co-repressor complex.

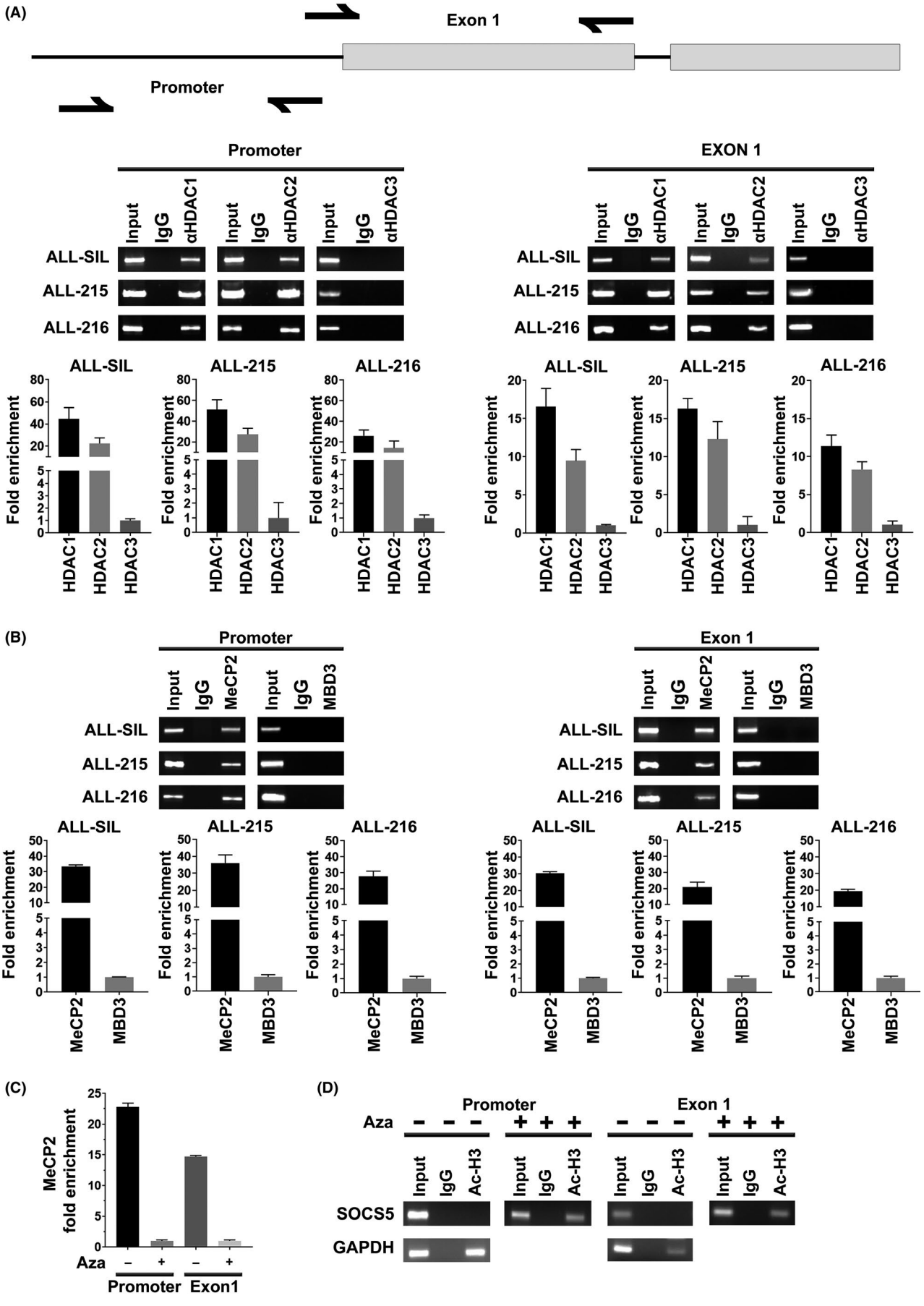
4 | DISCUSSION

A cytokine-inducible negative regulator of the JAK-STAT signaling pathway, SOCS5 is epigenetically deregulated in T-ALL, leading to a number of previously unappreciated effects on T-ALL cells. The downstream consequences include inhibition of cell proliferation and leukemia progression, negative regulation of cytokine receptor and JAK-STAT signaling.

Our analyses show that *SOCS5* is differentially expressed in primary T-ALL samples; however, we and others[1] did not identify mutations in the *SOCS5* gene that might explain its expression profiles. Although mutations in the *SOCS* family genes are very rare, aberrant *SOCS* signaling has been linked to the epigenetic deregulation of their expression.[22,23] For instance, reduced levels of *SOCS5* mRNA and its promoter hypermethylation were reported in hepatocellular carcinoma, cervical cancer, and thyroid tumors.[21,24] In this study, we dissected the mechanism of epigenetic deregulation of *SOCS5* expression in T-ALL through aberrant DNA methylation. We showed that *SOCS5* mRNA levels were closely associated with its promoter methylation. *DNMT3A* was identified to negatively regulate the *SOCS5* expression levels in T-ALL cells. Histone acetylation has also been implicated in the epigenetic regulation of the *SOCS* family genes in solid tumors.[21] In addition, early studies identified the CpG binding protein, MeCP2 as a transcriptional silencer.[49,50] Our ChIP analyses provide evidence that MeCP2 binds to the CpG islands of the *SOCS5* promoter/1st exon and that MeCP2 regulation of *SOCS5* expression is methylation-dependent. Furthermore, we identified the presence of HDAC1 and HDAC2 at the *SOCS5* promoter region, suggesting that MeCP2 recruits HDAC1/2 through SIN3 co-repressor complexes. Our results indicate that MeCP2 and histone deacetylation are mechanistically linked to the negative regulation of *SOCS5* expression in T-ALL. Other potential mechanisms of epigenetic deregulation of SOCS5 cannot be excluded and might be related to aberrant expression of microRNAs in T-ALL.[51]

There is a growing body of evidence indicating that genes involved in T cell differentiation play important roles in T-ALL pathobiology.[1,52] Our data suggest that *SOCS5* is differentially expressed among distinct molecular subtypes of T-ALL but we found no evidence for the association between *SOCS5* mRNA levels and specific stages of T-cell maturation arrest. Strikingly, we observed that *SOCS5* mRNA levels were lower in *HOXA*-and *TLX1*-deregulated cases but higher in the *TLX3*-deregulated subtype, which all represent the Homeobox family members. Moreover, *LMO2/LYL1*-deregulated samples, which represent the most immature T-ALL subsets, had elevated levels of *SOCS5* mRNA in contrast to normal $CD34^+$ progenitors, in which *SOCS5* expression levels were relatively lower. This interesting observation could be linked to aberrant JAK-STAT activation commonly found in ETP patients.[11,14] In our studies, *SOCS5* was not significantly elevated in ETP samples except for 1 dataset reported by Zhang et al[14] (Figure S7). Further studies are required to delineate the roles of ETP phenotype in modulating the activity of *SOCS5* in T-ALL. One of the intriguing observations was

FIGURE 8 Suppressor of cytokine signaling 5 (*SOCS5*) expression is regulated through histone deacetylation. A, Identification of specific histone deacetylases (HDAC) and methyl binding proteins at promoter/1st exon regions of the *SOCS5* gene. A schematic diagram of the *SOCS5* gene (upper) with half arrows indicating the primers used for ChIP analyses. ChIP (lower) with specific (A) α-HDAC1, α-HDAC2, α-HDAC3 and (B) α-methyl CpG binding protein-2 (α-MeCP2) and methyl-CpG-binding domain protein-3 (MBD3) Abs was carried out in ALL-SIL cells and 2 primary T-cell lineage acute lymphoblastic leukemia samples, ALL-215 and ALL-216. The immunoprecipitates were analyzed using quantitative real-time (qRT)-PCR and the amplification product was separated on 3% agarose gel. Data are means ± SD for 3 independent experiments. An IgG Ab was used as negative control. Signals are normalized to input DNA. C, The presence of MeCP2 at promoter/1st regions of *SOCS5* was tested in ALL-SIL cells untreated (–) or treated (+) with 10 μmol/L 5-azacitidine (Aza, 24 h). The immunoprecipitates were analyzed by qRT-PCR as described above. D, ChIP analyses for the acetylation status of histone H3 tails at *SOCS5* promoter/1st exon regions were done in ALL-SIL cells using α-acetylated H3 Ab. The cells were incubated in the absence (–) and in the presence (+) of 10 μmol/L Aza (24 h). Immunoprecipitates (Ac-H3) were subjected to qRT-PCR with primer pairs specific for *SOCS5* promoter/1st exon region and for *GAPDH*, as a positive control


(A)
Exon 1
Promoter
Promoter
EXON 1
Input
IgG
αHDAC1
αHDAC2
αHDAC3
ALL-SIL
ALL-215
ALL-216
Fold enrichment
HDAC1
HDAC2
HDAC3
(B)
Promoter
Exon 1
MeCP2
MBD3
(C)
MeCP2 fold enrichment
Aza
Promoter
Exon1
(D)
Promoter
Exon 1
Aza
Ac-H3
SOCS5
GAPDH

that *SOCS5* was downregulated in *KMT2A*-R T-ALL. In our study patients, ALL-144, ALL-138, and ALL-215 had *KMT2A*-R and almost undetectable SOCS5 protein levels (Figure 4E). We found that forced *KMT2A*-R expression lowered SOCS5 protein levels and led to increased activation of JAK-STAT signaling (Figure 5A). Our previous analyses on deregulated gene expression profiles in *KMT2A*-R T-ALL identified *SOCS5* within the most downregulated genes.[53] Whether *SOCS5* silencing is linked to *KMT2A*-R-driven oncogenesis in T-ALL and other acute leukemias remains unknown.

In line with previous studies, in which *SOCS5* was proposed as a putative tumor suppressor, our results show that forced *SOCS5* expression inhibited leukemic cell proliferation and cell cycle progression. Interestingly, apoptosis was not affected, suggesting that SOCS5 specifically regulates proliferation but not cell death in T-ALL. The results were consistent with our knockdown studies, in which *SOCS5* inactivation accelerated cell growth and cell cycle progression in T-ALL cells. *SOCS5* downregulation and its tumor suppressive role were reported in cervical and thyroid cancers.[21,30,31] However, in chronic lymphocytic leukemia patients, increased *SOCS5* expression governed the defective function of dendritic cells.[54] Moreover, elevated SOCS5 protein levels were associated with unfavorable prognosis in liver and ovarian cancer (www.proteinatlas.org), strongly suggesting that the roles exerted by *SOCS5* are likely tissue- and tumor-specific.

Interleukin-7 signaling is a critical determinant of normal T cell development and differentiation, and activating mutations in IL-7R have been shown to drive an oncogenic program in approximately 8%-10% T-ALL.[1,14,16,55-57] Here, we show for the first time that *SOCS5* negatively regulates the IL-7R signaling pathway in T-ALL cells. Because IL-7R is downregulated in the presence of IL-7 secreted in the bone marrow microenvironment,[55] it will be critical to further assess the roles of *SOCS5*-mediated signal transduction in IL-7 signaling. Our data provide evidence on the roles of SOCS5 downregulation in potentiating STAT5 activation in the presence of IL-7. In addition, IL-4 was also shown to stimulate T-ALL cell growth and proliferation.[58] In our study, *SOCS5* depletion upregulated both IL-4R and IL-4, raising an important question regarding the IL-4-dependent autocrine loop, which could self-induce T-ALL proliferation. These findings provide an important foundation for further research into the mechanistic link between *SOCS5* and cytokine receptor signaling in T-ALL as autocrine and/or paracrine effects were reported for IL-2 and IL-15 in adult T-cell leukemia.[59] In addition to IL-7R mutations, activating mutations in *JAK1*, *JAK2*, *JAK3*, and *STAT5B* were associated with aberrant JAK-STAT activation in T-ALL cells.[1,17,18] In this study, we showed that *SOCS5* negatively regulates the activation of STAT proteins and selectively regulates phosphorylation of JAK1, JAK2, and JAK3 but not TYK2. These results are of great interest considering that JAK-STAT activation was also reported in patients lacking mutations in IL-7R and JAK-STAT signaling molecules[11,56] suggesting that there are other mechanisms activating this pathway. Our data show that *SOCS5* downregulation potentiates JAK-STAT signal transduction and leukemia progression. Although we did not determine the mutational status of IL-7R and JAK-STAT molecules in the tested primary T-ALL samples (n = 24), our analyses of previously reported 264 T-ALL patients[1] show a lack of correlation between mutations in the JAK-STAT pathway and the levels of SOCS5 in T-ALL (Figure S8).

Finally, our work indicates that SOCS5 inactivation accelerated leukemia cell proliferation and engraftment in the T-ALL xenotransplantation model in vivo, emphasizing its critical role in T-ALL cell proliferation and leukemia progression. Strikingly, lower levels of SOCS5 facilitated extensive blast dissemination in bone marrow but also in brain and other organs, pointing towards proliferative and possibly migratory roles of *SOCS5* in T-ALL. Previous studies in solid tumors reported the roles of SOCS5 downregulation in promoting cell migration through epidermal growth factor receptor and JAK-STAT activation.[51]

In summary, we propose that epigenetic deregulation of SOCS5 expression impacts T-ALL cell proliferation and leukemic progression. We postulate that downregulation of *SOCS5* expression potentiates aberrant JAK-STAT signal transduction to govern T-ALL progression. Further studies are warranted to determine whether and how SOCS5 orchestrates with recurrent mutations in the IL-7R and JAK-STAT signaling pathway in T-ALL.

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DISCLOSURE

The authors have no conflicts to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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