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# Histone deacetylase inhibitors repress macrophage migration inhibitory factor (MIF) expression by targeting *MIF* gene transcription through a local chromatin deacetylation

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ABSTRACT

The cytokine macrophage migration inhibitory factor plays a central role in inflammation, cell proliferation and tumorigenesis. Moreover, macrophage migration inhibitory factor levels correlate with tumor aggressiveness and metastatic potential. Histone deacetylase inhibitors are potent antitumor agents recently introduced in the clinic. Therefore, we hypothesized that macrophage migration inhibitory factor would represent a target of histone deacetylase inhibitors. Confirming our hypothesis, we report that histone deacetylase inhibitors of various chemical classes strongly inhibited macrophage migration inhibitory factor expression in a broad range of cell lines, in primary cells and *in vivo*. Nuclear run on, transient transfection with macrophage migration inhibitory factor promoter reporter constructs and transduction with macrophage migration inhibitory factor expressing adenovirus demonstrated that trichostatin A (a prototypical histone deacetylase inhibitor) inhibited endogenous, but not episomal, *MIF* gene transcription. Interestingly, trichostatin A induced a local and specific deacetylation of macrophage migration inhibitory factor promoter-associated H3 and H4 histones which did not affect chromatin accessibility but was associated with an impaired recruitment of RNA polymerase II and Sp1 and CREB transcription factors required for basal *MIF* gene transcription. Altogether, this study describes a new molecular mechanism by which histone deacetylase inhibitors inhibit *MIF* gene expression, and suggests that macrophage migration inhibitory factor inhibition by histone deacetylase inhibitors may contribute to the antitumorigenic effects of histone deacetylase inhibitors.



## 1. Introduction

Macrophage migration inhibitory factor (MIF), one of the first cytokine activity identified [1,2], is a proinflammatory mediator that plays a central role in the development of inflammation and innate and adaptive immune responses [3]. MIF has been implicated in the pathogenesis of acute and chronic infectious, inflammatory and autoimmune diseases. In addition, recent studies strongly support an important role for MIF in the control of cell growth and tumorigenesis at multiple levels. MIF stimulates cell proliferation through a sustained activation of the ERK1/2 MAPKs [4] and the induction of cyclin D1 (CCND1) [5] and promotes cell survival by inhibiting the p53 and retinoblastoma/E2F tumor suppressor pathways [6–8] and by activating the phosphoinositide-3-kinase (PI3K)/Akt survival pathway [9]. Moreover, MIF promotes tumor-associated neovascularization [10] and inhibits antitumor natural killer (NK) and cytotoxic T-lymphocyte (CTL) responses [11–14]. MIF also plays a role in the control of the response to DNA damage [15] and regulates tumor cell motility and invasion in a Rac1-dependent cell manner [16]. Finally, human cancer tissues, such as prostate, breast, colon, brain, skin and lung-derived tumors, have been shown to overexpress MIF [17,18]. Altogether, an accumulating body of evidence indicates that MIF have a crucial role in the development of inflammatory diseases and neoplasia.

The structure of chromatin is influenced by covalent modifications of amino-terminal tails of histones, particularly acetylation at lysine residues [19–21]. Acetylation is regulated by the opposing actions of histone acteyl transferases and histone deacetylases (HDACs). Increased histone acetylation has been linked to gene overexpression [22]. Transcription coactivators like p300/CBP and PCAF display acetyltransferase activity whereas transcription co-repressor complexes contain HDAC activity [23,24]. The current view is that histone acetylation induces chromatin scaffolding to assume a more relaxed, open structure increasing the accessibility of regulatory factors to DNA and favoring active gene transcription. Yet, several observations have challenged this traditional view. First, genome-wide expression

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studies have revealed that HDAC inhibitors (HDIs) affect the expression of a small subset of cellular genes with similar proportions of genes down-regulated and up-regulated [25–28]. Second, an increasing number of non-histone proteins among which transcription regulators have been shown to be modified by acetylation [21].

An abundant literature indicates that deregulation of acetylation contributes to abnormal gene expression observed in many forms of cancer. Therefore, HDACs are considered among the most promising targets for intervention expected to reverse abnormal epigenetic status in cancer cells [19–21]. In agreement, HDIs have shown encouraging therapeutical results in preclinical studies [29] and the broad spectrum HDI suberoylanilide hydroxamic acid (SAHA) has been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma [30]. The powerful and specific anticancer activities of HDIs result, to a great extent, from their capacity to induce differentiation, growth arrest and/or apoptosis of transformed cells. HDIs may also affect cancer development by modifying tumor angiogenesis, metastasis and invasion and antitumor immunity [19–21].

Considering that MIF is overexpressed in malignant cells and the role played by MIF in tumorigenesis, we hypothesized that MIF could be a target of HDIs. Here we report that HDIs strongly inhibit MIF expression in various cell lines, primary cells and *in vivo*. Moreover, we describe a new molecular mechanism by which TSA, a broad spectrum HDI, inhibits *MIF* expression through a local and specific deacetylation of histones associated with the proximal MIF promoter. Altogether this study expands our comprehension of the mechanisms by which HDIs inhibit gene expression and suggests that MIF is a target of the antitumorigenic effects of HDIs.

## 2. Materials and methods

### 2.1. Cells, mice and reagents

Human HeLa cervix epithelial cells and HaCat keratinocytes and mouse B16-10 melanoma were cultured in DMEM containing 4.5 g/l glucose and 10% glutamax (Invitrogen). Human HL-60 promyelocytic, U-937 promonocytic and THP-1 monocytic cell lines, KG1a promyeloblasts and A549 airway epithelial cells were cultured in RPMI 1640 medium containing 2 mM L-glutamine (Invitrogen). All media contained 10% heat-inactivated fetal calf serum (Seromed).

Animal procedures were approved by the Office Vétérinaire du Canton de Vaud (authorizations no. 876.5 and 876.6) and performed according to institution guidelines for animal experiments. Eight to ten-week-old female BALB/c mice were purchased from Charles River Laboratories (L'Arbresle, France). Mouse bone-marrow-derived macrophages were obtained as previously described [31]. Mice (3 animals per group) were injected intraperitoneally with TSA (10 mg/kg) or an equal volume of diluent every 24 h. After 2 days, blood was collected. MIF in plasma (8 μl) was analyzed by Western blotting.

TSA and valproic acid (VPA) and mithramycin A were purchased from Sigma-Aldrich and SAHA from ALEXIS Biochemicals. Recombinant MIF and anti-human MIF antibodies isolated from the sera of MIF-immunized New Zealand White rabbits were obtained as previously described [32]. HeLa cells were transduced with the empty control and MIF-expressing adenoviruses [16,33] at a multiplicity of infection of 100. The medium was replaced by fresh medium 1 h after viral infection, and incubation prolonged for 18 h before the addition of TSA.

### 2.2. mRNA analysis

Total RNA was isolated using the RNeasy kit (Qiagen). MIF and $p21^{WAF1/CIP1}$ (p21 or cyclin-dependent kinase inhibitor 1A, CDKN1A) mRNA steady state levels were assessed by Northern blotting using PCR amplified DNA probes (primers are listed in Table 1). Quantification of specific signals was performed using an Instant Imager 2024 (Packard). Total RNA was reverse transcribed using the ImProm-II Reverse Transcription System (Promega). MIF and hypoxanthine phosphoribosyltransferase 1 (HPRT) mRNA expression was analyzed by PCR. Quantitative PCR was performed with a 7500 Fast Real-Time PCR System using the Power SYBR Green PCR Master Mix (Applied Biosystems) as previously described [34]. MIF mRNA expression was expressed relative to HPRT mRNA expression in arbitrary units.

### 2.3. Western blot and co-immunoprecipitation analyses

Cells were lysed in Mammalian Protein Extraction Reagent (Pierce). To detect histones, acid-soluble proteins were extracted as previously described [35]. Cell-lysates and acid-insoluble proteins were electrophoresed through polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated with antihuman MIF, α-tubulin (T 5168, Sigma-Aldrich) and acetylated histones H3 and H4 antibodies (06-755 and 05-858, Millipore), washed and incubated with horse radish peroxidase (HRP)-conjugated secondary antibodies. Signals were revealed using the ECL Western blotting analysis system (GE Healthcare).

For co-immunoprecipitation studies, total cell extracts were obtained by incubating $10^7$ HeLa cells in a lysis buffer containing 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 4 mM Na3VO4 and 1 μg/ml leupeptin. 200 μg of proteins were pre-absorbed with Protein G-Sepharose ™ 4FF beads (Amersham). Supernatants were incubated overnight at 4 °C with acetylated-Lysine antibody (9441S, Cell Signaling Technology) and then with Protein G-Sepharose™ 4FF beads for an additional 3 h. The beads were washed and heated 3 min at 100 °C in electrophoresis sample buffer. Samples were analyzed by SDS-PAGE and Western blotting using anti-Sp1 (sc-59x, Santa Cruz Biotechnology) and HRP-conjugated immunopure rabbit antigoat IgG antibody (31402, Pierce).

MIF protein decay was measured in HeLa cells pre-incubated for 1 h with or without TSA (1 μM) and then cultured with or without cycloheximide (CHI, 10 μg/ml, Sigma-Aldrich). Protein extracts were collected at intervals and analyzed by Western blotting as described above.

**Table 1**
Oligonucleotides used in this study.

| | Oligonucleotide | 5′ -> 3′ sequence |
|---|---|---|
| Probe synthesis | MIF S | CACGCTCGCAGTCTCTC |
| | MIF AS | GAGGCTCAAAGAACAGC |
| | p21 (CDKN1A) S | GACACCACTGGAGGGTGACT |
| | p21 (CDKN1A) AS | GGATTAGGGCTTCCTCTTGG |
| Nuclear run-on | MIF NRO S | CCGGACAGGGTCTACATCAA |
| | MIF NRO AS | AATTCTCCCCACCAGAAGGT |
| | ACTB NRO S | TAAGGAGAAGCTGTGCTAGG |
| | ACTB NRO AS | TTGCTGATCCACATCTGCTG |
| EMSA | $Sp1^P$ wt | GCCTCGCGGGGGCGGGGCCTGGCG |
| | $Sp1^P$ mt | GCCTCGCGGGTAGAGGGCCTGGCG |
| | CRE wt | GCGGTGGCGTCACAAAAGGCGG |
| | CRE mt | GCCGGCGGTGGATACACAAAAGG |
| PCR | MIF S | GGTCTCCTGGTCCTTCTGC |
| | MIF AS | TGCACCGCGATGTACTGG |
| | Ad-MIF S | GCCAGAGGGGTTTCTGTC |
| | Ad-MIF AS | GTTCGTGCCGCTAAAAGTCA |
| | HPRT S | GAACGTCTTGCTCGAGATGTG |
| | HPRT AS | CCAGCAGGTCAGCAAAGAATT |
| ChIP and CHART | MIF S3 | TCCCAGCATCCTATCCTCTT |
| | MIF S8 | CGGTGACTTCCCCACTCG |
| | MIF AS1 | GGCACGTTGGTGTTGTTTACGAT |
| | MIF AS3 | TGGGGATGCGGCGGTGAACC |

Base substitutions are underlined.
S: sense; AS: antisense; wt: wild-type; mt: mutant.

### 2.4. Whole blood MIF secretion assay

Whole blood (200 μl) from healthy donors was diluted one in five in RPMI medium with or without TSA and incubated 20 h at 37 °C in a humidified incubator containing 5% $CO_2$. Samples were centrifuged at 3000×*g* for 3 min. MIF levels in supernatants were measured by ELISA as previously described [36].

### 2.5. Nuclear run-on

Nuclei isolation and *in vitro* transcription using biotin-16-UTP (Roche) were performed as described [37]. RNA was isolated using TRIzol® (Invitrogen), extracted with chloroform, precipitated with isopropanol and resuspended in 50 μl sterile water. Total RNA (10 μl) was conserved to monitor RNA input. Purification of biotinylated RNA transcripts was performed using the μMACS Streptavidin Kit according to the manufacturer's instructions (Miltenyi Biotec). Input RNA and biotinylated RNA were used for cDNA synthesis with random hexamer primers. The level of MIF and β-actin cDNA in the preparations was determined by PCR using specific oligonucleotides (Table 1). The PCR conditions were as follows: 95 °C 15 min, followed by 40 cycles consisting of 95 °C 30 s, 56 °C 30 s and 72 °C 1 min. PCR products were analyzed by electrophoresis through agarose gels.

### 2.6. Transient transfection

The MIF promoter driven luciferase vectors have been described in details [38]. HeLa cells ($5\times10^4$) were platted in 24-well culture plates. The following day, cells were transiently transfected using Fugene™ 6 transfection reagent (Roche Diagnostics) and 100 ng of luciferase reporter vectors [39]. Six hours later, transfected cells were exposed to the drugs. After 18 h, cells were harvested. Luciferase activity was measured using the Dual-Luciferase™ Reporter Assay System (Promega).

### 2.7. Chromatin immunoprecipitation (ChIP)

Cells were treated with 1% formaldehyde for 30 min at 37 °C, harvested, washed twice with cold PBS containing 1 mM PMSF and 0.9 μg/ml aprotinin, lysed in 50 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, 1 mM PMSF and 0.9 μg/ml aprotinin (200 μl per $10^6$ cells) and sonicated using a Soniprep 150 (MSE) sonicator (5 × 30 s, amplitude 14). Cleared lysates (200 μl) were diluted 10-fold in 1% Triton X-100, 150 mM NaCl, 20 mM Tris–HCl pH8.0, 2 mM EDTA pH 8.0, 1 mM PMSF and 0.9 μg/ml aprotinin. Twenty microliters were kept for controls (input DNA). The rest of the preparation was incubated overnight at 4 °C with 2.5 μg of control antibody (sc-2027, Santa Cruz Biotechnology) or anti-acetylated histones H3 and H4 (06-599 and 06-866, Upstate), anti-phosphorylated CREB (9198, Cell Signaling Technology), anti-Sp1 (07-645, Upstate) and anti-RNA polymerase II (sc-9001x, Santa Cruz Biotechnology) antibody. One hundred microliters of Protein A Sepharose slurry (GE healthcare) were added to the lysates and the mixtures were incubated 2 h at 4 °C. Beads were extensively washed and immune complexes were eluted in 450 μl 1% SDS and 100 mM $NaHCO_3$. Input DNA and immunoprecipitated DNA were reverse-crosslinked, purified by phenol:chloroform:isoamyl alcohol extraction, precipitated with ethanol and resuspended in 50 μl of water. MIF promoter sequence was detected by PCR using the S8 and AS1 primers (Table 1). The PCR conditions were as for nuclear run-on. p21 promoter sequence was detected as previously described [40]. Signals were quantified using an Eagle eye video imaging system (Stratagene).

### 2.8. HDAC activity assay

HDAC activity in 50 μg of nuclear proteins was measured using the HDAC assay kit (Millipore and BioVision) according to the manufacturer's recommendations.

### 2.9. Chromatin accessibility by real-time PCR (CHART-PCR)

Cells were incubated 10 min on ice in 15 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 0.2% NP40 and 5% sucrose. The suspension was layered on a 10% sucrose cushion. After centrifugation, the nuclei were recovered in 15 mM NaCl, 10 mM Tris pH 7.4, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine and 8.5% sucrose. Nuclei from $10^6$ cells were diluted in the recommended 1× restriction enzyme dilution buffer and 1 U/μl of *Dra*I or *Not*I. The reaction mixture was incubated 1 h at 37 °C. A non-digested control was included in the assay as well as a purified DNA positive control. Genomic DNA was isolated using the DNeasy Tissue kit (Qiagen), eluted in water, digested to completion with *Xma*I and used in real-time PCR. Quantitative PCR was performed with a Light Cycler (Roche) using the SYBR®-Green PCR assay (Qiagen) and primer pairs S3/AS3 and S8/AS1 (Table 1) overlapping the region of DNA targeted by restriction enzymes (Fig. 6). PCR conditions were as for nuclear run-on. Results of restriction digest were normalized to the intact region and expressed as percentage of non-digested DNA.

## 3. Results

### 3.1. HDIs down-regulates MIF expression

We used TSA, a broad spectrum prototypical HDI [19], to examine whether HDIs affect MIF expression. Analyses of MIF mRNA steady state levels in HeLa epithelial cells revealed that TSA decreased MIF expression in a time- and dose-dependent manner (Fig. 1A and B). Of note, other HDIs such as SAHA and valproic acid (VPA) inhibited MIF mRNA expression in HeLa cells (Fig. 1C). TSA strongly reduced MIF mRNA expression also in HaCat keratinocytes, HL-60, KG1a and U-937 leukemic cell lines, THP-1 monocytic cells, A549 airway epithelial cells and B16 melanoma (Fig. 1D and data not shown). Conversely, TSA was shown to reactivate the expression of *p21* (*CDKN1A*) in all cell lines (Fig. 1A and D), indicating that the effect on MIF mRNA expression was specific. In line with a strong inhibition of MIF mRNA expression, TSA decreased intracellular MIF protein content in U-937, HaCat and A549 cells in a time-dependent manner (Fig. 1E and data not shown). TSA also strongly decreased MIF mRNA level in mouse bone-marrow-derived macrophages (Fig. 1F) and MIF release in human whole blood (Fig. 1G). Strengthening these *in vitro* observations, injection of TSA in mice (10 mg/kg intraperitoneally q24 h for 2 days) reduced MIF blood levels two-fold (Fig. 1H). Overall, these data demonstrated that HDIs inhibit MIF mRNA and protein expression in cells lines, primary cells and *in vivo*.

### 3.2. TSA decreases MIF gene transcription

Nuclear run-on assays were used to determine whether TSA inhibited MIF mRNA expression via a transcriptional mechanism. Nuclei of U-937 cells cultured with or without TSA were isolated and subjected to *in vitro* transcription using biotinylated UTP. Neo-synthesized biotinylated messengers were isolated, reversed transcribed and amplified by PCR using MIF and β-actin specific oligonuleotides. The level of nascent MIF, but not β-actin, mRNA was substantially reduced upon treatment with TSA (Fig. 2). These data definitively indicated that TSA decreased MIF mRNA expression by inhibiting the transcription of the *MIF* gene.

### 3.3. TSA targets endogenous MIF gene expression, but not ectopically expressed MIF

To test whether TSA impaired *MIF* transcription by interfering with MIF promoter activity, we first thought to analyze the transcriptional activity of MIF promoter (−858/+129, −308/+129,

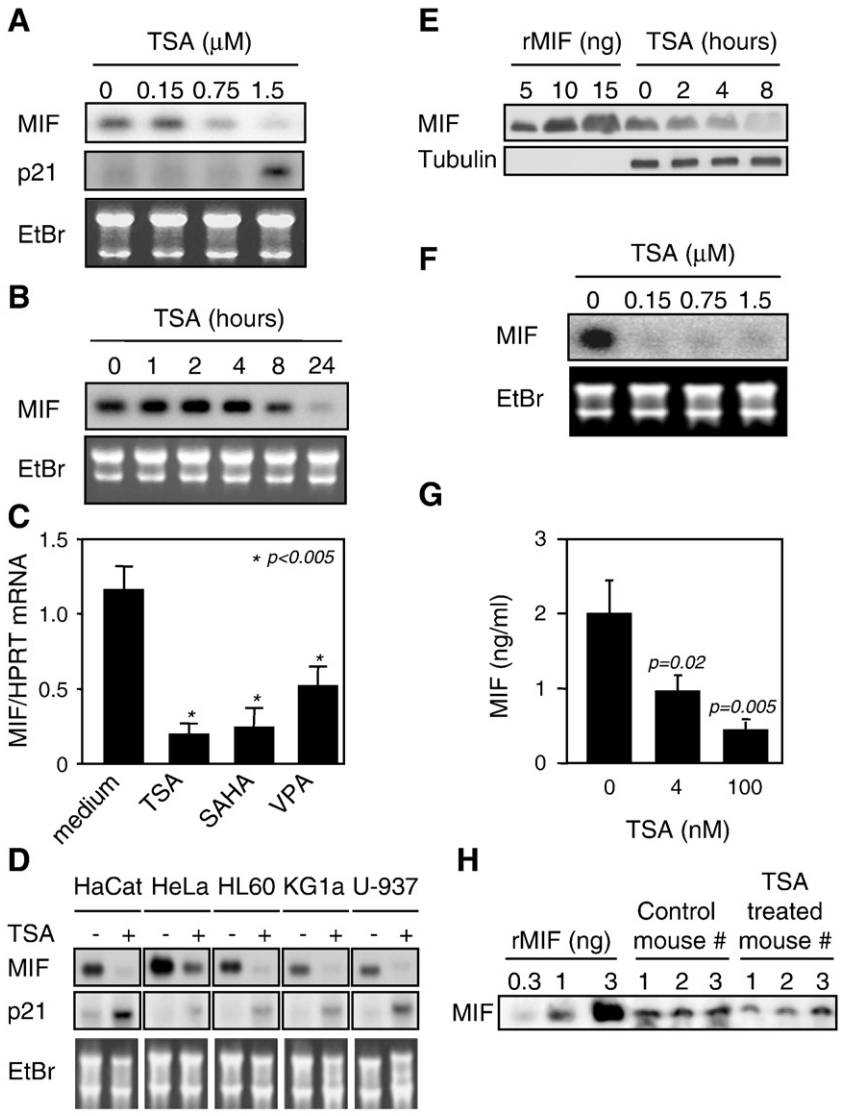


**Fig. 1.** TSA down-regulates MIF expression. (A, B) Northern blot analysis of MIF and p21 (CDKN1A) mRNA expression in HeLa cells cultured for 18 h with increasing concentrations of TSA (A) or cultured for increasing time with 1 μM TSA (B). Uniform RNA loading was verified by ethidium bromide (EtBr) staining of 28S and 18S RNA. (C) MIF mRNA expression in HeLa cells cultured 18 h with TSA (1 μM), SAHA (1 μM) and valproic acid (VPA, 1 mM) analyzed by real time-PCR. Results are expressed as the ratio of MIF to HPRT mRNA levels. Data are means ± S.D. of triplicate samples from one experiment ($p<0.005$ for HDIs versus medium by two-tailed paired Student's *t*-test). (D) MIF and p21 mRNA expression in HaCat, HeLa, HL60, KG1a and U-937 cells cultured for 18 h with or without TSA (1 μM). (E) Western blot analysis of intracellular MIF in U-937 cells treated for 0, 2, 4 and 8 h with TSA (1 μM). Recombinant MIF (rMIF) was used as a standard. (F) MIF mRNA expression in mouse bone-marrow-derived macrophages cultured for 24 h with increasing concentrations of TSA. Results are representative of at least two independent experiments (A-F). (G) Whole blood was incubated for 24 h with TSA. MIF secretion was measured by ELISA. Data are means ± S.D. of triplicate samples from one experiment ($p=0.02$ and 0.005 for 4 and 100 nM TSA compared to medium by two-tailed paired Student's *t*-test). (H) BALB/c mice ($n=3$) were injected with TSA (10 mg/kg i.p. q24h) or diluent (control) for 2 days. Blood was collected and a fixed volume of plasma was used to analyze MIF expression by Western blotting.

−157/+129, −81/+129 and +44/+129) luciferase reporter vectors in HeLa cells. In agreement with high MIF mRNA expression in resting HeLa cells, all but one construct (+44/+129) drove strong luciferase activity (Fig. 3A). In contrast to expectations, TSA treatment strongly increased luciferase activity driven by all MIF promoter reporter constructs active at baseline. The proximal region of the MIF promoter contains several DNA-binding sites among which are one c-Myb, two Sp1 ($Sp1^p$ and $Sp1^d$) and two cAMP response element ($CRE^p$ and $CRE^d$) sites (Fig. 3B). We previously reported that Sp1 and CREB bind to the $Sp1^p$ and $CRE^p$ sites to positively regulate MIF promoter activity [38]. To analyze their involvement in TSA-induced transcriptional activity of MIF promoter, the c-Myb, Sp1 and CRE sites were disrupted in −157/+129 construct (Fig. 3B). Mutation of the cMyb and CRE sites did not impact on TSA-induced MIF promoter activity. In contrast, disruption of the $Sp1^p$ site and, to a lesser extent, of the $Sp1^d$ site reduced MIF promoter induction by TSA. Furthermore, mithramycin A, an antibiotic that interferes with Sp1 binding to DNA, abrogated TSA-mediated increased activity of wild-type but not the $Sp1^p$ mutant MIF promoter (Fig. 3C), confirming that TSA increased episomal MIF promoter activity through Sp1 DNA binding. Overall, the effect of TSA on ectopically expressed MIF promoters did not recapitulate the inhibitory effect observed on endogenous *MIF* gene,

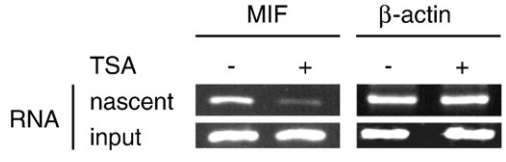


**Fig. 2.** TSA inhibits *MIF* gene transcription. Nuclei were isolated from U-937 cells treated for 8 h with or without TSA (1 μM) and subjected to *in vitro* transcription analysis. MIF and β-actin mRNA levels in elongated and initiated transcripts (nascent) and in total nuclear transcripts (input) were amplified by RT-PCR (see Materials and methods). PCR products were analyzed by gel electrophoresis.

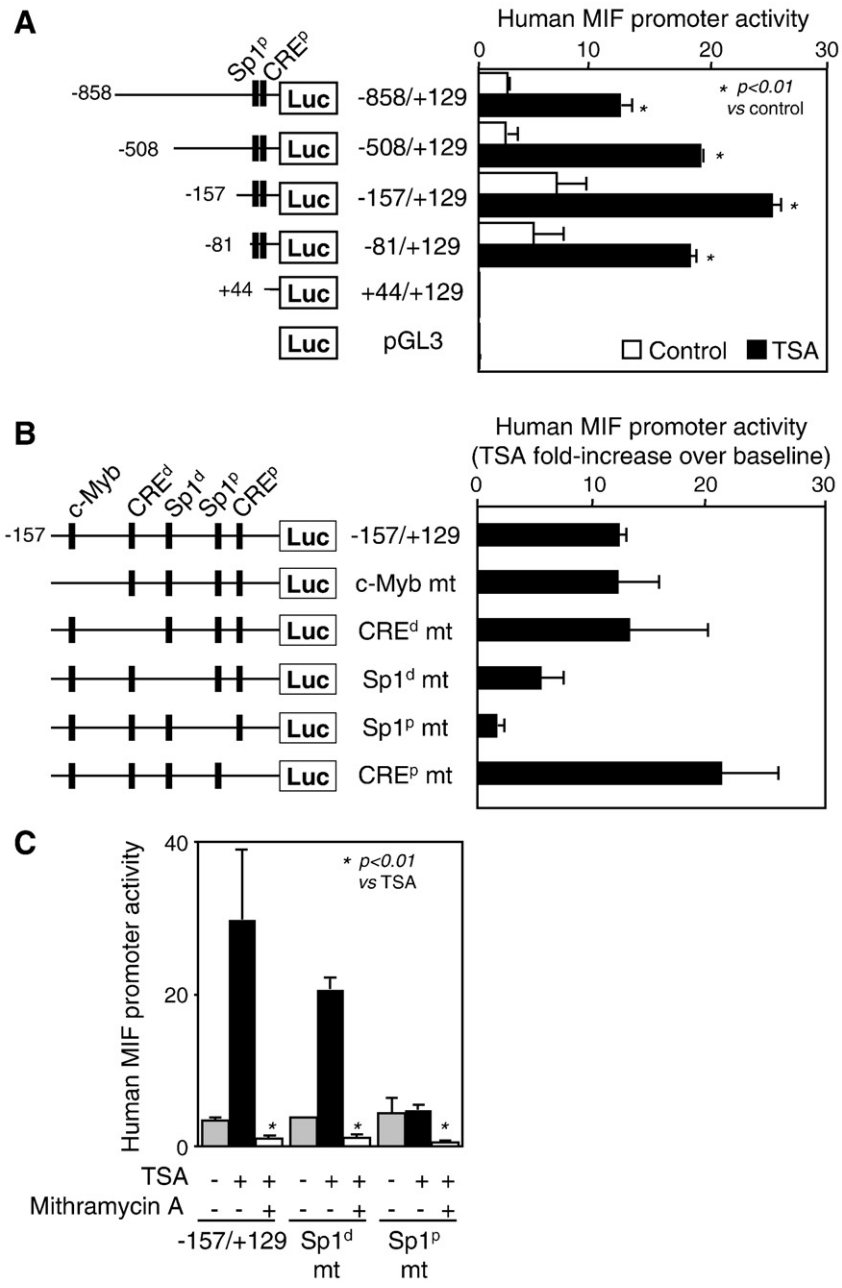


**Fig. 3.** TSA increases episomal MIF promoter activity. (A–C) HeLa cells were transfected with an empty pGL3 vector, human MIF promoter deletion constructs (−858/+129, −508/+129, −157/+129, −81/+129 and +44/+129) or the −157/+129 promoter construct with mutations (mt) in c-Myb, $CRE^d$, $Sp1^d$, $Sp1^p$ and $CRE^p$ DNA-binding sites. Of note, the Sp1 and CRE DNA-binding sites that control constitutive transcriptional activity of the *MIF* gene are deleted from construct +44/+129. After 6 h, cells were treated with or without TSA (1 μM) and mithramycin A (100 nM). Luciferase activity was determined 18 h latter (right panel). Data are means ± S.D. of six determinations from one experiment representative of two experiments. $p<0.01$ for medium versus TSA by two-tailed paired Student's *t*-test for −858/+129, −308/+129, −157/+129 and −81/+129 and for $Sp1^p$ mt versus the −157/+129.

which probably relies on the fundamental differences that exist between episomal reporter constructs and endogenous chromosomal genes.

To further demonstrate that TSA specifically inhibited endogenous MIF expression, we transduced HeLa cells with an adenoviral vector encoding *MIF* under the control of the CMV promoter. A PCR discriminating between endogenous MIF mRNA and adenoviral-derived MIF mRNA was used to evaluate the effect of TSA. As previously observed (Fig. 1), TSA dose-dependently inhibited the endogenous MIF mRNA steady state level (Fig. 4A). In contrast, TSA did not affect adenoviral-derived MIF mRNA expression. In agreement with these observations, TSA reduced MIF protein expression two-fold in cells transduced with the control adenovirus, whereas it slightly increased MIF protein level in cells transduced with the MIF-encoding adenovirus (Fig. 4B). Altogether, these data argued in favor of a direct inhibitory effect of TSA on endogenous *MIF* gene transcription.

### 3.4. TSA deacetylates the proximal MIF promoter

Because the association of trans-regulatory elements with promoters occurs in the context of chromatin, we tested whether TSA affected the acetylation status of histones H3 and H4 associated with the proximal MIF promoter using chromatin immunoprecipitation (ChIP) (Fig. 5A and B). Interestingly, TSA markedly reduced (two- to three-fold) the level of acetylated histones H3 and H4 associated with the MIF promoter in both U-937 and HeLa cells exposed to TSA. The

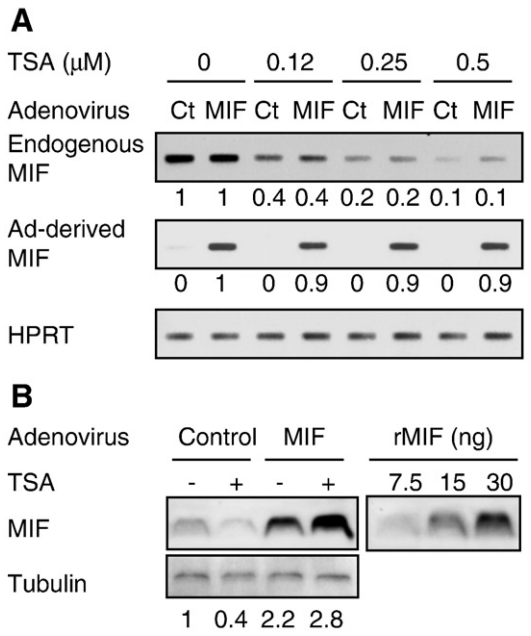


**Fig. 4.** TSA does not inhibit ectopically expressed MIF. HeLa cells were transduced with an empty control (Ct) or MIF-expressing adenovirus (Ad) and treated 18 h later with TSA (0.5 μM unless specified). RNA and proteins were recovered after 18 h. (A) Endogenous MIF, adenovirus (Ad)-derived MIF and HPRT mRNA expression levels were assessed by PCR. (B) MIF and tubulin expression was analyzed by Western blotting. Recombinant MIF (rMIF) was processed in parallel to estimate MIF content in HeLa cells. Results are representative of two independent experiments.

effect was specific of MIF promoter-associated histones since TSA increased the acetylation of histones H3 and H4 related to the p21 promoter (Fig. 5C). Moreover, as positive controls, we confirmed that TSA dose-dependently inhibited HDAC activity (Fig. 5D) at concentrations shown to inhibit MIF mRNA expression (Fig. 1) and raised global acetylation of histones H3 and H4 (5.2- and 8.3-fold for histones H3 and H4, Fig. 5E) in TSA-treated cells. Therefore, deacetylation of the histones H3 and H4 associated with the MIF promoter was an MIF-specific HDI effect.

To determine whether TSA-induced inhibition of MIF mRNA expression and MIF promoter histone acetylation required newly synthesized factors, U-937 cells were pretreated for 1 h with cycloheximide before exposure to TSA. Cycloheximide given alone gradually decreased MIF mRNA levels over time, indicating that MIF mRNA expression required protein synthesis. TSA alone decreased MIF mRNA levels similarly to CHI. The combination of cycloheximide plus TSA increased MIF mRNA levels at an early time point (1.6-fold increase after 2 h of treatment) but decreased MIF mRNA levels after 8 and 16 h as observed with cycloheximide or TSA (Fig. 6A). Cycloheximide did not modify the acetylation of MIF promoter-associated histones H3 and H4, whereas it counter-acted TSA-mediated inhibition of histone acetylation (Fig. 6B). Thus, TSA decreased MIF promoter histone acetylation and MIF mRNA expression through a molecular mechanism requiring de novo protein synthesis.

### *3.5. TSA does not modify chromatin accessibility to the proximal MIF promoter*

The proximal region of the MIF promoter contains several DNA-binding sites among which are one Sp1 (at −42 bp) and one cAMP response element (CRE, at −20 bp) sites that we previously reported to bind Sp1 and CREB to positively regulate MIF promoter activity [38]. We therefore developed a chromatin accessibility by real-time PCR (CHART-PCR) assay to test whether hypoacetylation of MIF-associated histones rendered the MIF promoter less accessible to Sp1 and CREB. Nuclei were isolated and incubated with *Dra*I and *Not*I that recognize sequences present in the distal and proximal regions of the MIF promoter (at nucleotide −528 and +41, respectively, Fig. 7A). Genomic DNA was subjected to real-time PCR amplification using primer pairs surrounding the *Dra*I and *Not*I restriction sites. The amount of material amplified was inversely proportional to the

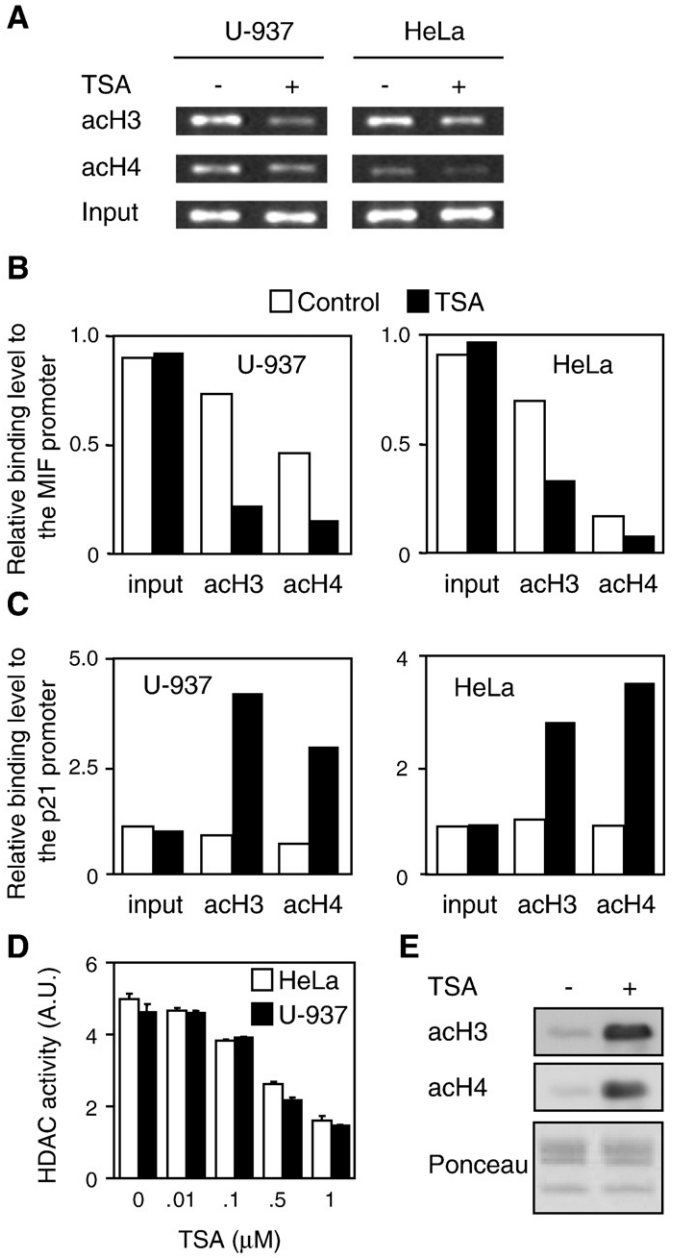


**Fig. 5.** TSA deacetylates the histones associated with the proximal region of the MIF promoter. (A–C) U-937 and HeLa cells were treated for 10 h with or without TSA (1 μM) and analyzed by ChIP assay using anti-acetylated (ac) H3 and anti-acH4 antibodies. Input and immunoprecipitated DNA were subjected to PCR amplification using primers specific of the promoter region of the *MIF* (A, B) and *p21* (C) genes. PCR were analyzed by gel electrophoresis (A) and signals quantified by densitometry (B, C). Results are representative of two independent experiments. (D) HDAC activity in nuclear extracts from HeLa and U-937 cells treated for 10 h with increasing concentrations of TSA (0–1 μM). Data are means ± S.D. of duplicate samples from one experiment. (E) Histones were extracted from U-937 cells treated for 10 h with or without TSA (1 μM) and subjected to Western blot analysis using anti-acH3 and anti-acH4 antibodies. The gel was colored with Ponceau Red to visualize total histones. Results are representative of three independent experiments.

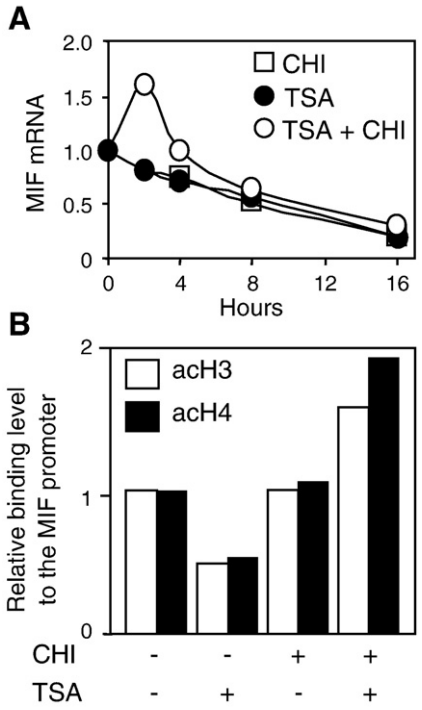


**Fig. 6.** TSA decreases MIF promoter histone acetylation through a molecular mechanism requiring de novo protein synthesis. Quantification of MIF mRNA expression determined by Northern blotting (A) and ChIP assay of acH3 and acH4 histones associated with the proximal MIF promoter (B) in U-937 cells cultured for 1 h with or without cycloheximide (CHI, 10 μg/ml) and then incubated for 10 h with or without TSA (1 μM). Results are representative of two independent experiments.

restriction digestion of DNA, and therefore to the accessibility of the chromatin. In agreement with the localization of important transcriptional regulatory elements in the vicinity of the *MIF* transcriptional start site, the proximal region of the MIF promoter (examined by *Not*I digestion) was highly accessible in both U-937 and HeLa cells. Conversely, the distal region (examined by *Dra*I digestion) was much less accessible (Fig. 7B and C). When cells were incubated with TSA, the accessibility to the proximal region of the MIF promoter slightly increased in U-937 cells, whereas it was not modified in HeLa cells (right panels in Fig. 7B and C). Finally, the accessibility to the distal region of the MIF promoter was not affected by TSA (left panels in Fig. 7B and C). Thus, remodeling of chromatin accessibility by TSA was not a major mechanism whereby TSA inhibited *MIF* gene transcription.

### *3.6. TSA decreases the recruitment of regulatory proteins to the proximal MIF promoter*

The hypoacetylation of the MIF proximal promoter induced by TSA could potentially affect the binding of transcription regulatory factors essential for basal *MIF* expression such as Sp1 and CREB [38] and thereby prevent efficient RNA polymerase II recruitment. To address this question, we analyzed by ChIP the level of Sp1, CREB and RNA polymerase II interacting with the proximal MIF promoter (Fig. 8A and B). Whereas Sp1, CREB and RNA polymerase II were shown to be bound to the MIF promoter in resting U-937 cells, TSA strongly reduced their binding to DNA, which provided a molecular mechanism by which TSA inhibited *MIF* gene transcription. Of note, Western blot analyses demonstrated that TSA did not reduce the quantity of nuclear Sp1 and CREB (Fig. 8C and D). Moreover, co-immunoprecipitation analyses confirmed that TSA increased Sp1 acetylation (Fig. 8D), which has been associated with Sp1 transcriptional activity [41]. Altogether, these results ruled out the possibility that TSA decreased Sp1 and CREB binding to the endogenous MIF promoter by affecting their expression level or Sp1 acetylation status.

## 4. Discussion

We report that HDIs down-regulate the expression of the proinflammatory and protumorigenic cytokine MIF through a new molecular mechanism involving a local deacetylation of MIF promoter-associated histones which does not modify chromatin accessibility but impairs the recruitment of the basal transcriptional machinery to the proximal MIF promoter. Although the relative contribution of specific HDACs in these process has not been determined, the observation that chemically unrelated HDIs inhibited MIF expression argues for an important contribution of HDAC in regulating MIF expression.

Histone acetylation has been traditionally seen as a positive marker for transcription. It is generally admitted that elevated histone acetylation level induces a more open, transcriptionally more active chromatin state. This notion is, however, hardly reconcilable with the fact that HDIs, albeit strongly raising global histone acetylation, induce transcriptional changes of only 2–10% of the transcriptome. Interestingly, comparison of the transcriptome of different cancer cell lines treated with HDIs has identified common subsets of genes being

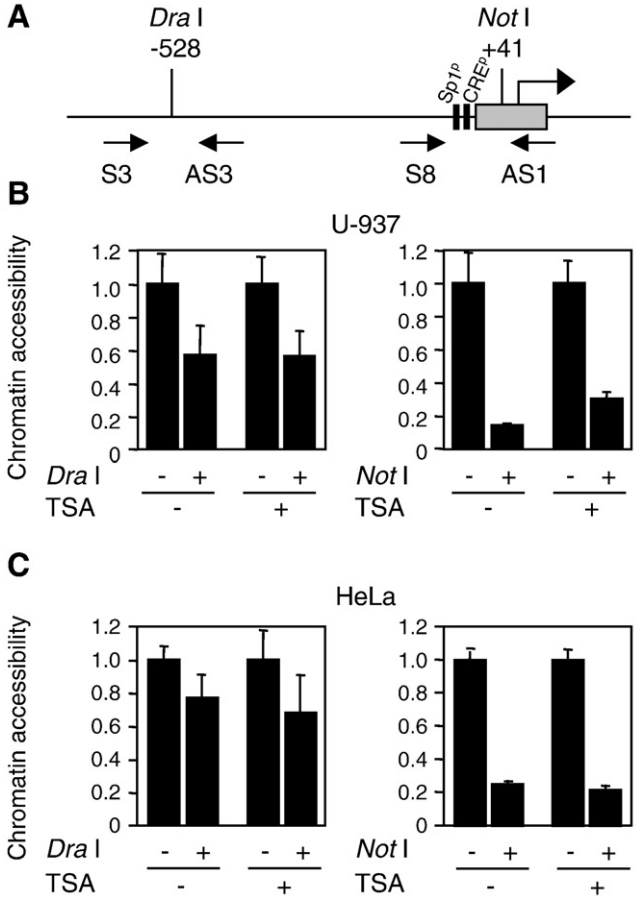


**Fig. 7.** TSA does not impair the accessibility to the chromatin associated with the MIF promoter. (A) Schematic representation of the MIF promoter showing MIF exon I (grey box), the translational start site (upper arrow), the Sp1 and CRE DNA-binding sites that regulate basal MIF promoter transcriptional activity, the *Dra*I and *Not*I restriction sites (nucleotide localization is relative to the transcriptional start site) and the relative localization of S3/AS3 and S8/AS1 primer pairs used to amplify by real-time PCR the DNA region surrounding the *Dra*I and *Not*I sites. (B, C) Nuclei isolated from U-937 cells (B) and HeLa cells (C) treated for 10 h with or without TSA (1 μM) were subjected to digestion with *Dra*I and *Not*I and analyzed by chromatin accessibility by real-time PCR. A non-digested control as well as a purified DNA positive control was included in the assays (data not shown). Data from restriction digests were normalized to those obtained without digestion set at 1. Data are means ± S.D. of three independent determinations. Similar results were obtained using cells incubated for 4, 10 and 24 h with TSA (data not shown).

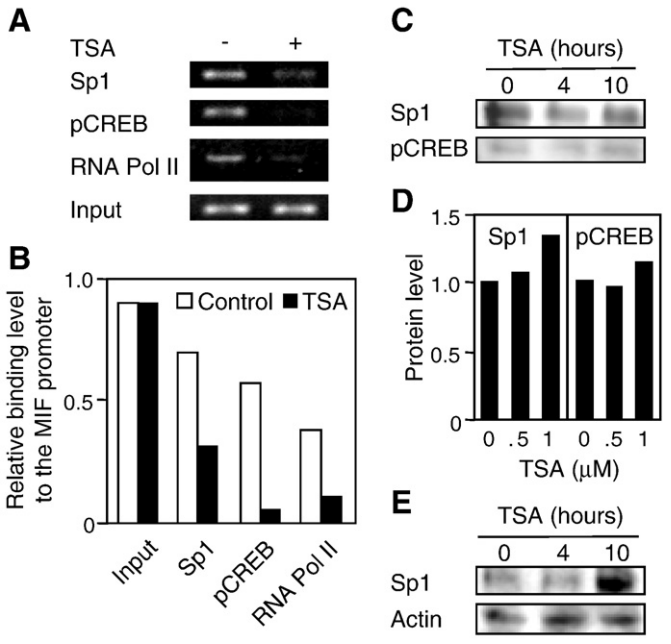


**Fig. 8.** TSA decreases the binding of Sp1, CREB and RNA polymerase II to the proximal region of the MIF promoter. HeLa cells were treated for 10 h with or without TSA (1 μM) and analyzed by ChIP assay using anti-Sp1, anti-phospho-CREB (pCREB) and anti-RNA polymerase II (RNA Pol II) antibodies. (A) The immunoprecipitated DNA was subjected to PCR amplification using primers specific for the proximal region of the MIF promoter. (B) Densitometric quantification of the gels shown in panel A. (C–E) Western blot analysis of Sp1 and phosphorylated CREB (pCREB) nuclear content (C, D) and co-immunoprecipitation study of acetylated Sp1 expression (D) in HeLa cells treated with TSA (1 μM in C, E) for the indicated time (C, E) or 10 h (D). Similar results were obtained in two independent experiments.

either activated or repressed, primarily genes encoding for proteins involved in cell cycle regulation, apoptosis, signaling and regulation of transcription [25–28]. The basis for this selectivity remains largely unknown and probably involves multiple components as HDIs modify the acetylation status of an increasing list of non-histone substrates including transcription factors and signal transduction mediators.

We found that MIF expression was strongly repressed by HDIs in a broad range of cell lines, including myeloid cells, epithelial cells, keratinocytes and melanoma. Considering that MIF is overexpressed in multiple kinds of tumors and that MIF expression levels have been associated with tumor aggressiveness and metastatic potential [12,42,43], MIF may represent a common target of the anticancer effects of HDIs.

While numerous studies have dissected the molecular mechanisms by which HDIs relieve gene expression, very little is known about how these drugs repress gene expression, particularly constitutive expression. Whereas TSA inhibited *MIF* gene transcription, it vigorously activated ectopically expressed MIF promoters concomitantly with an increased Sp1 acetylation. This effect was lost when the proximal Sp1 DNA binding site was mutated in MIF promoter reporter constructs, which is in fact in agreement with the observation that Sp1 acetylation increases its transcriptional activity [41]. Yet, of importance, these observations definitely illustrate the fundamental differences that exist between episomal reporter constructs and endogenous chromosomal genes and the risk of drawing conclusions from the analysis of transfected reporter gene exposed to chromatin remodeling agents [44].

In the context of HDAC inhibition, Sp1 regulatory elements, which we previously demonstrated to regulate MIF promoter activity [38], may have a dual role on ectopically expressed promoters. Indeed, Sp1 sites both increased (inhibitor of Cdk4, HMG-CoA synthase, CDKN1A and EC-SOD) [45–48] and inhibited (BCL2 and TGFβ type II receptor) [49,50] promoter activity upon TSA treatment. The dual role of Sp1 sites might reflect differences in promoter specific sequences allowing the recruitment of additional transcriptional co-regulators (activators or repressors), acetylation-dependent modifications of Sp1 activity, changes in chromatin architecture or even the cell type considered.

Considering that TSA strongly increased overall histone acetylation, it was unanticipated to detect a local deacetylation of histones associated with the proximal MIF promoter in cells exposed to TSA. MIF is one of the very rare examples of promoter deacetylation by HDIs, with that of *high-mobility-group A2* (*HMGA2*) in NIH-3T3 cells [51] and of *BCL2* in human lymphoma [49]. The local deacetylation of these promoters, while in apparent contradiction with the pro-acetylation properties of HDIs, is actually concordant with the fact that HDIs repressed *MIF*, *HMGA2* and *BCL2* gene expression. Genome-wide ChIP experiments (ChiP on Chip) combined with transcriptome analyses will be required to have a comprehensive view of the reciprocal influence of HDIs on histone acetylation and gene expression.

Transcriptome analyses have revealed that inhibition of protein synthesis by CHI prevents most of the effects of HDIs on gene expression [52]. In line with this observation, CHI prevented histone deacetylation by TSA. Co-treatment with TSA and CHI up-regulated MIF mRNA expression early on, whereas TSA-mediated repression of MIF mRNA expression required several hours. These data suggest that inhibition of MIF mRNA synthesis by TSA is not a direct effect but presumably belongs to a secondary response determined by *de novo* synthesis of transcriptional repressors that remain to be identified. Of note, we observed that TSA reduced MIF protein half-life from more than 18 h in HeLa cells cultured with CHI with or without pre-incubation for 1 h with TSA, to 4.5 h in cells cultured with TSA alone. Therefore, TSA not only affected *MIF* transcription, but also stimulated MIF protein degradation through a process requiring protein synthesis.

Considering that TSA reduced MIF promoter histone acetylation, we speculated that this DNA region would be less accessible to Sp1 and CREB which positively regulate constitutive *MIF* gene expression [38]. Whereas our ChIP experiments confirmed that TSA reduced the binding of Sp1, CREB and RNA polymerase II to the MIF promoter, analysis of chromatin accessibility by real-time PCR clearly demonstrated that the accessibility to the proximal region of the MIF promoter was not affected by TSA. This unique situation challenges the concept that hypoacetylated chromatin forms a compact structure [24]. Since TSA did not impact on Sp1 and CREB expression levels, further work will be required to test whether TSA displaces the binding of Sp1 and CREB from the MIF promoter through a direct modification of these proteins affecting their DNA binding capacity or through the recruitment of a transcriptional repressor competing with, or disrupting, Sp1 and CREB DNA binding.

MIF promotes cell proliferation and survival through activation of the PI3K/Akt survival pathway and through inhibition of the p53/CDKN1A and retinoblastoma/E2F tumor suppressor pathways [6–8]. Conversely, HDIs have been reported to induce tumor cell apoptosis by inhibiting Akt signaling which is constitutively activated in many types of cancer [53–55]. Moreover, HDIs most invariably increase tumor expression of CDKN1A in either a p53-dependent or a p53-independent manner. Therefore, it is tempting to speculate that inhibition of MIF expression by HDIs is a significant contributor of the pro-apoptotic effects of HDIs.

The inflammatory cells and mediators in the tumor microenvironment participate in neoplastic processes, orchestrating survival, proliferation and migration of malignant cells, promoting angiogenesis and altering antitumor adaptive immune responses and response to cancer therapy [56,57]. Besides its effect on cell proliferation and survival, MIF has been reported to promote tumor-associated angiogenesis [10] and to inhibit antitumor NK and CTL responses [11–14]. Following these observations and considering the well-established central role played by MIF in acute and chronic

inflammatory responses [3], inhibition of MIF-mediated inflammation by HDIs may represent an important facet by which HDIs interfere with tumorigenesis.

Recent studies converge to attribute MIF an important role in tumor development. Transplantation of neuroblastoma, ovarian cancer and melanoma cell lines engineered to express reduced levels of MIF resulted in delayed or reduced tumor establishment, progression and metastasis and improved animal survival [14,58–60]. Similarly, the development of chemically induced fibrosarcoma and muscle invasion of bladder cancer was impaired in $MIF^{-/-}$ compared to wild-type mice [61,62]. Considering that TSA reduced MIF blood levels in mice (Fig. 1H), it will be of interest to analyze whether the anticancer activity of HDIs is associated with decreased MIF expression *in vivo*.

In conclusion, we report that HDIs inhibit *MIF* gene expression through a local deacetylation of MIF promoter-associated histones that affects the recruitment of the basal transcription machinery. MIF has a unique spectrum of biological activities, which positions MIF as a central mediator of inflammation and innate immunity and as an important contributor to cell proliferation, malignant transformation and angiogenesis. Bringing together the observations that MIF expression levels correlate with tumor aggressiveness and metastatic potential, that altered expression of HDACs contributes to tumor development and that HDIs powerfully inhibit MIF expression, we propose that MIF could represent a candidate for potential targeted or adjunctive anticancer therapy.

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