Trichostatin A induces 5-lipoxygenase promoter activity and mRNA expression *via* inhibition of histone deacetylase 2 and 3

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Abstract

The 5-lipoxygenase (5-LO) is the key enzyme in the formation of leukotrienes. We have previously shown that the histone deacetylase (HDAC) inhibitor trichostatin A (TSA) activates 5-LO transcription *via* recruitment of Sp1, Sp3 and RNA polymerase II to the proximal promoter. To identify the HDACs involved in the regulation of 5-LO promoter activity isoform-specific HDAC inhibitors were applied. 5-LO promoter activity and mRNA expression were up-regulated by the class I HDAC inhibitors apicidin and MS-275 but not by class II inhibitors. Knockdown of HDAC 1, 2 and 3 revealed that HDAC2 and HDAC3 but not HDAC1 is involved in the up-regulation of 5-LO mRNA expression. To analyse the chromatin modifications at the 5-LO promoter associated with HDAC inhibition, the time course of 5-LO mRNA induction by trichostatin A was investigated and the concomitant changes in histone modifications at the 5-LO promoter in HL-60, U937 and Mono Mac6 cells were determined. Chromatin immunoprecipitation analysis revealed that trichostatin A increases acetylation of histones H3 and H4 at the 5-LO core promoter in HL-60 and U937 cells whereas no significant changes were observed in Mono Mac6 cells. The appearance of H3 and H4 acetylation preceded the 5-LO mRNA induction whereas in all three cell lines, induction of 5-LO mRNA expression correlated with histone H3 lysine 4 trimethylation (H3K4me3), a marker for transcriptional activity of gene promoters.

Keywords: 5-lipoxygenase ● epigenetics ● histone deacetylase inhibitor ● trichostatin A ●
chromatin immunoprecipitation ● histone acetylation

Introduction

The human 5-LO catalyses the formation of leukotrienes, which represent mediators of inflammation and allergy. The 5-LO pathway is involved in innate immune responses but also associated with diseases such as atherosclerosis, asthma and certain types of cancer. 5-LO expression is mainly restricted to B-lymphocytes and cells of myeloid origin such as granulocytes, monocytes, mast cells and dendritic cells [1, 2]. The 5-LO promoter possesses a unique GC-rich region which contains consensus sequences for the transcription factors Sp1 and Egr-1 (GC-boxes) and which are important for basal transcriptional activity [3]. The promoter region contains eight GC-boxes, but lacks TATA and CAAT boxes. As such, it resembles promoters of typical housekeeping genes, which is in contrast to the cell type specific expression of the gene. In studies with 5-LO positive (HL-60, Mono Mac6) and 5-LO negative myeloid cell lines (U937), it was found that DNA methylation strongly down-regulates 5-LO promoter activity [4]. In myeloid cells, 5-LO expression and activity is strongly up-regulated during cell differentiation by calcitriol and transforming growth factor-β (TGF-β) [5]. We found that these effects are promoter-independent and are due to stimulation of 5-LO transcript elongation and maturation [6]. In contrast to these observations, induction of 5-LO mRNA expression by the HDAC inhibitor TSA is mainly due to 5-LO promoter activation [7]. Induction of 5-LO promoter activity by TSA correlates with the recruitment of the transcription factors Sp1 and Sp3 to the 5-LO core promoter and depends on an Sp1

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binding site close to the transcription start site [8]. Histone deacetylase enzymes deacetylate histones as well as other proteins such as transcription factors and are divided into different classes, namely class I (comprising HDACs 1,2,3,8), class II (4,5,6,7,9,10) and class IV (11) [9].

Inhibition of classes I and II HDACs by TSA leads to profound changes in the acetylation status of histones and other proteins such as transcription factors involved in the regulation of gene transcription. Histone acetylation is the most prominent epigenetic marker for active transcription and the level of histone acetylation usually correlates with the transcriptional activity of genes [10]. Histone acetylation is catalysed by histone acetyl transferases (HATs), which promote gene activation. Histone deacetylases induce repression of gene expression by the reduction of histone acetylation and the dynamic interaction of HATs and HDACs determines the transcriptional activity.

In addition to acetylation, methylation of histones at certain amino acid residues also contributes to the so-called histone code that is linked to the regulation of gene expression. Histone H3 lysine 4 trimethylation (H3K4me3) represents an important marker for transcriptional activity in fully active promoters, whereas H3K4me2 is linked to basal transcription [3, 11]. H3K4 methylation is catalysed by MLL family of proteins [12]. Histone H3 lysine 9 monomethylation (H3K9me) is considered to mark transcriptional repression [13].

To identify the HDAC subtype that is involved in 5-LO gene activation, subtype-specific HDAC inhibitors were used and knockdown experiments were performed. Both approaches revealed that up-regulation of 5-LO promoter activity and mRNA expression by HDAC inhibitors is due to inhibition of HDAC2 and HDAC3. To analyse the changes in chromatin markers in the 5-LO promoter, histone H3 and H4 acetylation (acH3, acH4) and H3 lysine 4 trimethylation (H3K4me3) was determined at the 5-LO promoter after HDAC inhibition. An increase in the acetylation status of the core histone proteins H3 and H4 was found by chromatin immunoprecipitation (ChIP) assays in TSA-treated HL-60 and U937 but not in Mono Mac6 cells. This change in histone acetylation preceded the induction of 5-LO mRNA. In all three investigated cell lines the presence of H3K4me3, a marker for transcriptional activity, correlated with the induction of 5-LO mRNA suggesting that the recruitment of the MLL complex is a critical event in the up-regulation of 5-LO promoter activity.

Materials and methods

Cells and culture

HL-60 cells (DSMZ, ACC 3) and U937 cells (DSMZ, ACC 5) were grown at 37°C with 6% CO_2 in RPMI 1640 medium + 10% (v/v) foetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml). Mono Mac6 cells (DSMZ, ACC 124) were grown in RPMI 1640 medium supplemented with 10% (v/v) FCS, 1× non-essential amino acids, sodium pyruvate (1 mM),

oxalacetate (1 mM), insulin (10 μg/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). HeLa cells were grown in DMEM supplemented with 10% (v/v) FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml).

Reagents

Trichostatin A (#T8552; Sigma-Aldrich, Taufkirchen, Germany) and cycloheximide (#239763, CHX; Calbiochem, Darmstadt, Germany) were dissolved in ethanol 96%. apicidin (A8851; Sigma-Aldrich), MS-275 (ALX-270-378; Axxora, Lörrach) and SB-379278A (CAS# 167897-35-0; Best West Laboratories, Salt Lake City, USA) were dissolved in DMSO. MC-1568 was kindly provided by Prof. Antonello Mai, Rome, and dissolved in methanol.

RNA extraction and cDNA synthesis

Two micrograms of total RNA were extracted from U937, HL-60 or Mono Mac6 cells by the Total RNA Mini Kit from Omega, Darmstadt; (R6834-02). RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (170-8897; Biorad, Munich, Germany) according to the manufacturer's protocol.

Real-time PCR analysis for detection of time-dependent induction of 5-LO mRNA expression

Quantitative PCR analysis was performed with a MyiQ cycler (Biorad). The sequences of the 5-LO primers were 5′-GTTCCTGAATGGCTGCAAC (forward) and 5′- GGCAATGGGGACAATCTTG (reverse). Results were normalized to riboprotein P0 Ct values. Sequences of the riboprotein P0 primers were as follows: 5′-AGATGCAGCAGATCCGCAT (forward) and 5′-GTGGT-GATACCTAAAGCCTG (reverse). Each sample was set up in triplicates. The expression was quantified by comparative $\Delta\Delta C_t$ method.

Determination 5-LO mRNA expression in the HDAC knockdown cell lines

Quantitative PCR analysis of the knockdown cells was performed with a StepOne PCR system (Applied Biosystems, Darmstadt, Germany). The 5-LO primer pair is described earlier. Quantitative results for 5-LO mRNA levels were obtained using a 5-LO expression plasmid (pcDNA-5LO). These results were related to absolute β-actin mRNA levels that were obtained using a plasmid (pcDNA-actin). β-Actin primer sequences were: 5′-CGGGACCTGACTGACTACCTC (forward) and 5′-CTTCTCCTTATTGT-CACGCACG (reverse). Similarly, samples were quantified in triplicates.

Western blot analysis of HDAC knockdowns in mono Mac6 cells

For detection of the knockdown efficiency of HDACs, cell pellets (6×10^6 cells) were lysed by ultrasonification (3 × 15 sec.). The samples were mixed with the same volume of 2× SDS-PAGE loading buffer, heated for 5 min. at 95°C and analysed for HDAC proteins by SDS-PAGE and Western blotting using the following antibodies: HDAC1 (sc-7872, dilution 1:200;

Santa Cruz Biotechnology, Heidelberg, Germany), HDAC2 (sc-7899, dilution 1:200; Santa Cruz Biotechnology), HDAC3 (sc-11417, dilution 1:200; Santa Cruz Biotechnology) and β-actin (sc-1616, dilution 1:1000; Santa Cruz Biotechnology). For detection, the Odyssey® imaging system was used (LI-COR; Bioscience, Bad Homburg, Germany).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed as described previously with some modifications [8]. Cells were incubated with 330 nM TSA for the indicated times before harvest. The collected immune complexes were washed twice with RIPA buffer (10 mM Tris-HCl pH 8.1, 1% Triton-X, 0.1% SDS, 0.25% sodium deoxycholate) and equilibrated with TE buffer (10 mM Tris-HCl pH8, 1 mM EDTA pH 8) before harvest. The antibodies used for immunoprecipitation were anti-acetyl-histone H3 (06-599; Upstate, Millipore Schwalbach), anti-acetyl-histone H4 (Upstate 06-866), anti-histone H3-K4 trimethyl (Upstate 07-473), anti-histone H3-K9 monomethyl (Upstate 07-450) and normal rabbit IgG (Santa Cruz Biotechnology, Sc-2027). The DNA was analysed by PCR (32 cycles) using the following primers: 5′-AGGAACAGACACCTCGCTGAGGAG (forward) and 5′-GAGGCTGAGGTAGATGTAGTCGTCAGTG (reverse), which cover the 5-LO promoter from bp −286 to +78. PCR was visualized after separation on 1% agarose gels containing 0.5% ethidium bromide with the Gel Doc 1000 System (Biorad, Munich, Germany).

Calcium phosphate transfection and reporter gene assay

Twenty-four hours prior to transfection, HeLa cells were seeded at a density of 4×10^4 cells per well. 0.8 μg 5-LO luciferase reporter gene plasmid pN10 [7] which comprises 831 bp of the 5-LO promoter and 0.2 μg pCMVRenilla as internal standard were transfected using the calcium phosphate method. After 24 hrs, cells were assayed for reporter gene activity with the Dual-Glo™ Stop and Glow Luciferase Assay system following the manufacturer's protocol (Promega, Mannheim, Germany) and measured with a Tecan infinite® M200 reader. Renilla luciferase activity was used to normalize the luciferase activity to the transfection efficacy. Data are shown as relative light units.

shRNA knockdown of HDAC1, HDAC2 and HDAC3

For gene knockdown a lentiviral shRNA approach was used. MISSION shRNA plasmids for HDAC1 (NM_004965; TRCN 0000195103), HDAC2 (NM_001527; TRCN0000196590) and HDAC3 (NM_003883; TRCN 0000196925) were obtained from Sigma-Aldrich.

Generation of the lentiviral particles was performed in HEK293T cells. A total of 4×10^4 HEK293T cells per well were seeded in a 24-well plate in 1 ml complete DMEM medium. After 24 hrs, cells were transfected by calcium phosphate transfection. The DNA transfection mixtures contained 10 μg of the shRNA plasmid and 10 μg packaging plasmids (VSVG). After 4 hrs, the medium was changed and cells were cultured for 72 hrs. The supernatants containing the infectious lentiviral particles were filtered using a 0.22 μm filter. For transduction the Mono Mac6 cells were seeded at a concentration of 0.4×10^6/ml and supplemented with protamine sulfate (4 μg/ml). Ten microlitres of each viral supernatant were added to 500 μl of cell

Table 1 EC$_{50}$-values of selected HDAC inhibitors for 5-LO promoter activation as determined by reporter gene assays and comparison with IC$_{50}$-values reported for specific HDAC isoforms

Compound	5-LO promotor activation (EC$_{50}$)	HDAC inhibition reported in the literature
Apicidin	64 nM	>10 μM (HDAC1) [38]
		20 nM (HDAC2) [38]
		43 nM (HDAC3) [38]
MS-275	539 nM	181 nM (HDAC1) [38, 39]
		300 nM (HDAC1) [40]
		180 nM (HDAC1) [41]
		1.1 μM (HDAC2) [38, 39]
		2.3 μM (HDAC3) [38, 39]
		8 μM (HDAC3) [40]
		0.74 μM (HDAC3) [41]
SB-379278A	>1 μM	500 nM (HDAC8) [40]
MC-1568	>1 μM	220 nM (HDACs class IIa) [42]

suspension and centrifuged (2500 r.p.m., 90 min., 32°C). The transduced cells were grown in complete medium for 2 days before puromycin (0.75 μg/ml final concentration) was added to select transduced clones. After 3 weeks, knockdown efficiency was analysed by Western blot.

Statistics

Statistic evaluation of the data was performed by ANOVA, *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.3.

Results

Only class I HDAC inhibitors induce 5-LO promoter activity

To identify the HDACs which are involved in the transcriptional regulation of 5-LO, more specific HDAC inhibitors than TSA were tested for induction of 5-LO promoter activity using reporter gene assays [7]. MS-275 that preferentially inhibits HDAC1 but also affects HDAC2 and HDAC3 at micromolar concentrations, apicidin as HDAC2 and HDAC3 inhibitor, SB-379278A as HDAC8 inhibitor and MC-1568 as inhibitor of class IIa HDACs were tested (Table 1). Apicidin strongly increased 5-LO promoter activity at a concentration of 100 nM, which was almost comparable to TSA (330 nM).
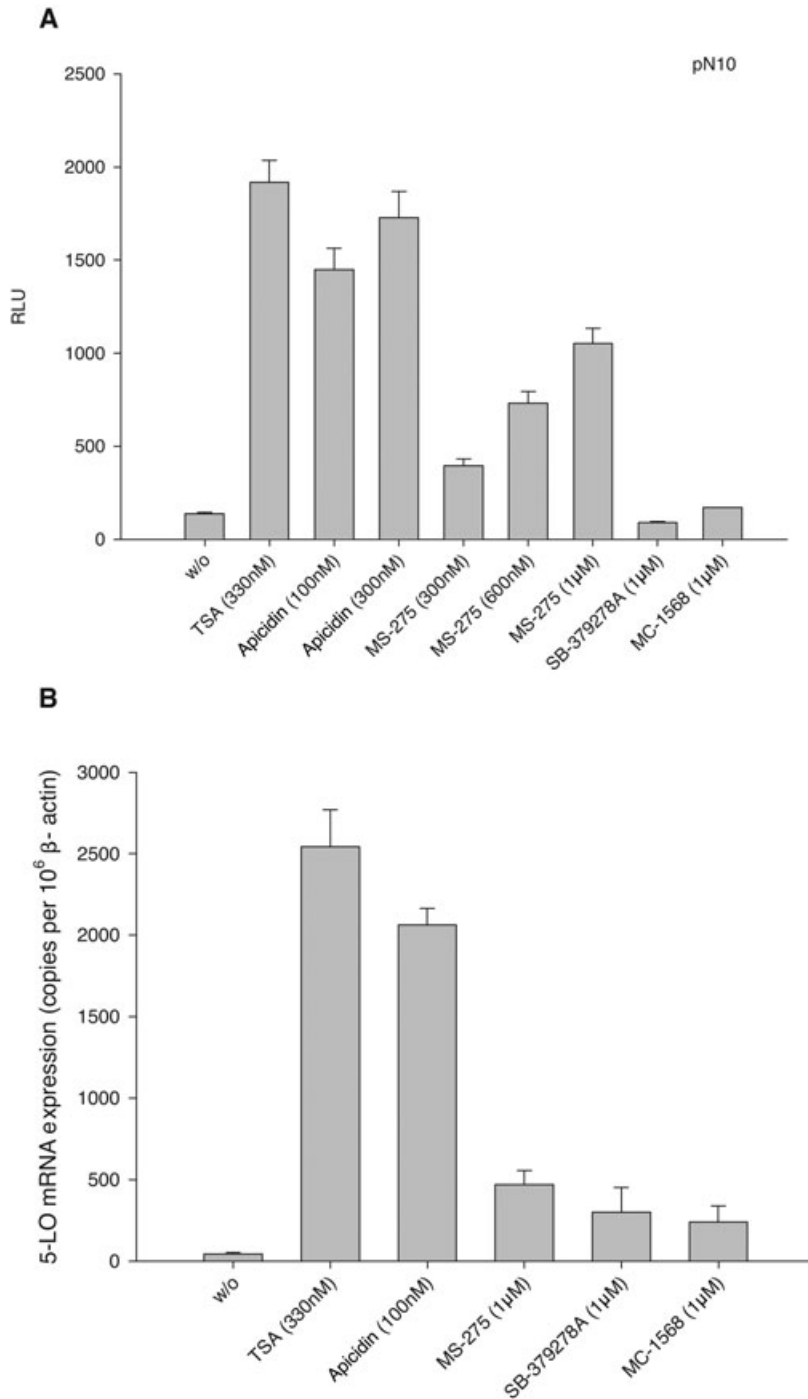
A

B

Fig. 1 Up-regulation of 5-LO promoter activity (**A**) and 5-LO mRNA expression (**B**) by histone deacetylase inhibitors. (**A**) 5-LO promoter activity was determined by reporter gene assays. HeLa cells were transfected with the 5-LO promoter construct pN10 (comprises bp −844 to −12 in relation to ATG) and cultured in the presence of the indicated HDAC inhibitors for 24 hrs. Then, reporter gene activity was determined. Results are expressed as relative light units (RLU) of the internal standard plasmid pCMVRenilla and are shown as mean + S.E. ($n = 3$). (**B**) Real-time PCR analysis of 5-LO mRNA expression in Mono Mac6 cells. Cells were treated with the indicated HDAC inhibitors for 24 hrs. Then, the cells were harvested, RNA was isolated, reverse transcribed into cDNA and 5-LO mRNA expression was determined by real-time PCR. Values are given as the mean + S.E. of three independent experiments.

In addition, MS-275 that inhibits HDAC1–HDAC3 activated the 5-LO promoter at 1 μM. The EC_{50}-values for induction of 5-LO promoter activity are given in Table 1. Of note, no 5-LO promoter activation was detectable with the compounds SB-379278A and MC-1568 (Fig. 1A).

Interestingly, similar results were obtained when the effects of these HDAC inhibitors on 5-LO mRNA expression were investigated in Mono Mac6 cells using quantitative RT-PCR. The cells were incubated with the HDAC inhibitors for 24 hrs at the indicated concentrations. Trichostatin A (330 nM) increased 5-LO mRNA

Fig. 2 Effects of HDAC 1, 2 and 3 knockdown on 5-LO mRNA expression in Mono Mac6 cells. (**A**) 5-LO mRNA expression in Mono Mac6 cells and the respective Mono Mac6 HDAC knockdown cell lines was determined by quantitative real-time PCR. Results are given as 5-LO mRNA copy number per 10^6 β-actin mRNA copies. Data are shown as mean + S.E. of six independent experiments. (**B**) Western blot analysis of HDAC expression in the respective HDAC knockdown cell lines. A total of 6×10^6 cells were harvested and analysed by Western blot for the indicated HDAC isoform.
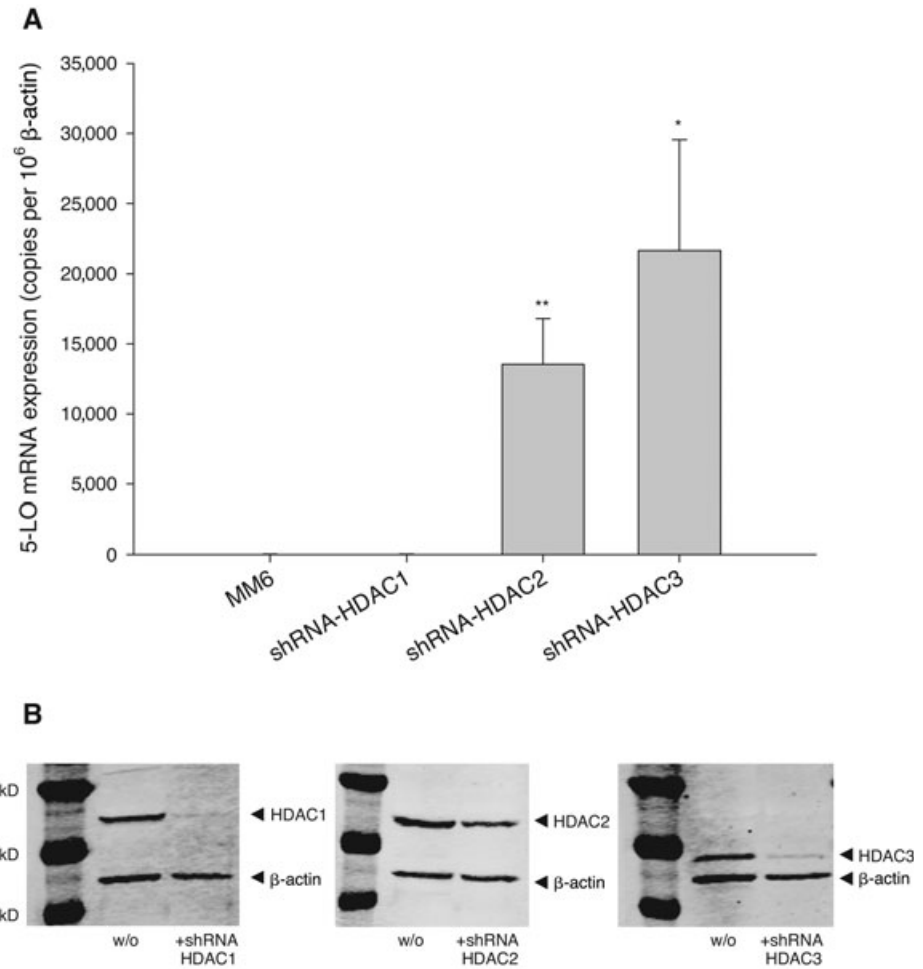
expression in Mono Mac6 cells at about 62-fold. Apicidin (300 nM) led to an up to 50-fold induction of 5-LO mRNA, MS-275 increased 5-LO mRNA about 12-fold at a concentration of 1 μM. Neither SB-379278A (1 μM) nor MC-1568 (1 μM) showed a strong effect on 5-LO mRNA expression (Fig. 1B).

Taken together, the data show that the HDAC2 and HDAC3 inhibitor apicidin as well as to a lower extent the HDAC1–HDAC3 inhibitor MS-275 can mimic the TSA effects on 5-LO mRNA expression and promoter activity.

Knockdown of class I histone deacetylases in Mono Mac6 cells

To further elucidate which class I HDAC isoenzyme is involved in the regulation of 5-LO transcription, HDAC1, HDAC2 and HDAC3 expression was knocked down by shRNA. Mono Mac6 cells were stably transfected using lentiviral shRNA constructs. The efficiency of the knockdown was tested by Western blot analysis

(Fig. 2B). The cell lines showed a strongly reduced protein expression of each HDAC that was targeted by the respective shRNA. 5-LO mRNA expression in the HDAC knockdown cell lines was determined by real-time PCR. Knockdown of HDAC2 as well as HDAC3 led to a strong induction of 5-LO mRNA expression, whereas the HDAC1 knockdown cell lines showed no up-regulation but a slight down-regulation of 5-LO expression (Fig. 2A). The data suggest that HDAC2 and HDAC3 are mainly involved in the up-regulation of 5-LO mRNA expression by HDAC inhibitors.

Effects of HDAC inhibitors on 5-LO mRNA expression in Mono Mac6 cells after HDAC knockdown

To further verify the contribution of the HDAC1–HDAC3 isoforms to 5-LO mRNA induction, the HDAC inhibitors TSA, apicidin and MS-275 where added to each Mono Mac6 HDAC knockdown cell line. As shown in Figure 3, 5-LO mRNA expression is low in
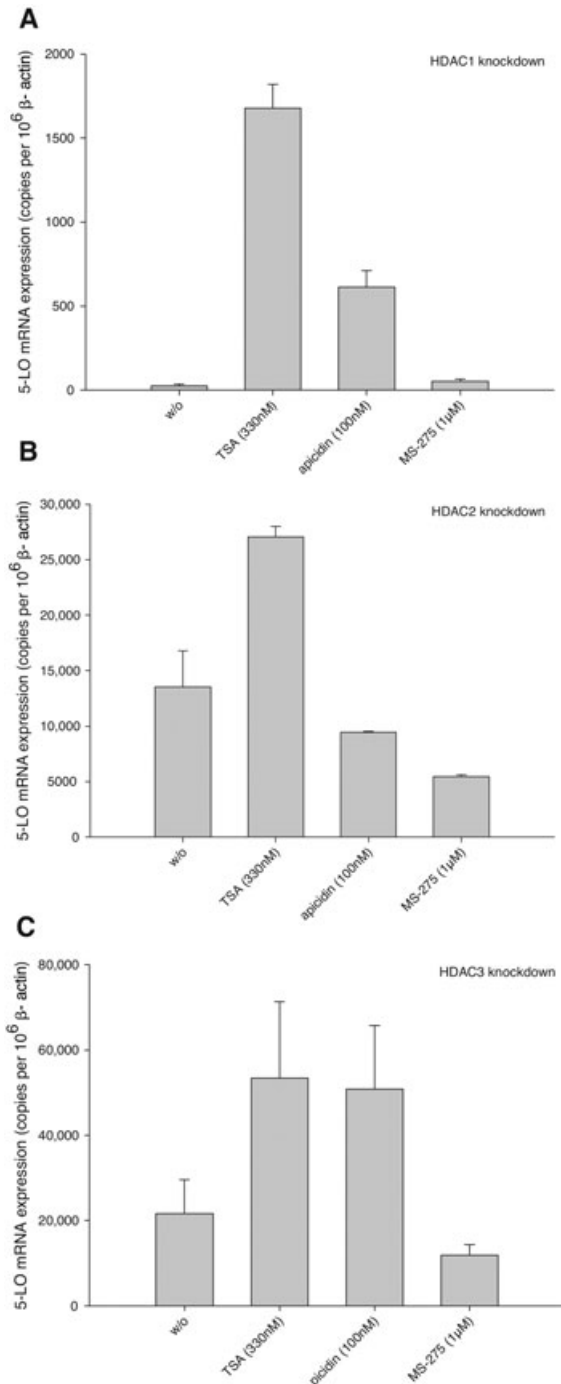
Fig. 3 Effects of HDAC inhibitors (TSA, apicidin and MS-275) on 5-LO mRNA expression in HDAC1, HDAC2 and HDAC3 knockdown cell lines of Mono Mac6. HDAC1 (**A**), HDAC2 (**B**) and HDAC3 (**C**) knockdown cells were treated for 24 hrs with the indicated HDAC inhibitors at the indicated concentrations. Then, 5-LO mRNA expression in the respective Mono Mac6 HDAC knockdown cell lines was determined by quantitative real-time PCR. Results are given as 5-LO mRNA copy number per 10^6 β-actin mRNA copies. Data are shown as mean + S.E. of three independent experiments.

HDAC1 knockdown cells and TSA and apicidin strongly induced 5-LO mRNA expression (67- and 25-fold, respectively), whereas the HDAC1 inhibitor MS-275 is without effect. In the HDAC2 and HDAC3 knockdown cell lines, the induction of 5-LO mRNA expression by TSA is modest (1.5- and 3.5-fold) compared to 62-fold in the wild-type cells (Fig. 2A) suggesting that there might be some additive effect when HDACs are inhibited by TSA in the HDAC2 knockdown cell line (Fig. 3B) or when HDAC2 is inhibited in the HDAC3 knock-down cells by apicidin (Fig. 3C). Taken together, knockdown of either HDAC2 or HDAC3 alone is sufficient for the strong up-regulation of 5-LO expression (compare Fig. 2A) and additional HDAC inhibition leads to an only modest 5-LO mRNA induction.

Induction of 5-LO mRNA expression by HDAC inhibition in HL-60 and U937 cells

To check whether HDAC inhibition induces 5-LO mRNA expression in other myeloid cell lines than Mono Mac6, we analysed 5-LO mRNA expression in HL-60 and U937 by quantitative RT-PCR. Cells were grown in the presence or absence of TSA (330 nM) for 24 hrs. Compared to Mono Mac6 cells, a lower increase in 5-LO mRNA expression was found in response to TSA treatment (about four- to fivefold) in HL-60 and U937 cells. The results suggest that induction of 5-LO mRNA expression by HDAC inhibitors is not restricted to Mono Mac6 cells but seems to occur in myeloid cells in general.

Time course of 5-LO mRNA induction by HDAC inhibition

To characterize the induction of 5-LO mRNA and the concomitant changes in histone modifications, the time course of up-regulation of 5-LO mRNA expression by HDAC inhibition was investigated and correlated with histone acetylation at the 5-LO promoter. Mono Mac6, HL-60 and U937 cells were incubated with TSA (330 nM) for the indicated times. Then, the cells were harvested and 5-LO mRNA was determined by quantitative RT-PCR. In Mono Mac6 and HL-60 cells, a marked increase in 5-LO mRNA expression was detected after 4 hrs, whereas in U937 cells the increase seems to start immediately after treatment (Fig. 4). In U937 cells, maximal 5-LO expression was obtained after 24 hrs of TSA treatment whereas in Mono Mac6 and HL-60 cells 5-LO mRNA expression peaked at 16 hrs and declined again at 24 hrs. To distinguish between primary or secondary effects which would require protein synthesis, Mono Mac6 cells were treated with TSA in the presence of the protein biosynthesis inhibitor CHX at 50 μM. CHX did not significantly affect induction of 5-LO mRNA expression by TSA up to 4 hrs but at later time-points it partially inhibited the TSA effect on 5-LO mRNA expression. This suggests that primary effects as well as secondary effects at later time-points requiring protein biosynthesis are involved in the up-regulation of 5-LO mRNA expression by TSA (Fig. 4).
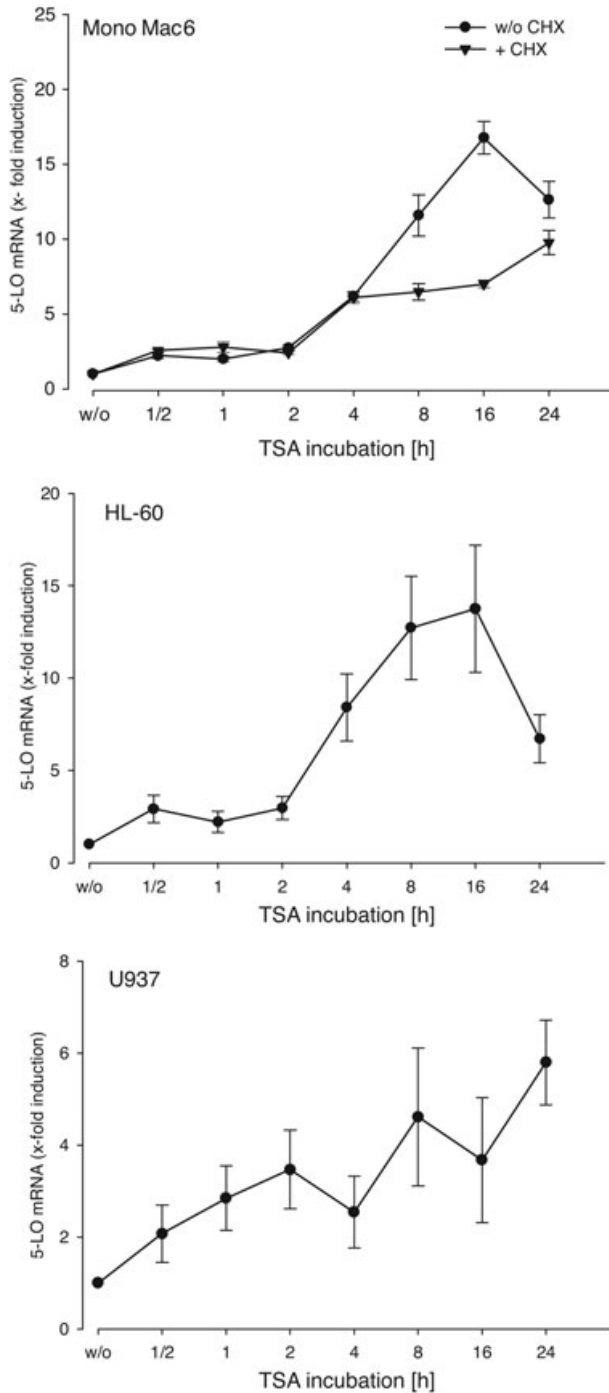
Fig. 4 Time course of 5-LO mRNA induction by TSA in Mono Mac6, HL-60 and U937 cells. The cells were cultured in the presence of TSA (330 nM) with or without CHX (50 μM) for the indicated times. Then, the cells were harvested and RNA was isolated, reverse transcribed into cDNA and analysed by quantitative real-time PCR for 5-LO mRNA expression. Data are shown as mean ± S.E. from three independent experiments.

Basal and TSA-induced H3 and H4 acetylation at the 5-LO promoter

Histone deacetylase inhibitors are known to increase the global histone acetylation status of many mammalian cells [14]. Here, we analysed the time-dependent changes in the acetylation status of histone proteins H3 and H4 during HDAC inhibition at the 5-LO promoter by ChIP assays. Under basal conditions (w/o TSA), Mono Mac6 cells showed higher levels of acetylated H3 and H4 proteins at the 5-LO core promoter compared to HL-60 and U937 cells (Fig. 5A). The lowest acetylation status of histone H3 and H4 at the 5-LO core promoter was observed in U937 cells where the promoter is methylated (Fig. 5A). To study the time course of the TSA effect on H3 and H4 acetylation, cells were incubated with TSA for the indicated times before harvest (Fig. 5B). An increase in acetylated histone proteins H3 and H4 was detectable in HL-60 and U937 cells (Fig. 5B). In U937 cells, maximal acetylation of histone H3 and H4 was detectable after 1–8 hrs and 8 hrs of TSA
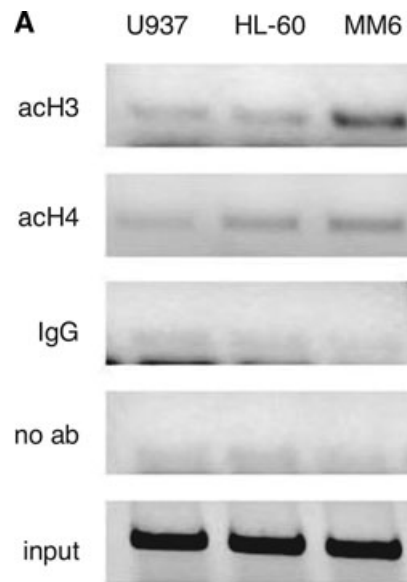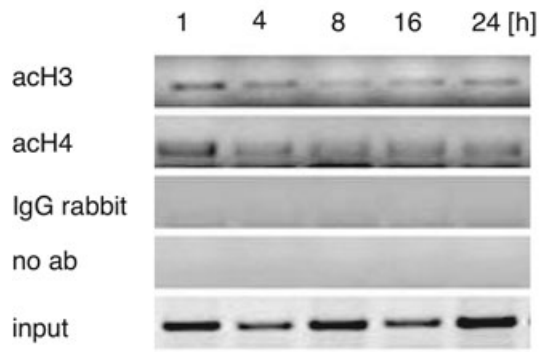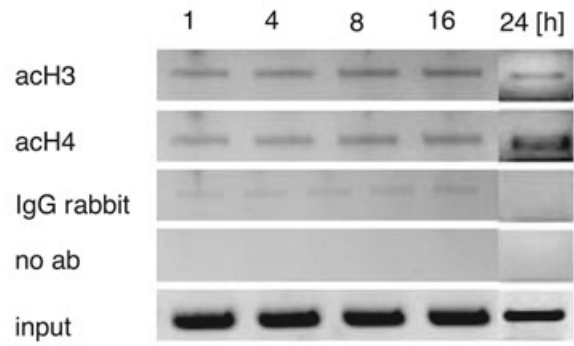

Fig. 5 Chromatin immunoprecipitation assay (ChIP) for acetylated histones H3 (acH3) and H4 (acH4) at the 5-LO core promoter in U937, HL-60 and Mono Mac6 cells. Normal rabbit IgG antibodies served as negative control to detect unspecific binding to histone proteins and the no antibody control (no ab) was used to detect unspecific binding to the immunoprecipitation beads. The input sample (sheared chromatin without immunoprecipitation) served as positive control for the PCR reaction. (**A**) Analysis of the acetylation status of the histone proteins H3 and H4 at the 5-LO promoter in U937, HL-60 and Mono Mac6 cells. 32 PCR cycles were applied. (**B**) Time course of H3 and H4 acetylation of the 5-LO promoter in the indicated cell lines. Cells were treated with TSA (330 nM) for the indicated times. Then, the cells were harvested and analysed by ChIP as described. Thirty-two PCR cycles were performed except for AcH4 in Mono Mac6 cells, where the cycle number was reduced to 28.
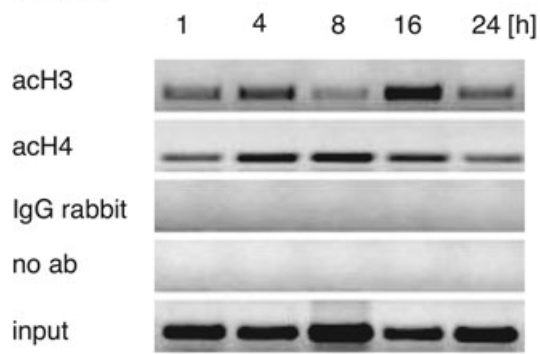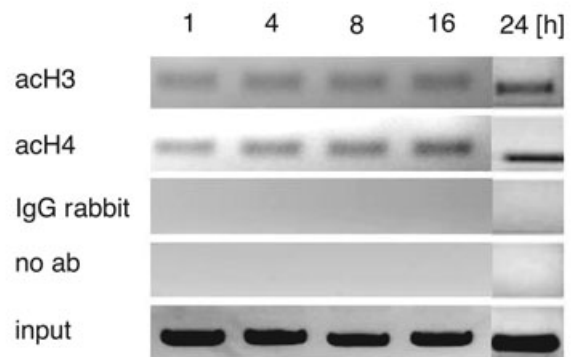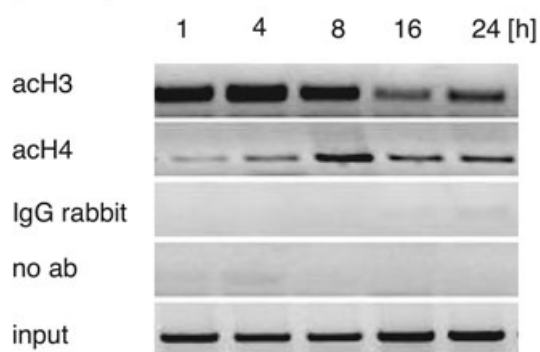
B

Mono Mac6

TSA incubation
(330nM)

w/o

	1	4	8	16	24 [h]
acH3					
acH4					
IgG rabbit					
no ab					
input					

	1	4	8	16	24 [h]
acH3					
acH4					
IgG rabbit					
no ab					
input					

HL-60

TSA incubation
(330nM)

w/o

	1	4	8	16	24 [h]
acH3					
acH4					
IgG rabbit					
no ab					
input					

	1	4	8	16	24 [h]
acH3					
acH4					
IgG rabbit					
no ab					
input					

U937

TSA incubation
(330nM)

w/o

	1	4	8	16	24 [h]
acH3					
acH4					
IgG rabbit					
no ab					
input					

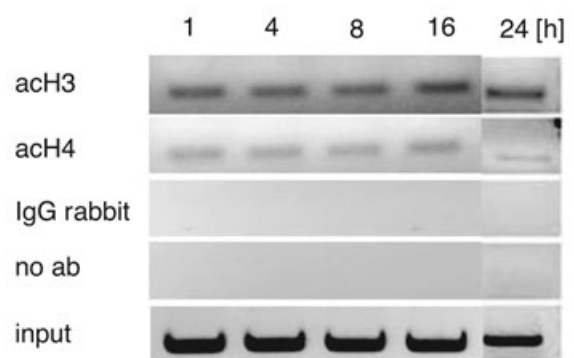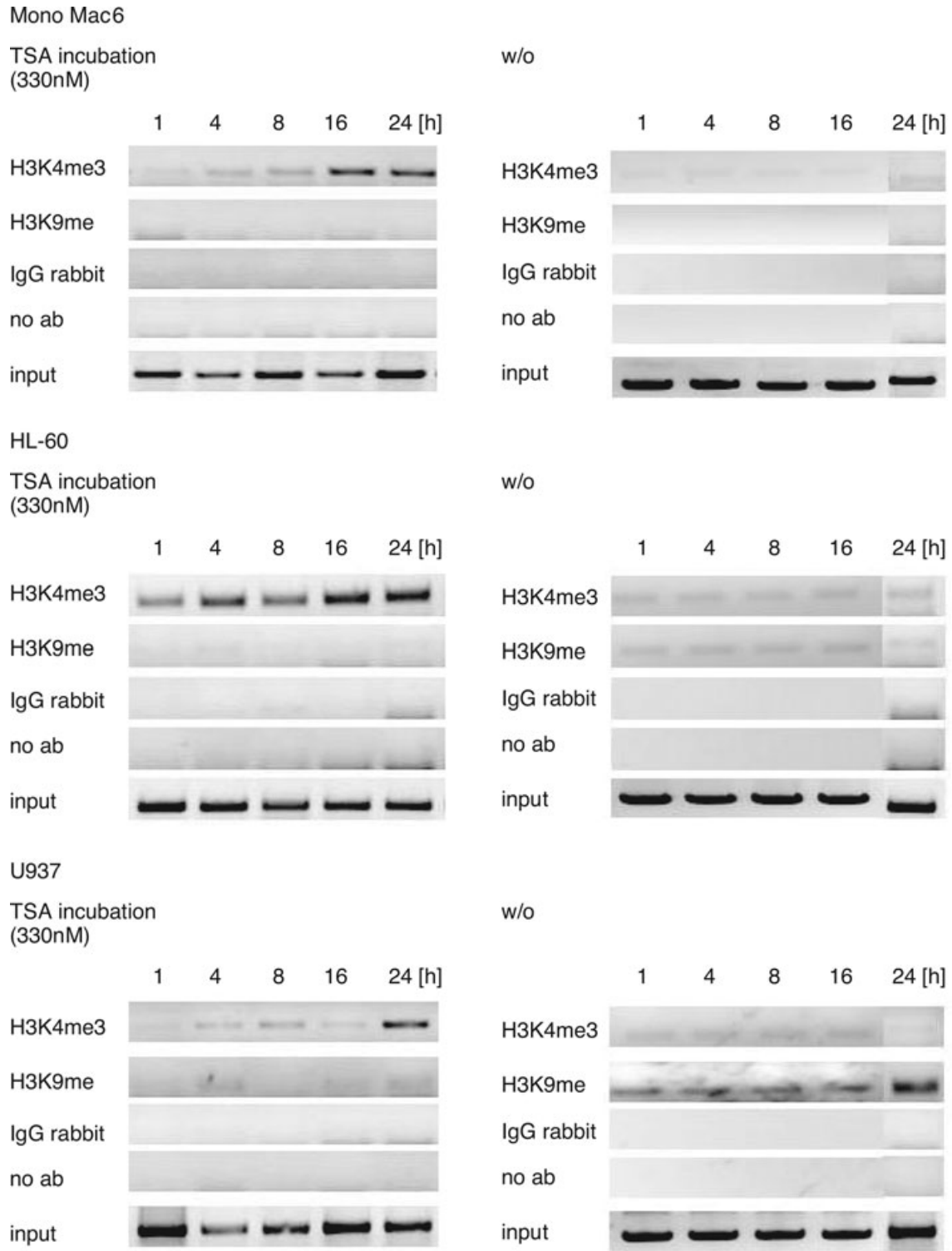|	1	4	8	16	24 [h]
acH3					
acH4					
IgG rabbit					
no ab					
input					

Fig. 5 Continued.

Fig. 6 Chromatin immunoprecipitation assay for histone H3K4 trimethylation (H3K4me3) and histone H3K9 monomethylation (H3K9me) at the 5-LO core promoter in Mono Mac6, HL-60 and U937 cells. Cells were grown with TSA (330 nM) for the indicated times. Then, the cells were harvested and subjected to ChIP analysis. Thirty-two PCR cycles were applied.
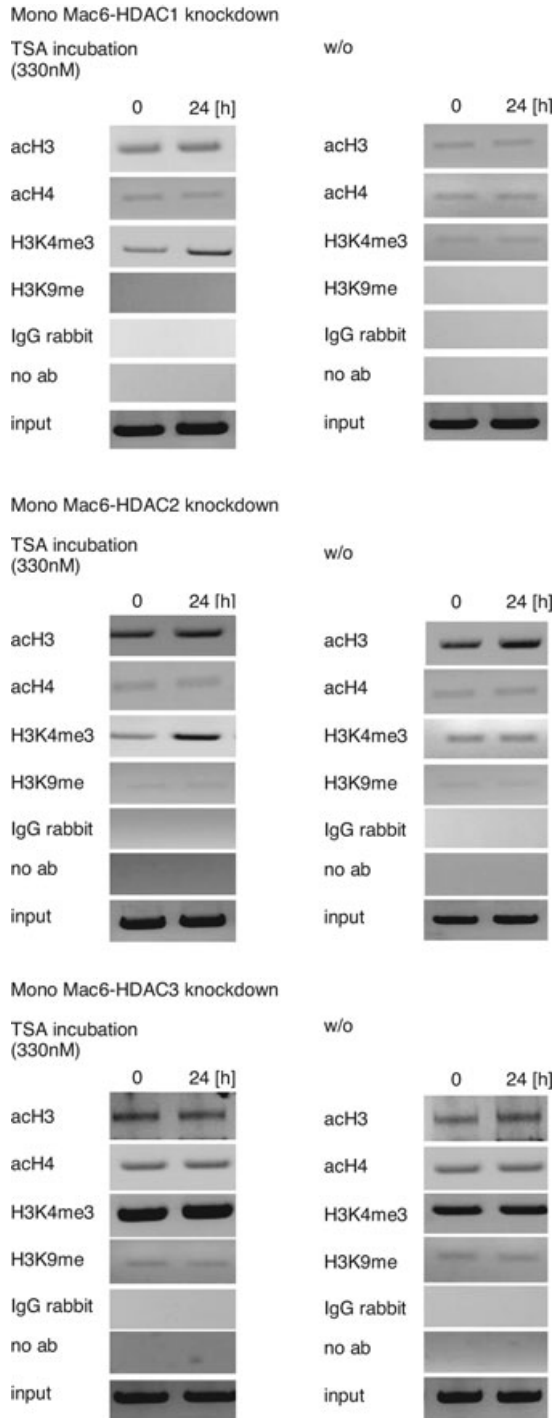
Fig. 7 Chromatin immunoprecipitation assay for histone acetylation (acH3, acH4), H3K4 trimethylation (H3K4me3) and histone H3K9 monomethylation (H3K9me) at the 5-LO core promoter in Mono Mac6 knockdown cell lines. Cells were grown with or without TSA (330 nM) for 24 hrs. Then, the cells were harvested and subjected to ChIP analysis. Thirty-two PCR cycles were applied.

incubation, respectively. In HL-60 cells, the highest histone H3 and H4 acetylation was observed after 16 and 8 hrs, respectively. Interestingly, strong increases in H3 acetylation already occurred in U937 and HL-60 cells 1 and 4 hrs after TSA addition which clearly preceded the induction of 5-LO mRNA (Fig. 4). In contrast, no significant changes in the H3 and H4 acetylation status were detectable in Mono Mac6 cells, a fact that was already reported for acetylated H4, but not for acetylated H3 [8].

HDAC inhibition induces histone H3 lysine 4 trimethylation at the 5-LO promoter

H3K4me3 is classified as a marker for transcriptionally active promoters, whereas H3K9me is a marker for transcriptional repression. Usually, the level of H3K4me3 correlates with the acetylation status of histone H3 [15]. To elucidate whether TSA-induced 5-LO expression correlates with H3 methylation, ChIP assays were performed to determine H3K9me and H3K4me3 levels after incubation of the cells with TSA for the indicated times. An increase in H3K4me3 levels was detectable in all three tested cell lines with a maximum reached after 16–24 hrs of TSA treatment (Fig. 6). H3K9me was only detectable in untreated U937 cells.

Effect of HDAC knockdown on histone acetylation and methylation at the 5-LO promoter

In accordance to our results in wild-type Mono Mac6 cells where HDAC inhibition did not change acH3 and acH4, TSA did not increase acH3 and acH4 in the HDAC knockdown cell lines (Fig. 7). An increase in H3K4me3 by TSA is clearly detectable in HDAC1 and HDAC2 knockdown cells. Interestingly, knockdown of HDAC3 leads to a very prominent H3K4 trimethylation, which corresponds to the very high expression of 5-LO mRNA in this cell line.

Discussion

High expression of 5-LO mRNA and protein is mainly restricted to myeloid cells such as mature granulocytes, monocytes and dendritic cells [2]. This tight regulation of 5-LO expression reflects the function of 5-LO as key enzyme in the formation of leukotrienes, which are mediators of inflammatory and host defence reactions [16]. We found that the unspecific HDAC inhibitor TSA induces 5-LO mRNA and protein expression in the human monocytic cell line Mono Mac6 which is obviously due to the up-regulation of 5-LO promoter activity as we could show with reporter gene assays in HeLa cells [7]. Trichostatin A is a reversible HDAC inhibitor of HDACs class I and class II. HeLa cells are known to express HDAC1 [17], HDAC2 [18], HDAC3 [19] and HDAC8 [20] from the HDAC class I group and at least HDAC4 and HDAC7 of class IIa [21, 22] which suggests that one of these isoenzymes

could be responsible for the induction of 5-LO promoter activity observed with reporter gene assays in HeLa cells by TSA. Previous studies have demonstrated that HDAC inhibition by TSA leads to the recruitment of Sp1 to an Sp1 binding site in close proximity to the 5-LO transcription start site as well as accumulation of RNA polymerase II [8]. This is in line with enhanced transcription initiation. To get more insight into the regulation of the 5-LO promoter, we aimed to identify the HDAC responsible for the TSA effects on 5-LO gene expression.

By the use of subtype-specific HDAC inhibitors, we found that the HDAC2 and HDAC3 inhibitor apicidin as well as the HDAC1–HDAC3 inhibitor MS-275 can mimic the TSA effects on 5-LO mRNA expression and promoter activity whereas the HDAC8 inhibitor SB-379278A and the HDAC class II inhibitor MC-1568 were inactive. Regarding MS-275, the reported IC_{50}-values for HDAC1 are 180–300 nM whereas those for HDAC2 and HDAC3 are 1.1 μM and between 0.7 and 8 μM, respectively. Thus, MS-275 preferentially inhibits HDAC1 but can also reduce HDAC2 and HDAC3 activity at the concentrations required for induction of 5-LO promoter activity (Table 1). Apicidin, which inhibits HDAC2 and HDAC3 but not HDAC1 also induced 5-LO mRNA expression. Of note, apicidin was able to achieve similar effects on 5-LO promoter activation and mRNA induction as TSA (Fig. 1A and B).

Taken together, our HDAC inhibitor studies suggest that either HDAC2 or HDAC3 or both are involved in the regulation of 5-LO expression. This conclusion was confirmed by knockdown experiments in Mono Mac6 cells. Knockdown of HDAC2 or HDAC3 strongly induced cellular 5-LO mRNA expression, whereas HDAC1 knockdown did not induce 5-LO mRNA expression. The results suggest that HDAC3 and to a somewhat lesser extend HDAC2 are involved in the regulation of 5-LO promoter activity. However, one should consider that we were not able to get an as efficient knockdown of HDAC2 as with HDAC3. Thus, the slightly lower induction in 5-LO mRNA expression achieved with the HDAC2 knockdown could also be at least in part related to the differences in knockdown efficiency. Of note, addition of the HDAC inhibitor TSA to the HDAC2 or HDAC3 knockdown cell lines revealed an only weak additive effect of the drug suggesting that both knockdowns were efficient (Fig. 3). The data suggest that inhibition of either HDAC2 or HDAC3 is sufficient for 5-LO up-regulation and that the benefit of combined HDAC2 and HDAC3 inhibition is limited.

Previously, we have shown that HDAC inhibition increases 5-LO promoter activity by recruitment of Sp1 to the core promoter [8]. Thus, it seems that HDAC2/3 inhibition is the critical step that leads to Sp1 recruitment. Interestingly, a similar phenomenon has been described for the induction of the cdk inhibitor p21 [23].

In this study, we found that the 5-LO mRNA induction by HDAC inhibition is not restricted to Mono Mac6 cells but can also be observed in other myeloid cell lines such as HL-60 and U937. Of interest, the lowest 5-LO mRNA induction by TSA was observed in U937 cells. In comparison to Mono Mac6 or HL-60 cells, U937 cells show low basal 5-LO expression, due to methylation of the 5-LO core promoter in these cells [4]. This correlates with the presence of H3K9me, a marker for transcriptional repression, which was detectable in U937 but not in Mono Mac6 or HL-60

cells (Fig. 6). DNA methylation is often connected to histone hypoacetylation [24] and for some genes it was shown that HDAC inhibitors, such as TSA, are able to demethylate and reactivate silenced genes [25–27]. In U937 cells, we found that TSA reduces the H3K9 marker and leads to a strong acetylation of H3, but the effects of the drug on 5-LO mRNA expression are modest. In HL-60 and U937 cells but not in Mono Mac6 cells, TSA increased H4 acetylation. H3 and H4 acetylations are considered as markers of actively transcribed genes. Of note, the turnover of histone acetylations is rather fast and the half-life of the histone acetylation marks is in the range of a few minutes. This fact points to rapid acetylation/deacetylation cycles where HDAC inhibitors shift the equilibrium towards the acetylated forms. In HL-60 and Mono Mac6 cells, the changes in H3 and H4 acetylation by TSA as well as the time course of the TSA effect does not directly correlate with the induction of 5-LO mRNA expression. For example, in Mono Mac6 cells there is almost no effect of TSA on H3 as well as H4 acetylation (Fig. 5B), whereas 5-LO mRNA is induced up to 62-fold. On the other hand, a good correlation between up-regulation of 5-LO mRNA expression and H3K4me3 is observed after HDAC inhibition in all three cell lines (Figs 4 and 6). In contrast to acetylation, histone methylation markers represent rather stable signals with half-lives in the range of 0.5–1 day and our data suggest that HDAC inhibition leads to a slow but prominent increase in H3K4me3 at the 5-LO promoter.

However, at the moment, the precise link between HDAC inhibition and H3K4 trimethylation and the recruitment of Sp1 to the promoter is unknown. Methylation of H3K4 is catalysed by the MLL protein family and H3K4 trimethylation by the MLL complex is stimulated by acetylated histone H3 [15]. This observation provides a functional link between histone H3 acetylation and H3K4 trimethylation and could thus be responsible for the increase in H3K4me3 in response to HDAC inhibition. On the other hand, induction of 5-LO mRNA expression by TSA was partially inhibited by cycloheximide, which suggests that protein biosynthesis is required for full up-regulation and that TSA obviously induces the expression of proteins that are involved in the up-regulation of the cellular 5-LO mRNA level. Similar observations were made with TGF-β and 1,25(OH)$_2$D$_3$, which induce 5-LO mRNA expression in myeloid cell lines [5, 6, 28]. There, cycloheximide acted as partial inhibitor of TGF-β-induced 5-LO mRNA expression [29, 30].

Another interesting function of HDAC3 is its interference with the P-TEFb complex. P-TEFb is a heterodimeric complex composed of cyclin-dependent kinase 9 (cdk9) and a regulatory cyclin subunit of the T family [31, 32]. It is a transcription elongation factor that enhances transcriptional elongation by phosphorylating RNA polymerase II at Ser2 of the carboxy-terminal domain. The kinase activity of P-TEFb is regulated by cdk9 acetylation where p300 was identified as acetylase and HDAC3 was shown to be a pTEFb deacetylase [32]. This observation provides a mechanistic link between HDAC3 inhibition and the stimulation of transcript elongation. Interestingly, 5-LO transcript elongation was found to be a critical step in the regulation of 5-LO mRNA expression. We showed that the strong induction of 5-LO mRNA expression by TGF-β and 1,25-dihydroxyvitamin D$_3$ is mainly due

to the stimulation of transcript elongation [5, 6, 28]. Thus, the prominent up-regulation of 5-LO mRNA expression observed after pharmacological inhibition of HDAC3 or after HDAC3 knockdown certainly involves the stimulation of 5-LO promoter activity as shown by reporter gene assays, but additional effects of HDAC3 inhibition on 5-LO transcript elongation *via* P-TEFb activation could also contribute.

Another interesting observation is that knockdown of HDAC2 leads to a significant increase in 5-LO mRNA expression in Mono Mac6 cells (Fig. 2). HDAC2 has been shown to be involved in the suppression of the expression of proinflammatory genes and reduced HDAC2 expression was observed in alveolar tissue from patients with chronic obstructive pulmonary disease (COPD) [33–35]. Because leukotrienes are major players in inflammatory diseases in the lung such as asthma and COPD and because elevated levels of leukotrienes have been found in COPD patients [36], our data suggest that it might be of interest to check whether there is an increased expression of 5-LO in lung tissue of COPD patients.

Histone deacetylase inhibition has also been linked to cell differentiation. The HDAC inhibitor butyrate has been shown to induce cell differentiation in myeloid cell lines and this effect is enhanced by the combination with DMSO and hexamethylene bisacetamide [37]. Butyrate has also been found to induce 5-LO expression in colorectal carcinoma Caco-2 cells and silencing of HDAC3 and other class I HDACs in colon cancer cell lines leads to cell growth inhibition, differentiation and apoptosis [23]. Thus, regulation of 5-LO promoter activity by HDAC2 and/or HDAC3 seems to link 5-LO expression with cell differentiation.

Acknowledgements

Conflict of interest

The authors confirm that there are no conflict of interest.

References

1. **Rinaldo-Matthis A, Haeggstrom JZ.** Structures and mechanisms of enzymes in the leukotriene cascade. *Biochimie*. 2010; 92: 676–81.

2. **Radmark O, Werz O, Steinhilber D**, *et al*. 5-Lipoxygenase: regulation of expression and enzyme activity. *Trends Biochem Sci*. 2007; 32: 332–41.

3. **Bernstein BE, Humphrey EL, Erlich RL,** *et al*. Methylation of histone H3 Lys 4 in coding regions of active genes. *Proc Natl Acad Sci USA*. 2002; 99: 8695–700.

4. **Uhl J, Klan N, Rose M**, *et al*. The 5-lipoxygenase promoter is regulated by DNA methylation. *J Biol Chem*. 2002; 277: 4374–9.

5. **Brungs M, Radmark O, Samuelsson B,** *et al*. Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor beta and 1,25-dihydroxyvitamin D3. *Proc Natl Acad Sci USA*. 1995; 92: 107–11.

6. **Stoffers KL, Sorg BL, Seuter S**, *et al*. Calcitriol upregulates open chromatin and elongation markers at functional vitamin D response elements in the distal part of the 5-lipoxygenase gene. *J Mol Biol*. 2010; 395: 884–96.

7. **Klan N, Seuter S, Schnur N**, *et al*. Trichostatin A and structurally related histone deacetylase inhibitors induce 5-lipoxygenase promoter activity. *Biol Chem*. 2003; 384: 777–85.

8. **Schnur N, Seuter S, Katryniok C,** *et al*. The histone deacetylase inhibitor trichostatin A mediates upregulation of 5-lipoxygenase promoter activity by recruitment of Sp1 to distinct GC-boxes. *Biochim Biophys Acta*. 2007; 1771: 1271–82.

9. **Yang XJ, Seto E.** The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men. *Nat Rev Mol Cell Biol*. 2008; 9: 206–18.

10. **Pokholok DK, Harbison CT, Levine S,** *et al*. Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell*. 2005; 122: 517–27.

11. **Guenther MG, Levine SS, Boyer LA**, *et al*. A chromatin landmark and transcription initiation at most promoters in human cells. *Cell*. 2007; 130: 77–88.

12. **Guccione E, Bassi C, Casadio F,** *et al*. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. *Nature*. 2007; 449: 933–7.

13. **Hake SB, Allis CD.** Histone H3 variants and their potential role in indexing mammalian genomes: the "H3 barcode hypothesis." *Proc Natl Acad Sci USA*. 2006; 103: 6428–35.

14. **Yoshida M, Kijima M, Akita M**, *et al*. Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J Biol Chem*. 1990; 265: 17174–9.

15. **Nightingale KP, Gendreizig S, White DA,** *et al*. Cross-talk between histone modifications in response to histone deacetylase inhibitors: MLL4 links histone H3 acetylation and histone H3K4 methylation. *J Biol Chem*. 2007; 282: 4408–16.

16. **Peters-Golden M.** Expanding roles for leukotrienes in airway inflammation. Curr Allergy Asthma Rep. 2008; 8: 367–73.

17. **Grozinger CM, Chao ED, Blackwell HE,** *et al*. Identification of a class of small molecule inhibitors of the sirtuin family of NAD-dependent deacetylases by phenotypic screening. *J Biol Chem*. 2001; 276: 38837–43.

18. **Huang BH, Laban M, Leung CH**, *et al*. Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell Death Differ*. 2005; 12: 395–404.

19. **Ishii S, Kurasawa Y, Wong J**, *et al*. Histone deacetylase 3 localizes to the

mitotic spindle and is required for kineto-chore-microtubule attachment. *Proc Natl Acad Sci U S A*. 2008; 105: 4179–84.

20. **Lee H, Sengupta N, Villagra A, *et al.*** Histone deacetylase 8 safeguards the human ever-shorter telomeres 1B (hEST1B) protein from ubiquitin-mediated degradation. *Mol Cell Biol*. 2006; 26: 5259–69.

21. **Liu F, Dowling M, Yang XJ, *Kao GD*.** Caspase-mediated specific cleavage of human histone deacetylase 4. *J Biol Chem*. 2004; 279: 34537–46.

22. **Gao C, Li X, Lam M, *et al.*** CRM1 mediates nuclear export of HDAC7 independently of HDAC7 phosphorylation and association with 14-3-3s. *FEBS Lett*. 2006; 580: 5096–104.

23. **Wilson AJ, Byun DS, Popova N, *et al.*** Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer. *J Biol Chem*. 2006; 281: 13548–58.

24. **Razin A.** CpG methylation, chromatin structure and gene silencing-a three-way connection. *EMBO J*. 1998; 17: 4905–8.

25. **Ou JN, Torrisani J, Unterberger A, *et al.*** Histone deacetylase inhibitor Trichostatin A induces global and gene-specific DNA demethylation in human cancer cell lines. *Biochem Pharmacol*. 2007; 73: 1297–307.

26. **Wu LP, Wang X, Li L, *et al.*** Histone deacetylase inhibitor depsipeptide activates silenced genes through decreasing both CpG and H3K9 methylation on the promoter. *Mol Cell Biol*. 2008; 28: 3219–35.

27. **D'Alessio AC, Weaver IC, Szyf M.** Acetylation-induced transcription is required for active DNA demethylation in methylation-silenced genes. *Mol Cell Biol*. 2007; 27: 7462–74.

28. **Harle D, Radmark O, Samuelsson B, *et al.*** Calcitriol and transforming growth factor-beta upregulate 5-lipoxygenase mRNA expression by increasing gene transcription and mRNA maturation. *Eur J Biochem*. 1998; 254: 275–81.

29. **Harle D, Radmark O, Samuelsson B, *et al.*** Transcriptional and posttranscriptional regulation of 5-lipoxygenase mRNA expression in the human monocytic cell line Mono Mac 6 by transforming growth factor-beta and 1,25-dihydroxyvitamin D3. *Adv Exp Med Biol*. 1999; 469: 105–11.

30. **Seuter S, Sorg BL, Steinhilber D.** The coding sequence mediates induction of 5-lipoxygenase expression by Smads3/4. *Biochem Biophys Res Commun*. 2006; 348: 1403–10.

31. **Kohoutek J.** P-TEFb- the final frontier. *Cell Div*. 2009; 4: 19.

32. **Fu J, Yoon HG, Qin J, *et al.*** Regulation of P-TEFb elongation complex activity by CDK9 acetylation. *Mol Cell Biol*. 2007; 27: 4641–51.

33. **Barnes PJ.** Targeting histone deacetylase 2 in chronic obstructive pulmonary disease treatment. *Expert Opin Ther Targets*. 2005; 9: 1111–21.

34. **Barnes PJ.** Role of HDAC2 in the pathophysiology of COPD. *Annu Rev Physiol*. 2009; 71: 451–64.

35. **Barnes PJ, Adcock IM, Ito K.** Histone acetylation and deacetylation: importance in inflammatory lung diseases. *Eur Respir J*. 2005; 25: 552–63.

36. **Montuschi P, Kharitonov SA, Ciabattoni G, *et al.*** Exhaled leukotrienes and prostaglandins in COPD. *Thorax*. 2003; 58: 585–8.

37. **Breitman TR, He RY.** Combinations of retinoic acid with either sodium butyrate, dimethyl sulfoxide, or hexamethylene bisacetamide synergistically induce differentiation of the human myeloid leukemia cell line HL60. *Cancer Res*. 1990; 50: 6268–73.

38. **Khan N, Jeffers M, Kumar S, *et al.*** Determination of the class and isoform selectivity of small-molecule histone deacetylase inhibitors. *Biochem J*. 2008; 409: 581–9.

39. **Itoh Y, Suzuki T, Miyata N.** Isoform-selective histone deacetylase inhibitors. *Curr Pharm Des*. 2008; 14: 529–44.

40. **Hu E, Dul E, Sung CM, *et al.*** Identification of novel isoform-selective inhibitors within class I histone deacetylases. *J Pharmacol Exp Ther*. 2003; 307: 720–8.

41. **Beckers T, Burkhardt C, Wieland H, *et al.*** Distinct pharmacological properties of second generation HDAC inhibitors with the benzamide or hydroxamate head group. *Int J Cancer*. 2007; 121: 1138–48.

42. **Mai A, Massa S, Pezzi R, *et al.*** Class II (IIa)-selective histone deacetylase inhibitors. Synthesis and biological evaluation of novel (aryloxopropenyl)pyrrolyl hydroxyamides. *J Med Chem*. 2005; 48: 3344–53.