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Gfi-1 is the transcriptional repressor of *SOCS1* in acute myeloid leukemia cells

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ABSTRACT

Silencing of *SOCS1*, a TSG, has been detected in various malignancies, including AML. However, the underlying mechanism of *SOCS1* inactivation remains elusive. In this study, we explored the role of histone methylation in *SOCS1* expression in AML cells. By ChIP assay, we demonstrated that G9a and SUV39H1, two enzymes catalyzing H3K9 methylation, were physically associated with the *SOCS1* promoter, and treatment with chaetocin, a histone methyltransferase inhibitor, suppressed H3K9 methylation on the *SOCS1* promoter and enhanced *SOCS1* expression. Furthermore, knockdown of *G9a* and *SUV39H1* by siRNA could also induce *SOCS1* expression. On the other hand, *SOCS*1 knockdown by shRNA eliminated chaetocin-induced cell apoptosis. To investigate further whether any transcription factor was involved in H3K9 methylation-related *SOCS1* repression, we scanned the sequences of the *SOCS1* gene promoter and found two binding sites for Gfi-1, a transcription repressor. By DNA pull-down and ChIP assays, we showed that Gfi-1 directly bound the *SOCS1* promoter, and ectopic Gfi-1 expression suppressed STAT5-induced *SOCS1* promoter activation. In contrast, *Gfi-1* knockdown by shRNA enhanced SOCS1 expression and inhibited STAT5 expression. Moreover, the knockdown of *G9a* completely rescued the repressive effect of Gfi-1 on STAT5A-induced *SOCS1* promoter activation. Collectively, our study indicates that the expression of Gfi-1 contributes to *SOCS1* silencing in AML cells through epigenetic modification, and suppression of histone methyltransferase can provide new insight in AML therapy. *J. Leukoc. Biol.* 95: 000–000; 2014.

Introduction

Epigenetic modification, including DNA methylation, histone acetylation, and methylation, modulates chromosome structures and gene transcription and plays an important role in control of cell proliferation and differentiation. Aberrant epigenetic modifications are found frequently in malignant diseases [1], implying that epigenetic deregulations are ideal targets for cancer therapies [2]. Methylations at different lysine residues of histones render distinct effects on gene expressions. The methylation on H3K9 is recognized as an inactive gene mark, associated with transcription repression [3]. H3K9 methylation is catalyzed primarily by two HKMTs, including G9a, which is responsible for the mono- and dimethylation of H3K9, and SUV39H1, which is mainly related to trimethylation of H3K9. Chaetocin is a HKMT inhibitor and specifically inhibits the enzymatic activities of HKMTs of the SUV39 family, including SUV39H1 and G9a [4]. Chaetocin repressed H3K9 methylation and induced expression of tumor-suppressor genes, such as *p21* and *p15*, leading to cell-cycle arrest and cell death in microglial and AML-193 cells, respectively [5, 6]. In addition, it was demonstrated that H3K9 trimethylation patterns could predict transcription factor activity and survival in AML patients [7]. These findings suggest that H3K9 methylation might play an important role in tumorigenesis.

The SOCS protein family consists of eight members, SOCS1–7 and cytokine-inducible Src homology 2 protein. SOCS1 is a negative regulator of the JAK/STAT pathway [8]. It inhibits

Abbreviations: 7-AAD=7-amino-actinomycin D, ChIP=chromatin immunoprecipitation, CoREST=corepressor of repressor element 1-silencing transcription factor, FasL=Fas ligand, Gfi-1=growth factor independent-1, H3K9=histone 3 lysine 9, HDAC=histone deacetylase, HKMG=10 mM HEPES (pH 7.9), 30 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 1% Nonidet P-40, HKMT=histone lysine methyltransferase, HSC=hematopoietic stem cell, LSD1=lysine-specific demethylase 1, PARP=poly ADP-ribose polymerase, pmoCMV=plasmid with modified CMV promoter, q=quantitative, sh=short hairpin, siRNA=small interfering RNA, SOCS1=suppressor of cytokine signaling 1, SUV39H1=suppressor of variegation 3–9 homolog 1, TSG=tumor suppressor gene

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intracellular signal transduction activated by cytokines, thus playing an important role in controlling the proliferation and differentiation of hematopoietic precursor cells [9]. *SOCS1* expression is tightly regulated at the levels of transcription, translation, and post-translation modification [10–13]. Abnormal expression of *SOCS1* promotes cell transformation and plays a role in carcinogenesis [14]. Silencing of *SOCS1* has been detected in various cancers, including AML [14], a hematological malignancy with uncontrolled proliferation and impaired differentiation of hematopoietic progenitors. However, the detailed mechanism underlying the regulation of *SOCS1* expression in AML cells remains unclear.

Gfi-1 and Gfi-1b function as DNA-binding transcriptional repressors. Their N-terminal Snail/Gfi-1 domain is required for nuclear localization. In contrast, their C-terminal zinc-finger domain is required for the binding DNA element with the consensus sequences of 5′-TAAATCAC(A/T)GCA-3′ [15]. Moreover, both domains are responsible for interaction with transcriptional corepressors, consisting of eight-twenty-one, CoREST, histone demethylase LSD1, HDAC isoforms 1–3, HKMT G9a, and SUV39H1 [16]. Association of multiple epigenetic modification enzymes with Gfi-1/Gfi-1b leads to the silencing of their target genes. Gfi-1 and Gfi-1b counter each other's expression in hematopoietic cells [17]. Gfi-1b expression is limited mainly to cells of erythroid and megakaryocytic lineages, and it was found that Gfi-1b may repress EPO-induced SOCS1 expression [13]. However, most AML are non-erythroid leukemias and are originated from HSCs or early progenitor cells [18]. Therefore, the mechanism of *SOCS1* regulation in AML cells, beside erythroleukemia cells, is still not clear. In this study, we investigated the role of histone methylation in *SOCS1* expression in AML cells. We demonstrated that *SOCS1* transcription was controlled by Gfi-1, and the repressive function of Gfi-1 on *SOCS1* transcription is dependent on histone methyltransferase in AML cells.

MATERIALS AND METHODS

Cell cultures

The 293T cells were maintained in DMEM, and human AML cell lines OCI-AML3 (kindly provided by Dr. Mark Minden, Princess Margaret Hospital, Toronto, Canada), HL60, and U937 were cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA). The medium was supplemented with 10% heat-inactivated FBS (Gibco-BRL) and 1% penicillin/streptomycin solution (Gibco-BRL). The cells were grown in a humidified incubator containing 5% CO_2 at 37°C.

Cellular apoptosis analysis

The percentage of apoptotic cells in chaetocin-treated HL60 and U937 cells was determined using the Annexin V-FITC apoptosis detection kit (MBL International, Woburn, MA, USA), according to the manufacturer's protocol. Briefly, chaetocin-treated cells were washed by PBS buffer twice and incubated with 10 μl Annexin V-FITC and 10 μl PI staining solutions for 15 min at room temperature. After PBS washing, apoptotic cells were determined by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). For apoptotic analysis of GFP-expressing U937 cells whose *SOCS1* is knocked down, the Annexin V-PE apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) was used.

Real-time qRT-PCR analysis

To quantify the endogenous mRNA expression levels, real-time qRT-PCR was performed. Total RNAs of leukemic cells were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA (1 μg) was used to synthesize the cDNA using SuperScript III RT (Invitrogen) with Oligo-dT primers. qPCR was performed using the SYBR Fast qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA). The primer sequences for qPCR are shown in Supplemental Table 1. The qPCR reaction was run in a final volume of 20 μl containing 1 μl RT product, 10 μl 2× SYBR Fast qPCR Master Mix (Kapa Biosystems), and 0.6 μl each primer (10 μM). The qPCR mixtures were incubated at 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. All qPCR was performed in triplicates by using an iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA, USA).

Gfi-1 knockdown by shRNA

To knock down the *Gfi-1* in U937 cells, shRNA against human *Gfi-1* (clone TRCN0000020467; Open Biosystems, Huntsville, AL, USA) [19] was used. Lentiviral particles were produced by cotransfecting 293T cells with the shRNA construct along with the packaging plasmids (Delta 8.9 and vesicular stomatitis virus G glycoprotein), using Arrest-In transfection reagent (Open Biosystems). A virus-containing supernatant was harvested 24 and 48 h later. U937 cells were infected with the viral supernatant in the presence of 8 μg/ml polybrene and selected in 1 μg/ml puromycin, 48 h after infection.

Knockdown of G9a, SUV39H1, and SOCS1 RNA

To knock down *G9a* or *SUV39H1* RNA expression, SMARTpool siRNA targeting G9a or *SUV39H1* (Dharmacon, Lafayette, CO, USA) was delivered into leukemic cells using MicroPorator-100 (Digital Bio Technology, Seoul, Korea). Forty-eight hours after incubation in cell culture, the knockdown efficiencies were determined by mRNA and protein levels of the targeted genes using qRT-PCR and Western blot assay. To knock down human *SOCS1*, shRNA against human *SOCS1* (clone ID: V2LHS_23983; Open Biosystems) was used. The plasmid was delivered into U937 cells by MicroPorator-100 and selected in 1 μg/ml puromycin, 48 h after electroporation.

Western blot analysis

To evaluate the protein expression levels of AML cells, Western blot analysis was performed. The cells were dissolved in a lysis buffer containing 10 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 0.1 mM sodium orthovanadate, 50 mM sodium fluoride, and 1 mM PMSF. Protein (50 μg) was fractionated via 8% or 12% SDS-PAGE and transferred to Immobilon membranes (Millipore, Billerica, MA, USA). Primary antibodies used in this study included those against β-actin (A4416; Sigma-Aldrich, St. Louis, MO, USA), trimethyl-H3K9 (07-442; Upstate Biotechnology, Lake Placid, NY, USA), PARP (#9542), G9a (C6H3; Cell Signaling Technology, Beverly, MA, USA), SUV39H1 (05–615; Millipore), histone 3 (FL-136), SOCS1 (C-20), Gfi-1 (N-20), Gfi-1b (B-7; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and STAT5a/b (C2C3; GeneTex, Irvine, CA, USA). The HRP-conjugated second antibodies recognizing mouse-, rabbit-, or goat-IgG were purchased from Santa Cruz Biotechnology. The chemiluminescence signal was recorded using the FluorChem Xplor (Alpha Innotech, San Leandro, CA, USA).

Plasmid construction

For generation of a reporter plasmid carrying the full-length of the *SOCS1* promoter, the −2238- to +10-bp region of the human *SOCS1* promoter was amplified from U937 genomic DNA by PCR and cloned into pGL3-basic vector (Promega, Madison, WI, USA). Other serial deletion constructs of the *SOCS1* promoter were then generated from the full-length *SOCS1* promoter reporter plasmid by subsequent PCR. In addition, the Gfi-1-binding site of the human *SOCS1* promoter reporter plasmid was mutated using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technolo-

gies, Santa Clara, CA, USA); the "AATC" core sequence of Gfi-1-binding site was mutated to "GGTC".

The CMV promoter of the pcDNA3.1/myc-His-A vector (Invitrogen) contains two Gfi-1-binding sites. To avoid the CMV promoter activity of the vector being repressed by Gfi-1when this protein was present, both sites were mutated by site-directed mutagenesis, and the modified plasmid was named pmoCMV. The cDNA of *Gfi-1* and *Myc* were amplified from U937 cDNA by PCR and then cloned into the pmoCMV vector to generate pmoCMV-*Gfi*-1 and pmoCMV-*Myc* plasmids. Another pmoCMV-*Gfi-1* (N382S) plasmid, which contained mutant Gfi-1 (N382S) that produces protein without DNA-binding activity, was generated from the pmoCMV-*Gfi*-1 plasmid using a site-directed mutagenesis kit. The constitutively activated STAT5A plasmid STAT5A1*6 (carrying mutations of H299R and S711F), kindly provided by Toshio Kitamura and coworkers [20], was also subcloned into the pmoCMV vector to generate the pmoCMV-*STAT5A1*6* plasmid.

Plasmid transfection by electroporation

All of the plasmids were transfected into AML cell lines by electroporation using MicroPorator-100 (Digital Bio Technology), according to the manufacturer's instructions. Plasmid (1 μg) was mixed with 1×10^6 cells in 10 μl electroporation buffer. After electroporation, cells were transferred to culture media at 37°C immediately.

Luciferase promoter assay

U937 cells were cotransfected with the *SOCS1* promoter luciferase reporter plasmid together with the Renilla luciferase reporter control plasmid (Promega) by using MicroPorator-100. Twenty-four hours after incubation, cellular protein lysates were processed to measure dual luciferase activity, according to the manufacturer's instructions. *SOCS1* promoter activity was normalized by the level of Renilla luciferase activity.

DNA pull-down assay

To investigate the association of the Gfi-1 and *SOCS1* promoter, the oligonucleotide pull-down assay was performed, as described previously [21] with slight modification. Biotinylated oligonucleotides containing the Gfi-1-binding site of the *SOCS1* promoter were generated by PCR using the *SOCS1* reporter plasmids containing the WT or mutant Gfi-1-binding site as the templates. Primer sequences are shown in Supplemental Table 2. PCR products were purified by the gel extraction method and quantitated by NanoVue spectrophotometer (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The pmoCMV-*Gfi-1* and pmoCMV-Myc were electroporated into 293T cells, respectively, to generate Gfi-1 and Myc overexpression cells. The cells were then washed by PBS buffer and lysed in HKMG buffer. The biotinylated oligonucleotides were preincubated initially with streptavidin-agarose beads in HKMG buffer with 10% FBS for 30 min at room temperature. The whole-cell lysates were then incubated overnight at 4°C with the beads conjugated with biotinylated oligonucleotides in the presence of 25-fold excess of polydeoxyinosinic:polydeoxycytidylic acid. DNA-bound proteins were then washed and dissolved in a loading buffer, followed by Western blot analysis.

ChIP assay

To explore the interaction of protein and DNA, ChIP was performed using a commercially available Magna ChIP A kit (Millipore), according to the manufacturer's instructions. Briefly, cells were fixed in 1% formaldehyde. The chromatin was then sonicated to smaller DNA fragments with an average length of 300–500 bp. Equal amount of soluble chromatin and protein A-conjugated magnetic beads were then incubated with anti-G9a (07-551; Upstate Biotechnology), anti-SUV39H1 (05-615; Millipore), anti-Gfi-1 (N-20; Santa Cruz Biotechnology), anti-dimethyl-H3K9 (07-441; Upstate Biotechnology), anti-trimethyl-H3K9 (07-442; Upstate Biotechnology), anti-histone 3 (FL-136; Santa Cruz Biotechnology), or normal IgG (12-371B and PP64B, Millipore; and N-13, Santa Cruz Biotechnology). After overnight antibody incubations, DNA protein cross-linking was reversed, and DNA fragments were eluted by 50 μl distilled water. DNA fragments (3 μl), obtained with each antibody, were subjected to qPCR analysis by using SYBR Fast qPCR Master Mix (Kapa Biosystems). Primer sequences are shown in Supplemental Table 3. DNA fragments obtained without antibody were used as the input controls, whereas DNA fragments obtained with normal IgG were applied as negative controls.

Statistical analysis

Two-tailed Student's *t*-test was used to analyze the results, and $P < 0.05$ was considered statistically significant.

RESULTS

Chaetocin treatment decreased H3K9 methylation on the *SOCS1* promoter accompanied by an increase of *SOCS1* mRNA expression in AML cells

To assess whether H3K9 methylation affected the *SOCS1* gene transcription, AML cell lines HL60 and U937 were first treated with 50 and 100 nM chaetocin, a H3K9 methyltransferase inhibitor, for 16 and 24 h. Cell viability was analyzed by Annexin V and PI staining, followed by flow cytometry. We found that chaetocin induced leukemia cell apoptosis (**Fig. 1A**); meanwhile, *SOCS1* mRNA expression, determined by qRT-PCR assay, was also enhanced in a dose- and time-dependent manner (Fig. 1B and C). Next, the changes of global H3K9 trimethylation and H3K9 methylation on the *SOCS1* promoter of U937 cells were evaluated by Western blot assays and ChIP-qPCR, respectively. As shown in Fig. 1D, the global H3K9 trimethylation was not affected after chaetocin treatment for 16 h. In contrast, dimethylated and trimethylated H3K9, which could be detected in the *SOCS1* promoter before chaetocin treatment, was eliminated significantly, 16 h after the treatment (Fig. 1E), suggesting that the *SOCS1* transcription was modulated by the local H3K9 methylation change on the *SOCS1* promoter in AML cells.

SOCS1 knockdown eliminated chaetocin-induced cell apoptosis

To explore the role of *SOCS1* induction in chaetocin-induced cellular apoptosis, *SOCS1* knockdown U937 cells were generated, and the efficiency of *SOCS1* knockdown was determined by qRT-PCR assay. As shown in **Fig. 2A**, there was no apparent difference in the basal levels of *SOCS1* mRNA between the knockdown and control cells. However, chaetocin-induced *SOCS1* mRNA expression in knockdown cells was inhibited significantly relative to shRNA control cells, whereas chaetocin-induced *FasL* (one of the chaetocin-activated genes) mRNA was not altered (Fig. 2B). Subsequently, cellular viability, 16 h after chaetocin treatment, was evaluated by Annexin V-PE and 7-AAD staining. As shown in Fig. 2C, in the *SOCS1* knockdown cells, chaetocin-induced cellular apoptosis was attenuated significantly relative to the shRNA control cells. Furthermore, the results were confirmed by decreased caspase-dependent cleavage of PARP in Western blot in the *SOCS1* knockdown cell assay compared with control cells (Fig. 2D). These results indi-

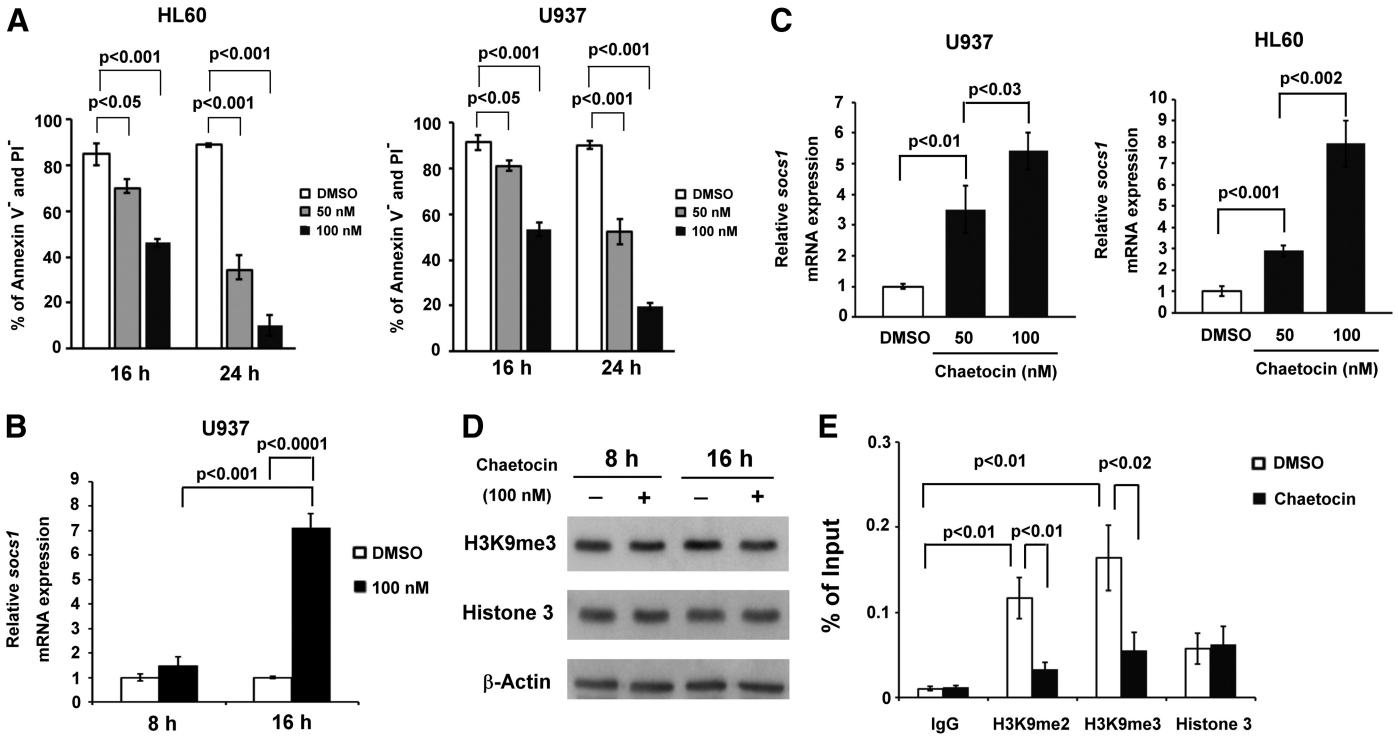

Figure 1. Chaetocin treatment decreased H3K9 methylation on the *SOCS1* promoter accompanied by the increase in the *SOCS1* mRNA expression. (A) HL60 and U937 cells were treated with 50 or 100 nM chaetocin for 16 and 24 h. The cellular apoptosis was analyzed by Annexin V-FITC and PI staining. (B) U937 cells were treated with 100 nM chaetocin or DMSO as a vehicle control for 8 and 16 h, and (C) U937 and HL60 cells were treated with chaetocin (50 and 100 nM) or DMSO for 16 h. The *SOCS1* mRNA expression was determined by qRT-PCR assays, and data were normalized by *GAPDH* mRNA expression. (D) Western blot analysis of H3 histone modifications in cell protein lysates of U937 cells treated with chaetocin (100 nM) or DMSO for 8 or 16 h. One of three representative experiments is shown. (E) U937 cells were treated with 100 nM chaetocin or DMSO for 16 h and then harvested for ChIP assay using the specific antibodies recognizing histone 3 and di- and trimethylated H3K9 (H3K9me2 and H3K9me3, respectively). The methylation levels of H3K9 were determined by the qPCR assay of the *SOCS1* promoter using the specific primers for amplification of the −485- to −331-bp region. (A–C, and E) Data were presented as mean ± SEM of three independent measurements.

cated that *SOCS1* induction might mediate chaetocin-induced cell apoptosis.

Knockdown of G9a and SUV39H1 elevated *SOCS1* mRNA expression in AML cell lines

To assess whether G9a and SUV39H1, two primary H3K9 methyltransferases, are involved in the regulation of *SOCS1* expression in AML cells, the *SOCS1* mRNA level was measured after *G9a* or *SUV39H1* was suppressed by siRNA in OCI-AML3, HL60, and U937 leukemic cells, respectively. As shown in **Fig. 3A–C**, the knockdown efficiencies of *G9a* and *SUV39H1*, determined by qRT-PCR and Western blot assay, were 70–80%, 48 h after the siRNA transfection. Meanwhile, the *SOCS1* mRNA expressions were augmented 1.7- to 3.2-fold in the knockdown cells (Fig. 3D–F), suggesting that *SOCS1* transcription was regulated by G9a and SUV39H1 in AML cells.

Gfi-1 bound the *SOCS1* promoter and modulated its activity in AML cells

Alternation of H3K9 methylation levels may result from deregulated expression of transcriptional factors. Moreover, the function of G9a and SUV39H1 in the regulation of target genes is dependent on their interaction with other specific DNA-binding transcription factors. To identify the critical regions responsible for the repression of the *SOCS1* promoter, the luciferase reporter plasmids with serial deletions of the *SOCS1* promoter were constructed, followed by measurement of the luciferase reporter activities. As shown in **Fig. 4A**, deletion of the promoter from −2238 to −1493 bp obviously did not alter *SOCS1* promoter activity. In contrast, deletion from −1493 to −979 bp apparently elevated *SOCS1* promoter activity. The highest promoter activity was detected in the construct containing the *SOCS1* promoter sequence from −491 to + 10 bp ($P<0.01$). Taken together, −1493 to −491 bp at the *SOCS1* promoter in AML cells might contain the binding sites for unidentified, repressive transcription factors.

By the Matinspector software tool, we found two potential binding sites of the Gfi-1 protein family at −1109 bp and −796 bp of the human *SOCS1* promoter, respectively. We next investigated whether Gfi-1 family proteins could modulate *SOCS1* gene transcription in AML cells. As the expression levels of mRNA and protein of Gfi-1 were more abun-

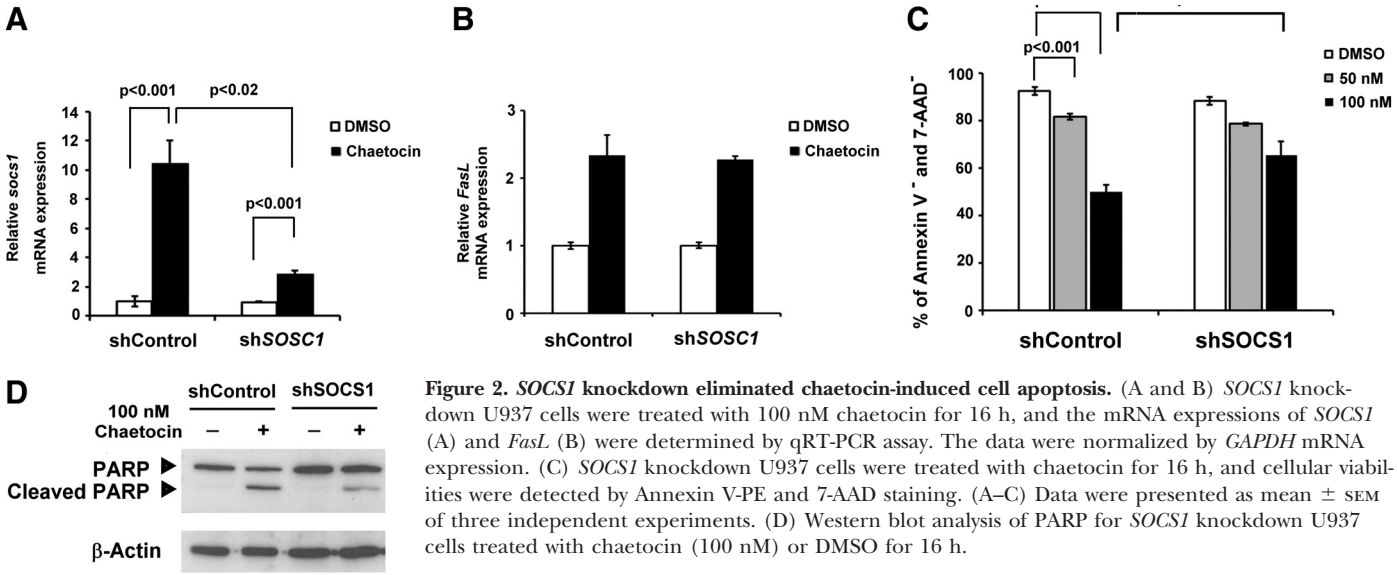

Figure 2. *SOCS1* knockdown eliminated chaetocin-induced cell apoptosis. (A and B) *SOCS1* knockdown U937 cells were treated with 100 nM chaetocin for 16 h, and the mRNA expressions of *SOCS1* (A) and *FasL* (B) were determined by qRT-PCR assay. The data were normalized by *GAPDH* mRNA expression. (C) *SOCS1* knockdown U937 cells were treated with chaetocin for 16 h, and cellular viabilities were detected by Annexin V-PE and 7-AAD staining. (A–C) Data were presented as mean ± SEM of three independent experiments. (D) Western blot analysis of PARP for *SOCS1* knockdown U937 cells treated with chaetocin (100 nM) or DMSO for 16 h.

dant than Gfi-1b in AML cell lines OCI-AML3, HL60, and U937 (Fig. 4B–D), and G9a was shown to interact with Gfi-1 in a previous study [16], we therefore focused on studying the Gfi-1 effect on *SOCS1* promoter activation in AML cells.

First, we mutated one or both of the potential Gfi-1-binding sites in the *SOCS1* reporter plasmid. We found that mutation of either one of Gfi-1-binding sites enhanced *SOCS1* promoter activity ($P<0.003$, and $P<0.05$), and the activity was augmented further when both sites were mutated ($P<0.001$; Fig. 4E). Moreover, chaetocin treatment decreased Gfi-1 expression, whereas it increased SOCS1 expression (Fig. 4F). The suppressor effect of Gfi-1 on *SOCS1* expression was confirmed further by shRNA knockdown of *Gfi-1* in U937 cells, in which mRNA and protein of SOCS1 were increased compared with those in wild U937 cells (Fig. 4G). Taken together, these results indicated that Gfi-1 plays a suppressor role in SOCS1 expression in U937 cells.

To assess whether Gfi-1 directly bound the two binding sites at −1109 bp and −796 bp, respectively, we performed DNApull-down assays on the cell lysates from Gfi-1-overexpressed 293T cells using the biotinylated oligonucleotides G1 and G2, corresponding to the two potential Gfi-1-binding sites (**Fig. 5A**). The oligonucleotides, containing the mutant sequence of the Gfi-1-binding site, were used as controls. The locations and part of the sequences of wild and mutated oligonucleotides for Gfi-1-binding assays were shown in Fig. 5A. In vitro DNA pull-down assays demonstrated that the oligonucleotides G1 and G2 exhibited binding affinity for Gfi-1-binding sites, although oligonucleotide G1 had higher affinity than oligonucleotide G2 (Fig. 5B). On the contrary, the oligonucleotides containing mutant sequences (G1 and G2 mutants) showed significantly reduced binding capacity to Gfi-1-binding sites (Fig. 5B). The similar finding could be seen in HL60 cells with ectopic Gfi-1 overexpression (data not shown). As the G1 probe contains the Myc-binding site (CACGTG), DNA pull-down assay for Myc was used as a positive control (Fig. 5B). These findings indicated an association between Gfi-1 and the human *SOCS1* promoter in vitro. We next used ChIP-qPCR assays using the anti-Gfi-1 antibody to test whether Gfi-1 directly bound on these sites in vivo. The Gfi-1-binding capacity to the human *SOCS1* promoter was determined by qPCR of the *SOCS1* promoter in the precipitate. As shown in Fig. 5C and D, the PCR product of −1167 to −1015 bp of the *SOCS1* promoter, amplified from the precipitate using the Gfi-1 antibody, was more abundant than that using the control antibody ($P<0.002$). On the other hand, there was no statistical difference in the quantity of the PCR product of +926 to +1064 bp of the *SOCS1* gene exon between the two precipitates using the Gfi-1 or control antibody. Taken together, these findings implicated that Gfi-1 indeed directly bound the human *SOCS1* promoter and modulated its activity in AML cell lines.

Gfi-1 repressed STAT5-activated *SOCS1* promoter activity

SOCS1 is a negative-feedback controller in the JAK/STAT signaling pathway. To examine whether Gfi-1 exhibited the ability to repress basal or STAT5A- activated *SOCS1* promoter activity in human AML cells, U937 cells were transfected with the *SOCS1* promoter luciferase reporter plasmid along with the expression plasmid of Gfi-1, STAT5A1*6, or both by electroporation. As shown in **Fig. 6A**, Gfi-1 expression decreased the basal level of *SOCS1* promoter activity ($P<0.02$), whereas STAT5A1*6 apparently enhanced *SOCS1* promoter activity ($P<0.0001$). Notably, Gfi-1 repressed STAT5A1*6-mediated *SOCS1* promoter activation in a dose-dependent manner ($P<0.001$). Furthermore, the expression of the Gfi-1 N382S mutant, which lost DNA-binding activity [22], was not able to repress STAT5A1*6-mediated *SOCS1* promoter activation ($P<0.03$; Fig. 6B). To explore further the effect of Gfi-1 on JAK/STAT signaling, STAT5a/b protein levels of *Gfi-1* knockdown U937 cells were examined, and the results indicated that there was less of an amount

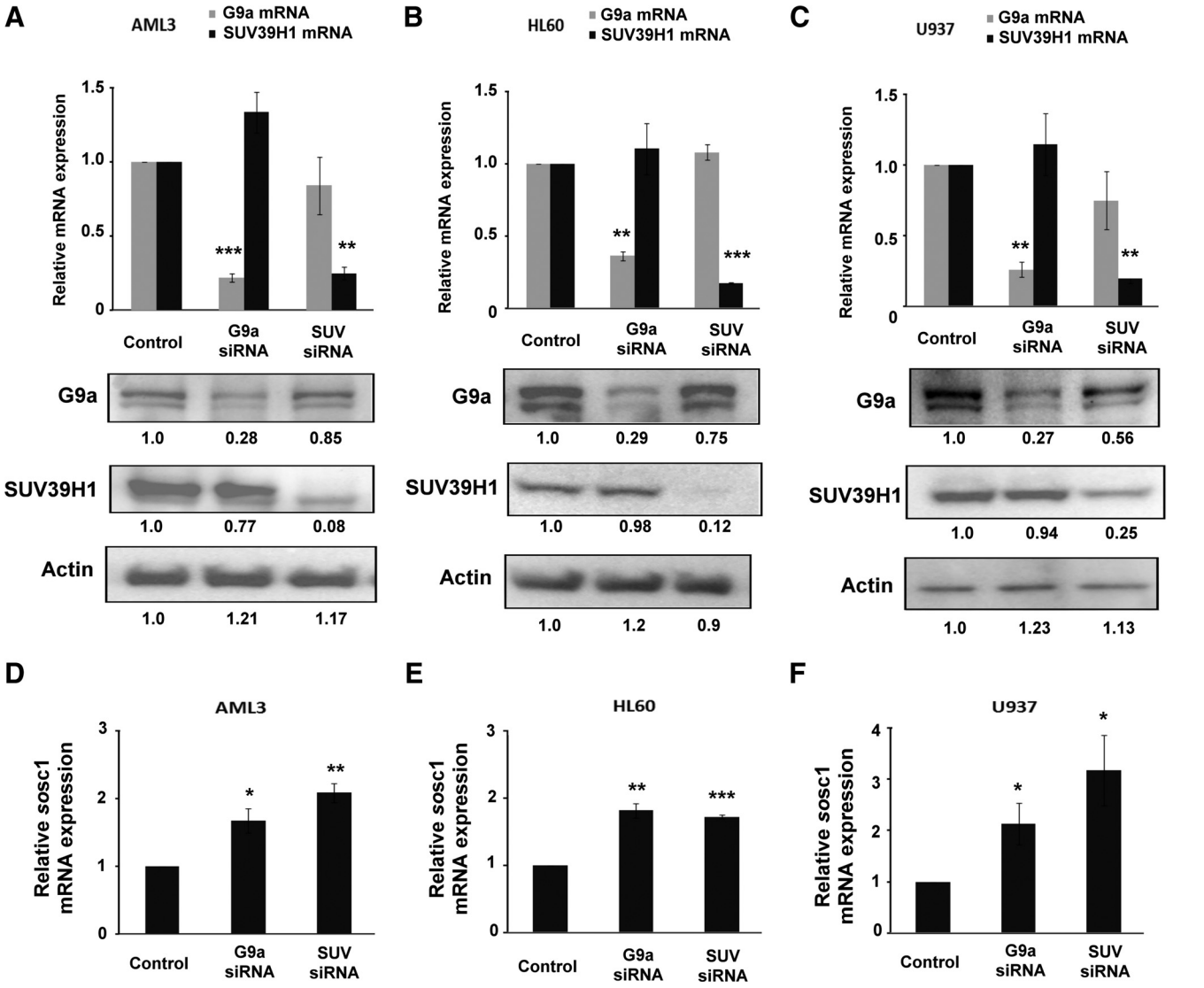

Figure 3. *G9a* or *SUV39H1* knockdown by siRNA enhanced *SOCS1* mRNA expression in AML cell lines. (A–C) *G9a* and *SUV39H1* (SUV) expressions of OCI-AML3 (AML3), HL60, and U937 cells were knocked down by transfection with their specific siRNA using electroporation. Scramble RNA was used as a control. After 48 h, total RNA and proteins were extracted to quantify the expressions of *G9a* and *SUV39H1* by qRT-PCR assays and Western blot assay. The mRNA levels of *G9a* and *SUV39H1* were normalized by *GAPDH* mRNA expression. For Western blot assay, relative levels of G9a and SUV39H1 were determined by densitometry and normalized by the coresponding β-actin amount. (D–F) The *SOCS1* mRNA expression levels were also determined by qRT-PCR. Data were presented as mean ± SEM of three independent experiments (*$P<0.05$ vs. control; **$P<0.01$ vs. control; ***$P<0.001$ vs. control).

of STAT5a/b proteins in the *Gfi-1* knockdown cells compared with the shRNA control cells (Fig. 6C).

G9a and SUV39H1 were required for Gfi-1-mediated transcriptional repression of *SOCS1*

To examine whether G9a and SUV39H1 regulate *SOCS1* transcription directly or indirectly, the interaction of G9a and SUV39H1 with the *SOCS1* promoter in U937 cells was examined by ChIP-qPCR assays using the specific antibodies against G9a and SUV39H1, respectively. We found that the PCR products of −1167 to −1015 bp, upstream of the transcription start site of *SOCS1*, were more abundant in the precipitate obtained using antibody to G9a or SUV39H1 than that using IgG control antibody (Fig. 6D and E). To assess further whether Gfi-1 modulates the association of G9a and SUV39H1 with the *SOCS1* promoter, *Gfi-1* knockdown U937 cells were subjected for ChIP assay. As shown in Fig. 6F, there was less of an amount of Gfi-1 associated with the *SOCS1* promoter in the *Gfi-1* knockdown cells; meanwhile, the association of G9a and SUV39H1 at the *SOCS1* promoter was also decreased (Fig. 6D and E). These findings indicated that the binding of G9a and SUV39H1 on the *SOCS1* promoter was modulated, at least partially, by Gfi-1 in U937 cells.

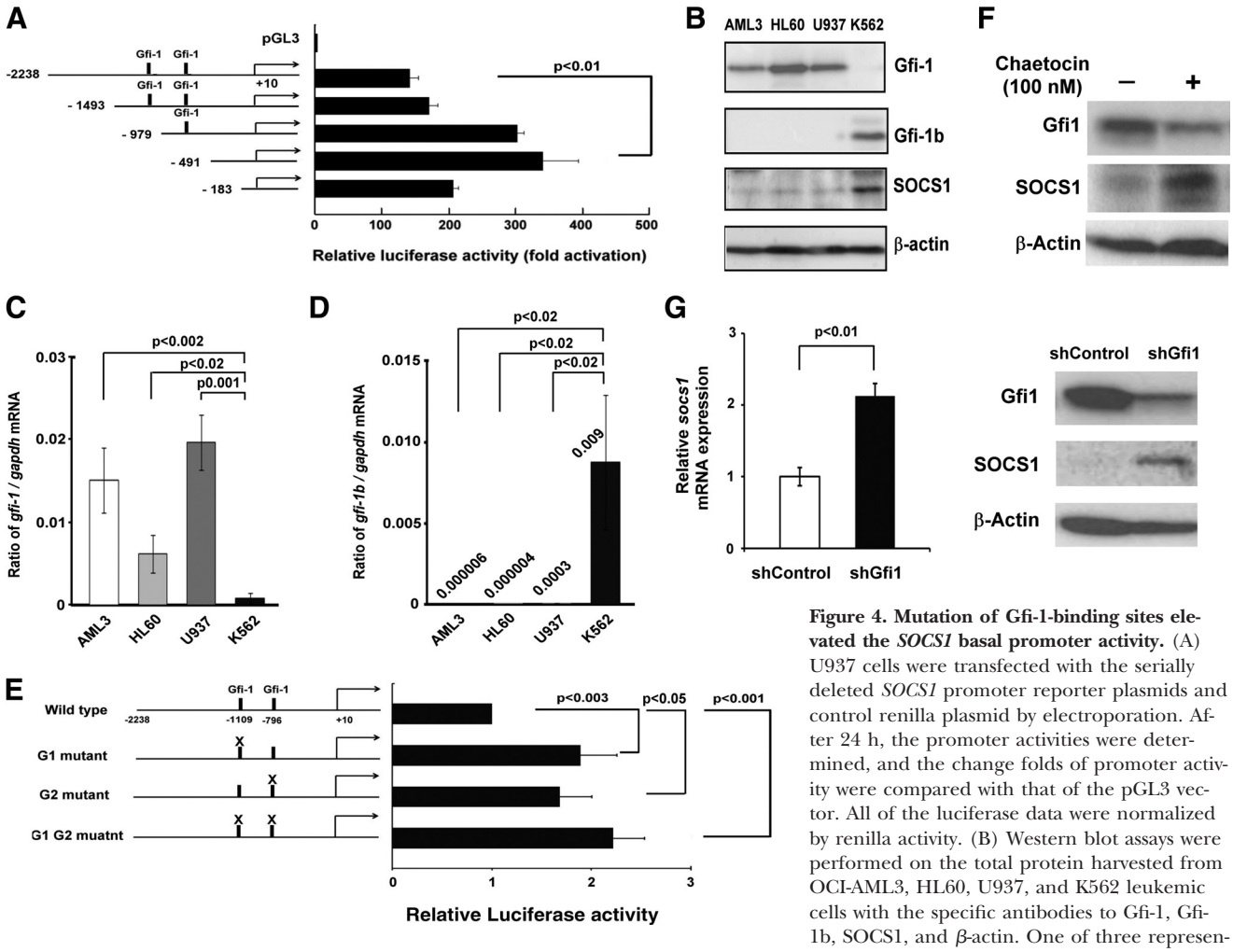

Figure 4. Mutation of Gfi-1-binding sites elevated the *SOCS1* basal promoter activity. (A) U937 cells were transfected with the serially deleted *SOCS1* promoter reporter plasmids and control renilla plasmid by electroporation. After 24 h, the promoter activities were determined, and the change folds of promoter activity were compared with that of the pGL3 vector. All of the luciferase data were normalized by renilla activity. (B) Western blot assays were performed on the total protein harvested from OCI-AML3, HL60, U937, and K562 leukemic cells with the specific antibodies to Gfi-1, Gfi-1b, SOCS1, and β-actin. One of three representative experiments is shown. (C and D) The mRNA expression of Gfi-1 (C) and Gfi-1b (D) of AML3, HL60, and U937 cells was determined by qRT-PCR assays. The data were normalized by *GAPDH* mRNA expression. (E) One or both Gfi-1-binding sites in the *SOCS1* promoter reporter plasmid were mutated by the site-directed mutagenesis method. The plasmids of the wild or mutant *SOCS1* promoter reporter and control renilla were electroporated into U937 cells. After 24 h, the promoter activities of the mutant *SOCS1* promoter constructs were analyzed and compared with that of the wild one. All of the luciferase data were normalized by renilla activity. (F) U937 cells were treated with chaetocin (100 nM) for 16 h, and Gfi-1 and SOCS1 expressions were assessed by Western blot assay. One of three representative experiments is shown. (G) The Gfi-1 and SOCS1 expressions of *Gfi-1* knockdown U937 cells were analyzed by Western blot assay. One of three representative experiments is shown. The *SOCS1* mRNA expression levels were also determined by qRT-PCR. The data were normalized by *GAPDH* mRNA expression. (A, C–E, and G) Data were presented as mean ± SEM of three independent experiments.

To assess whether G9a and SUV39H1 were required for the Gfi-1-mediated suppression of *SOCS1* promoter activity, the effect of Gfi-1 on *SOCS1* promoter activity was analyzed in U937 cells with knockdown of *G9a* or *SUV39H1* expression. We noted that in U937 cells with *G9a* and *SUV39H1* knockdown by siRNA for 48 h exhibited more apoptotic cells from 13.8% to 31% and 28%, respectively (Supplemental Fig. 1). These knockdown cells were used subsequently to perform the promoter assay, 24 h after electroporation. As shown in Fig. 6G, in the *G9a*-depleted U937 cells, Gfi-1 abrogated almost completely the ability to repress the STAT5A1*6-activated *SOCS1* promoter and did so partially in *SUV39H1*-depleted U937 cells. These data indicate that G9a and SUV39H1 were involved in the regulatory mechanism of Gfi-1-mediated repression of *SOCS1* promoter activity in AML cells.

DISCUSSION

Transcriptional silencing of TSGs through DNA methylation is a common epigenetic event in hematologic malignancies [23], but the role of histone methylation in the TSG expression remains to be explored. H3K9 methylation is a hallmark of repressive chromatin structure. Recently, it was reported that the profile of H3K9 trimethylation levels could distinguish AML cells from normal $CD34^+$ cells and predict the event-free survival in AML patients [7], suggesting that abnormal H3K9 methylation might play a role in leukemogenesis. H3K9 methylation may alter chromatin structure around the promoter of TSG, thus leading to gene silencing. For instance, in solid cancer cells, H3K9 methylation can be detected in promoter regions of some TSGs, such as *p21*, *p16*, *human MutL homolog 1*,

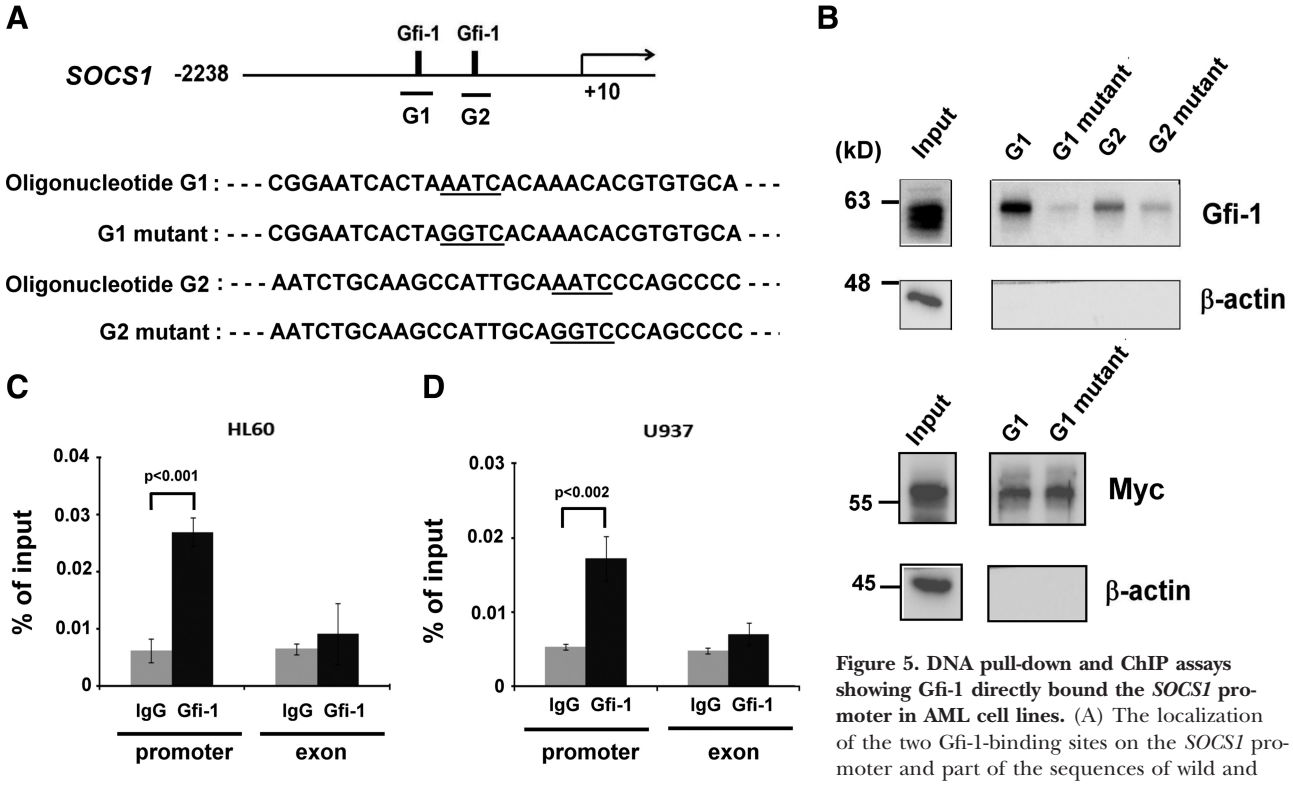

Figure 5. DNA pull-down and ChIP assays showing Gfi-1 directly bound the *SOCS1* promoter in AML cell lines. (A) The localization of the two Gfi-1-binding sites on the *SOCS1* promoter and part of the sequences of wild and mutant oligonucleotides for these two binding sites in DNA pull-down assay were shown. The core sequences of the Gfi-1-binding site for the mutant oligonucleotides were mutated from AATC to GGTC. (B) DNA pull-down assays were performed on the total protein extracted from ectopic Gfi-1 or Myc-overexpressed 293T cells using biotinylated oligonucleotides containing the wild or mutant Gfi-1-binding site. The precipitates were subjected to Western blot assay using the antibodies to Gfi-1, Myc, and β-actin. (C and D) ChIP assays were performed on formaldehyde-fixed HL60 and U937 cells using the specific anti-Gfi-1 antibody. Normal IgG antibody was used as a negative control. Binding efficiency of Gfi-1 on the *SOCS1* promoter was determined by the qPCR assay of the *SOCS1* promoter. The data were presented as mean ± SEM of three independent experiments.

and *O-6-methylguanine-DNA methyltransferase* [5, 24, 25], and is associated with the transcriptional silencing. Moreover, TSG *p16* could be silenced by H3K9 hypermethylation, independent of DNA methylation [25]. In AML cells, treatment with chaetocin, a H3K9 methyltransferase inhibitor of the SUV39 family, caused H3K9 demethylation on the promoters of *p15* and *E-cadherin* and reactivated their gene transcriptions without altering their DNA methylation [6], implying that H3K9 methylation alone contributed to *p15* and *E-cadherin* silencing.

To the best of our knowledge, this is the first report to demonstrate that *SOCS1* expression can be modulated by H3K9 methylation levels in AML cells. Decreased H3K9 methylation of the *SOCS1* promoter by chaetocin treatment enhanced *SOCS1* transcription. Moreover, the ChIP-qPCR assay demonstrated the presence of abundant H3K9 methylation on the *SOCS1* promoter, compatible with the results of ChIP-on-ChIP assay reported by Carsten Muller-Tidow et al. [7]. According to their microarray data, the level of H3K9 trimethylation at the *SOCS1* core promoter region is more abundant (3.8×) in AML than that in $CD34^+$ cells ($P<0.0019$) [7]. These findings further demonstrate the association of H3K9 methylation and silencing of TSG and provide new insight into developing histone methyltransferase inhibitors as anti-cancer drugs.

The sequential H3K9 methylation, from mono- and di- to trimethylation, is chiefly catalyzed by G9a and SUV39H1. It has been shown that a subset of the H3K9 methyltransferases SUV39H1, G9a, glucagon-like peptide, and set domain, bifurcated 1, participates in a multimeric complex to regulate expressions of their target genes located at euchromatin and heterochromatin [26]. We, for the first time, found that G9a and SUV39H1 were associated with the *SOCS1* promoter in AML cells, and depletion of *G9a* or *SUV39H1* by siRNA elevated *SOCS1* transcription, further indicating that *SOCS1* is one of the target genes affected by the deregulated H3K9 methylation in AML cells.

Gfi-1 and Gfi-1b counter each other's expression in hematopoietic cells [17]. Gfi-1 is detectable in HSCs, common lymphoid progenitors, and granulo/monocytic progenitors but is absent in common myeloid progenitors and megakaryocyte/erythroid progenitors [27]. In contrast, Gfi-1b expression is mainly limited to cells of erythroid and megakaryocytic lineages [28]. *Gfi-1* knockout mouse studies indicate that loss of Gfi-1 results in defects in the myeloid B and T cell lineages and HSCs [29], implying that Gfi-1 plays a pivotal role in different cell lineages. The first report about *SOCS1* regulation by the Gfi-1 protein family indicated that EPO-induced Gfi-1b bound to the *SOCS1* promoter and repressed *SOCS1* expression in erythropoiesis [13]. In addition, another report showed that the *SOCS1* expression was decreased in Gfi-1-infected Th2 cells [30]; however, the detail mechanism is still unclear. Fur-

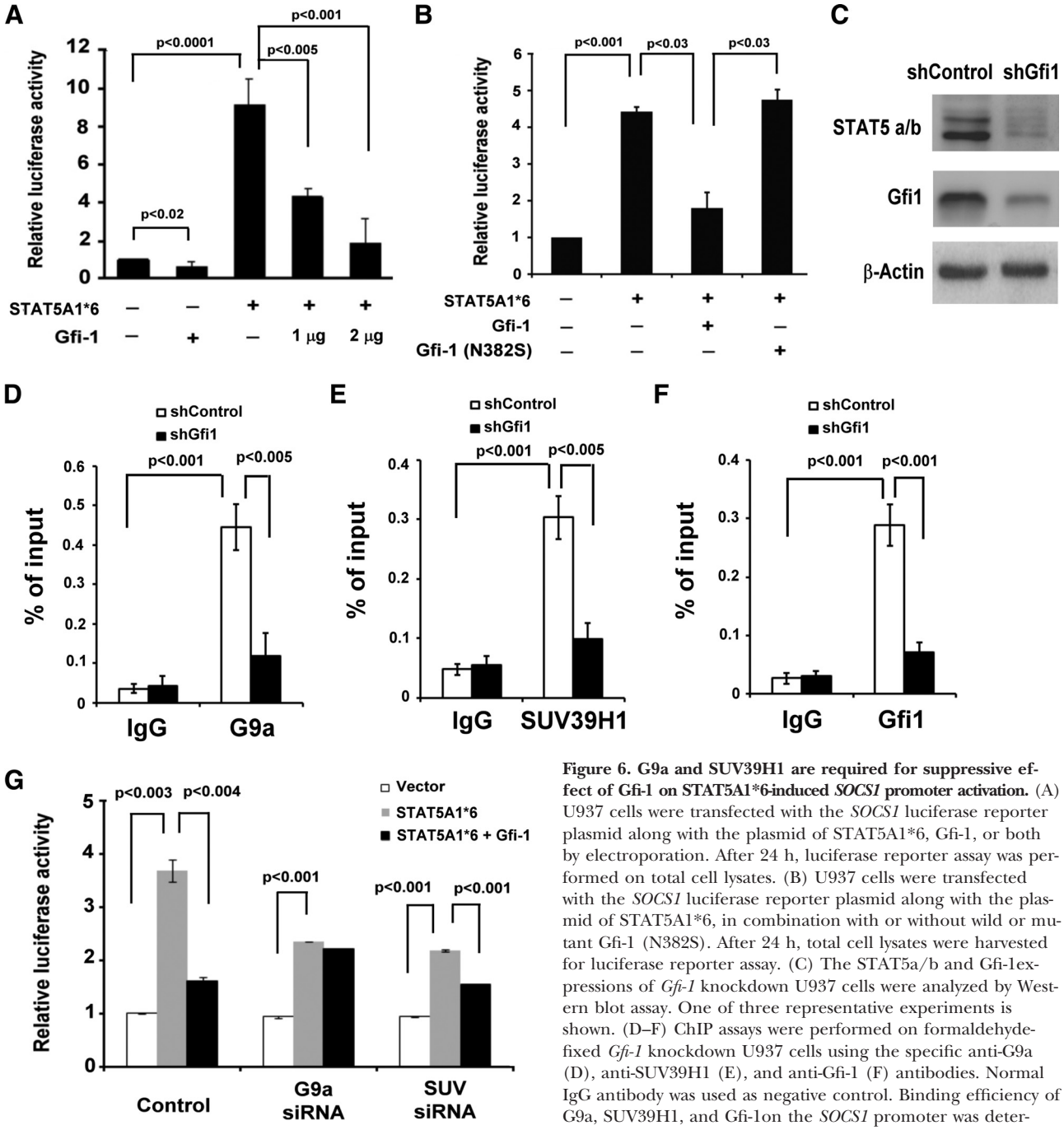

Figure 6. G9a and SUV39H1 are required for suppressive effect of Gfi-1 on STAT5A1*6-induced *SOCS1* promoter activation. (A) U937 cells were transfected with the *SOCS1* luciferase reporter plasmid along with the plasmid of STAT5A1*6, Gfi-1, or both by electroporation. After 24 h, luciferase reporter assay was performed on total cell lysates. (B) U937 cells were transfected with the *SOCS1* luciferase reporter plasmid along with the plasmid of STAT5A1*6, in combination with or without wild or mutant Gfi-1 (N382S). After 24 h, total cell lysates were harvested for luciferase reporter assay. (C) The STAT5a/b and Gfi-1expressions of *Gfi-1* knockdown U937 cells were analyzed by Western blot assay. One of three representative experiments is shown. (D–F) ChIP assays were performed on formaldehyde-fixed *Gfi-1* knockdown U937 cells using the specific anti-G9a (D), anti-SUV39H1 (E), and anti-Gfi-1 (F) antibodies. Normal IgG antibody was used as negative control. Binding efficiency of G9a, SUV39H1, and Gfi-1on the *SOCS1* promoter was determined by the qPCR assay. Data were presented as mean ± SEM of three independent measurements. (G) *G9a* or *SUV39H1* mRNA of U937 cells was knocked down by siRNA for 48 h, followed by electroporation again with the *SOCS1* promoter reporter plasmid, along with the plasmid of STAT5A1*6 or both STAT5A1*6 and Gfi-1. After 24 h, *SOCS1* promoter activities were analyzed. (A, B, and G) Data were normalized by renilla activity and presented as mean ± SEM of three independent measurements.

thermore, the *SOCS1* regulation in myeloid cells has not been explored.

Different HSCs/progenitor populations are characterized by distinctive transcription factor expression states [17]. In this study, we found that Gfi-1 expression is more abundant than Gfi-1b in nonerythroleukemia cells, such as OCI-AML3, HL60, and U937. Therefore, we focused our studies on the investigation of the Gfi-1 effect on *SOCS1* transcription in AML cells. The oncogenic potential of Gfi-1 is low, but it may cooperate with Myc and Pim-1 to induce T cell lymphoma [31]. In Gfi-1 knockdown mice, T and B cell development and granulocyte maturation are defective. These findings indicate that appropriate expression of Gfi-1 is essential for normal hematopoiesis. In this study, we demonstrated that the mutation of Gfi-1-

binding sites of the *SOCS1* promoter increased basal *SOCS1* promoter activity, whereas Gfi-1 overexpression repressed it. We also showed that Gfi-1 directly bound the *SOCS1* promoter in AML cells, and *Gfi-1* knockdown enhanced SOCS1 expression, implying that Gfi-1 acts as a repressive transcription factor for *SOCS1* promoter activation. Based on the fact that Gfi-1 may interact with many corepressors, including G9a, CoREST, HDAC, and LSD1, to regulate target gene expression [16, 21, 32], and the findings from this study that *Gfi-1* knockdown decreased the association of G9a and SUV39H1 on the *SOCS1* promoter, we suggest that Gfi-1 recruits G9a and SUV39H1 to form a repressive complex on the *SOCS1* promoter and subsequently, leads to *SOCS1* silencing in AML cells.

The constitutive activation of STAT3 and STAT5 is detected frequently in hematopoietic tumors [20, 33]. In addition, the sustained STAT5 activation in HSCs promoted self-renewal of HSCs—a process crucial to the pathogenesis of myeloproliferative diseases [20]. *SOCS1* expression is induced by STAT proteins to negatively control the JAK-STAT signal pathway. Therefore, *SOCS1* silencing may contribute to aberrant JAK-STAT pathway signaling [20]. In this study, we found that ectopic Gfi-1 overexpression inhibited STAT5A1*6-activated *SOCS1* promoter activation in U937 cells but not in those cells with depletion of *G9a* or *SUV39H1*. These findings suggest that Gfi-1, through its interaction with G9a and SUV39H1, may keep the *SOCS1* promoter in a close chromatin structure and block the effect of STAT5A. The cause that *SOCS1* promoter induction by STAT5A1*6 was lower in *G9a*- or *SUV39H1*-depleted cells compared with control cells was possibly a result of cell apoptosis related to *G9a* or *SUV39H1* suppression, which was demonstrated by previous reports [6, 34] and our study (Supplemental Fig. 1).

In summary, our study indicates that Gfi-1 is a repressor of *SOCS1* gene transcription in AML cells, and G9a and SUV39H1 are involved in the mechanism of Gfi-1-mediated suppression of *SOCS1* promoter activity. In addition, the histone methyltransferase inhibitor may be of value to use as a potential targeted therapy for AML, as it can activate TSGs, such as *p21* and *p15*, as shown in previous reports, and *SOCS1*, as shown in this study.

AUTHORSHIP

M.C.L. designed the research, performed experiments, analyzed results, and prepared the manuscript. Y-Y.K., W-C.C., and M.H. helped with the material preparation and performed part of the experiments, experiment design, and manuscript preparation. H-A.H. helped with the manuscript preparation. H-F.T. designed and oversaw the research and helped prepare the manuscript.

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DISCLOSURES

The authors declare no conflict of interest.

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G9a · SUV39H1 · H3K9 methylation