Inhibition of class I HDACs abrogates the dominant effect of MLL-AF4 by activation of wild-type MLL

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The ALOX5 gene encodes 5-lipoxygenase (5-LO), a key enzyme of inflammatory reactions, which is transcriptionally activated by trichostatin A (TSA). Physiologically, 5-LO expression is induced by calcitriol and/or transforming growth factor-β. Regulation of 5-LO mRNA involves promoter activation and elongation control within the 3′-portion of the ALOX5 gene. Here we focused on the ALOX5 promoter region. Transcriptional initiation was associated with an increase in histone H3 lysine 4 trimethylation in a TSA-inducible manner. Therefore, we investigated the effects of the MLL (mixed lineage leukemia) protein and its derivatives, MLL-AF4 and AF4-MLL, respectively. MLL-AF4 was able to enhance ALOX5 promoter activity by 47-fold, which was further stimulated when either vitamin D receptor and retinoid X receptor or SMAD3/SMAD4 were co-transfected. In addition, we investigated several histone deacetylase inhibitors (HDACi) in combination with gene knockdown experiments (HDAC1-3, MLL). We were able to demonstrate that a combined inhibition of HDAC1-3 induces ALOX5 promoter activity in an MLL-dependent manner. Surprisingly, a constitutive activation of ALOX5 by MLL-AF4 was inhibited by class I HDAC inhibitors, by relieving inhibitory functions deriving from MLL. Conversely, a knockdown of MLL increased the effects mediated by MLL-AF4. Thus, HDACi treatment seems to switch ‘inactive MLL’ into ‘active MLL’ and overwrites the dominant functions deriving from MLL-AF4.

INTRODUCTION

The human 5-lipoxygenase (5-LO), which is encoded by the ALOX5 gene, is an enzyme that catalyzes the first two steps in the biosynthesis of leukotrienes from arachidonic acid. In the pathophysiological context, leukotrienes are associated with inflammatory, allergic and cardiovascular diseases, as well as certain types of cancer.1

The human ALOX5 gene is organized by 14 exons.2 The ALOX5 promoter contains eight GC boxes, but lacks TATA and CAAT boxes.3 As such, the ALOX5 promoter resembles a promoter structure, which is typically found for housekeeping genes. Expression of the ALOX5 gene is regulated by transcriptional initiation as well as elongation. 5-LO transcript elongation and mRNA maturation are strongly stimulated by calcirol (1,25(OH)2D3) and transforming growth factor-β (TGFβ), respectively, and is controlled by regulatory elements outside the promoter in a ligand-dependent manner, whereas regulatory elements in the promoter region seem to act ligand independent.4–8 In addition to this promoter-independent mechanism, induction of 5-LO mRNA transcripts in undifferentiated myeloid cells can be strongly enhanced by the pan-histone deacetylase inhibitor (HDACi) trichostatin A (TSA).9,10 Furthermore, we observed that upregulation of ALOX5 promoter activity by TSA correlates with the recruitment of the transcription factors Sp1 and Sp3, to a promoter proximal Sp1-binding site next to the transcript initiation site.11

The status of histone acetylation, as a marker for active gene transcription, is regulated by histone acetyl transferases and counteracted by HDACs.11 HDACs deacetylate histones as well as other proteins such as transcription factors and can be 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).11 In addition to acetylation, phosphorylation and ubiquitination, the methylation of histones at certain residues contributes to a sophisticated system called the ‘histone code’ that is directly linked to the regulation and transcriptional memory of cellular gene expression.14–16 Histone H3 lysine 4 trimethylation (H3K4me3) represents the general signature for active promoters. The enzymatic reaction of H3 lysine 4 trimethylation is catalyzed, for example, by the SET domain of the MLL (mixed lineage leukemia) protein.17,18 For the MLL gene, a large number of chromosomal rearrangements are described. In particular, the chromosomal translocation t(4;11)(q21;q23) with the AF4 gene is the most frequently diagnosed reciprocal chromosomal translocation of the human MLL gene.19 The resulting fusion proteins MLL-AF4 (der11) and AF4-MLL (der4) are able to induce and maintain the onset of high-risk acute lymphoblastic leukemia. Possible mechanisms that explain the strong oncogenic behavior has been recently summarized in several publications.20–23

In this study, we demonstrate that HDAC inhibition induces 5-LO mRNA expression, which is concomitantly associated with H3K4 trimethylation of the ALOX5 promoter by the MLL protein. We also show evidence that the MLL-AF4 fusion protein acts as a strong, HDAC-independent transcriptional activator, which acts in a dominant-positive manner over endogenous or transfected MLL. Interestingly, when endogenous MLL becomes activated by HDAC inhibition, the high constitutive activity of MLL-AF4 is diminished to the level of wild-type MLL. We conclude from our study that the
ALOX5 promoter/gene system provides a unique tool to dissect regulatory properties of MLL and its derivative proteins.

RESULTS
Time-dependent induction of ALOX5 mRNA expression and histone H3K4me3 after HDAC inhibition
We recently demonstrated that ALOX5 promoter activity is upregulated by HDAC inhibition. Here we analyzed the TSA-mediated induction of 5-LO mRNA expression in a time-dependent manner in MM6 and HL-60 cells. Cells were grown in the presence or absence of TSA (330 nM) for the indicated time points (Figure 1a). Cells were harvested and the amount of ALOX5 mRNA was determined by quantitative reverse transcription–PCR. In MM6 as well as HL-60 cells, a distinct increase in 5-LO mRNA was detected already after 4 h. Maximal 5-LO mRNA expression was observed after 16 and 24 h of TSA treatment in HL-60 and MM6 cells, respectively. Only HL-60 cells displayed a decline at 24 h (Figure 1a). We additionally investigated the H3K4me3 signature as a surrogate marker for an activated promoter by chromatin immunoprecipitation (ChIP) assays after various time points of TSA treatment (Figure 1b). An increase in H3K4me3 levels was detectable in both tested cell lines, with a maximum after 16–24 h of TSA treatment. Moreover, H3K4me3 levels correlated with the amount of ALOX5 mRNA. Therefore, we concluded that TSA treatment seems to induce the recruitment of the H3K4 histone methyltransferase MLL to the ALOX5 promoter.

MLL-AF4 (der11) shows a strong, constitutive 5-LO promoter activation, whereas 5-LO promoter activation by MLL is HDAC dependent
In order to recapitulate the previous published data with TSA and its regulatory function on the ALOX5 promoter, we used the pN10 (ALOX5 promoter-luciferase) reporter construct. This construct covers the ALOX5 promoter from −778 to +53 relative to the transcriptional start site. In addition, we used the MLL fusion proteins, MLL-AF4 and AF4-MLL, which were known to strongly enhance transcription (MLL-AF4/der11) or transcriptional elongation (AF4-MLL/der4). All three expression constructs were co-transfected with the corresponding reporter construct into HeLa cells. Vehicle treatment (dimethyl sulfoxide) or TSA (330 nM) were used to analyze the effects of all three proteins on the ALOX5 promoter. As shown in Figure 2, MLL-AF4 strongly induced transcription from the ALOX5 promoter by about 47-fold. However, the addition of TSA caused an upregulation of MLL-mediated transcription, while the strong activation of the 5-LO promoter by MLL-AF4 disappeared, reaching a final activity level that was quite similar to that one observed for transfected MLL.

MLL and t(4;11) fusion proteins stimulate 5-LO promoter activity in the presence of VDR/RXR and of SMAD3/4 in a ligand-independent manner
As ALOX5 is a vitamin D3/TGFβ target gene, we investigated possible interactions of the vitamin D3 and TGFβ signaling...
Next, we wanted to identify the HDAC isoform, which is involved in the regulation of the ALOX5 promoter. HeLa cells were transfected with the ALOX5 promoter construct pN10 as well as pTarget or the expression vector for MLL, der4 or der11. Sixteen hours after transfection, cells were cultured in the presence of TSA (330 nM) for 24 h. Then, 5-LO promoter activity was determined by reporter gene assays. Values are given as relative light units (RLUs) and represent the mean ± s.e.m. of three independent experiments. Two-tailed t-test was performed to determine the significance of the TSA effect in reference to the control.

pathways with MLL on the ALOX5 promoter. HeLa cells were transfected with the pN10 reporter construct in conjunction with either pTarget (empty vector control) or pTarget-based expression vectors for MLL, AF4-MLL or MLL-AF4. As the ALOX5 promoter contains vitamin D response elements and SMAD-binding sites, cells were additionally co-transfected with the nuclear receptors vitamin D receptor/retinoid X receptor (VDR/RXR) or SMAD3/SMAD4 and incubated with or without 1,25(OH)2D3 or TGFβ.

In the absence of VDR/RXR or SMAD3/SMAD4, neither empty vector, MLL nor der4 stimulated the ALOX5 promoter (Figure 3a, white bars), while MLL-AF4 expression activated the ALOX5 promoter by about 10-fold. Interestingly, when VDR/RXR (Figure 3a) or SMAD3/SMAD4 (Figure 3b) were co-expressed, MLL, der4 and der11 stimulated 5-LO promoter activity, whereas no effect of VDR or SMADs was observed with the empty vector. The up-to-six-fold induction of ALOX5 promoter activity by VDR/RXR or SMAD3/SMAD4 is displayed in the right panels of Figures 3a and b, respectively.

Interestingly, neither the addition of 1,25(OH)2D3 nor TGFβ enhanced the VDR/RXR or SMAD3/SMAD4-mediated effects. This indicated that the observed effects are ligand independent. In contrast, when we used the VDR/RXR or SMAD3/SMAD4 luciferase reporter control plasmids, a ligand-dependent stimulation of about 4- to 8-fold was observed (Figures 3c and d). Thus, the MLL-mediated regulation of the ALOX5 promoter is stimulated by VDR/RXR and SMAD3/SMAD4, but does not depend on the presence of the respective ligand.

Inhibition of HDAC1-3 indirectly upregulates the ALOX5 promoter via the activation of the MLL protein

Next we wanted to identify the HDAC isoform, which is responsible for the MLL-dependent upregulation of 5-LO promoter activity. To address this question, our reporter construct was co-transfected with pTarget or the pTarget expression vectors for MLL, AF4-MLL or MLL-AF4. Transfected cells were then incubated with different HDACi that display distinct inhibitory profiles for the various HDAC isoforms: MC-1568 is an inhibitor of class Ia HDACs; Apicidin acts on HDAC1, HDAC2, HDAC3 and HDAC8; MS-275 (Entinostat) preferentially inhibits HDAC1, but also inhibits HDAC2 and HDAC3 at micromolar concentrations; PCI-34051 is a known HDAC8 inhibitor; Mocetinostat (MGCD0103) is an HDAC1, HDAC2 and HDAC3 inhibitor; and Droxinostat is an HDAC3, HDAC6 and HDAC8 inhibitor.24-26 Similar to TSA, all tested inhibitors that interfere with HDACs 1–3 strongly increased ALOX5 promoter activity, whereas the inhibition of class Ia HDACs or HDAC8 had no effect (Figures 4a and b). Co-transfection of AF4/MLL leads to similar promoter activity as pTarget (negative control), suggesting that in contrast to MLL and MLL-AF4, the AF4/MLL fusion protein does not activate the basal ALOX5 promoter. Interestingly, some HDACi caused an activation of the reporter construct (see Figure 4a) in the control cells (transfected with pTarget) and in cells where MLL was co-transfected. For example, Apicidin at 100 nM, which inhibits HDAC1, HDAC2 and HDAC3, leads to full promoter activation when MLL is co-transfected. Similar observations were made with MS275 at 1 µM, which inhibits HDAC1 and HDAC2. When the inhibitors were added at concentrations that inhibit HDAC1–3, for example, MS275 (10 µM) and Mocetinostat (1 and 10 µM), the ALOX5 promoter activation was comparable under all four conditions (control, AF4-MLL, MLL and MLL-AF4). Interestingly, co-transfection of MLL-AF4 led to strong activation of the ALOX5 promoter and the addition of HDACi did not enhance this effect. This suggests that the observed activation of the ALOX5 promoter was independent of any HDACi. Most important, all HDACi that lead to an MLL-dependent ALOX5 promoter activation (Apicidin, MS-275, Mocetinostat and Droxinostat) reduced the effect of the MLL-AF4 fusion protein to ~ 50%, similar to what was observed before with TSA (Figure 2). These results suggested again that a functional activation of (endogenous) MLL by distinct HDACi reduces the non-physiological effects of the MLL-AF4 fusion protein on gene transcription, possibly by a competition of MLL that disables binding of MLL-AF4 at the ALOX5 promoter.

Knockdown of HDACs1-3 induces 5-LO promoter activity

As the available HDACi only displayed a limited selectivity for HDAC1–3, transient knockdown experiments for these HDACs were performed to directly test their influence on ALOX5 promoter activity. Knockdown of each HDAC was performed by co-transfection of the respective small interfering RNA (siRNA) and the ALOX5 promoter-luciferase reporter construct. As displayed in Figure 5a, siRNA co-transfection leads to a reduction of HDAC1–3 mRNA in the range of about 40–75%. Unfortunately, we could not verify the knockdown of the HDACs at the protein level, as HDAC expression was too low to be detected by conventional western blotting experiments (data not shown). This HDAC knockdown experiment revealed that 5-LO promoter activity was per se not induced by the knockdown of single HDAC enzymes (Figure 5b). However, the combined knockdown of HDAC3 in conjunction with either HDAC1 or HDAC2 leads to slightly higher promoter activity (Figure 5b), supporting the assumption that HDAC3 is important for the function of the VDR/RXR that binds to ALOX5 promoter fragment, while HDAC1 or HDAC2 probably modulate genuine MLL functions.

Next, we wanted to confirm the synergistic effects between the various HDACs. To this end, we combined the HDAC knockdown with a TSA treatment in order to inhibit remaining HDAC activity and to assess this effect on ALOX5 promoter activation (Figure 5c). Interestingly, the combination of HDAC3 knockdown and TSA treatment led to the highest 5-LO promoter induction, indicating...
that HDAC3 has a key role in the regulation of 5-LO promoter activity but seems to require inhibition of additional HDACs (here, inhibited by TSA) for the maximal activation of the ALOX5 promoter. The lowered induction of the ALOX5 promoter in the presence of TSA (Figure 5c) was due to the higher basal activity of the ALOX5 promoter under the knockdown conditions (compare with Figure 5b). Taken together, our data suggest that inhibition of HDAC1–3 upregulates the ALOX5 promoter activity, presumably in an MLL-dependent manner.

Concentration-dependent effects of HDACi on 5-LO promoter activity and SEM cells

Finally, we used three different HDACi and recorded dose–response curves to compare their MLL-AF4-blocking activities with the concurrent activation of the endogenous MLL protein. For this experiment we used three representative inhibitors: Apicidin (3–300 nM) inhibits HDAC1, 2 and 3), MS-275 that preferentially inhibits HDAC1 (30 nM–3 μM), but at micromolar concentrations also HDAC2 and 3. Finally, we used Mocetinostat (3–300 nM) that inhibits all three HDACs. As shown in Figure 6a, all three inhibitors were able to decrease the promoter-stimulating activity of MLL-AF4 (pT-der11), while the inhibitors increased 5-LO promoter activity with endogenous MLL (pTarget) or the combination of endogenous and transfected MLL (pT-MLL), but all ending at similar promoter activities (compare Figure 4a). Interestingly, reduction of MLL-AF4 activity correlates with HDAC1 inhibition by the three inhibitors, respectively, whereas 5-LO promoter activation correlates with inhibition of all three HDACs. The data suggest an HDAC-dependent mechanism that blocks the promoter-stimulating activity of endogenous or transfected MLL, whereas MLL-AF4 is constitutively active and overrides endogenous MLL. However, if the intrinsic blocking mechanism of MLL is relieved by the addition of HDAC1/2 inhibitors, endogenous or transfected MLL is able to outcompete and thus inhibit the MLL-AF4 functions, thereby blocking its ectopic properties on gene transcription.

This observation was also tested on cells that bear the t(4;11) translocation. In this case, we used the SEM cell line that expresses...
both MLL fusion proteins, MLL-AF4 and AF4-MLL, together with wild-type MLL and AF4. As summarized in Figure 6b, cell counting kit-8 (CCK-8) assays and growth analysis demonstrated that the HDAC1–3 inhibitors Droxinostat and Apicidin display the most potent effects on the viability of these leukemic cells. The HDAC1–3 inhibitors Droxinostat (at 100 μM), Apicidin (300 nM) and MS-275

![Diagram](image)

**Figure 4.** Effect of HDAC inhibition on MLL-, der4- and der11-dependent 5-LO promoter activity. HeLa cells were transfected with the 5-LO promoter construct pN10 as well as pTarget or the expression vector for MLL, der4 or der11. Sixteen hours after transfection, cells were cultured in the presence of the indicated HDACi for 24 h. Then, 5-LO promoter activity was determined by reporter gene assays. (a) Values are given as relative light units (RLUs) and represent the mean ± s.e.m. of three independent experiments. (b) 5-LO promoter activity is indicated as x-fold induction over dimethyl sulfoxide treatment.

![Diagram](image)

**Figure 5.** Effect of HDAC1, 2 and 3 knockdown on 5-LO promoter activity. (a) Real-time PCR analysis of HDAC mRNA expression after siRNA-mediated knockdown in HeLa cells. The cells were transfected with the indicated siRNA and medium was changed after 16 h. RNA was extracted after 48 h. After cDNA synthesis, the knockdown efficiency of each siRNA and the influence of the negative control on mRNA expression (HDAC1, 2, 3 or GAPDH) was determined by quantitative PCR, using acidic riboprotein P0 as housekeeping gene. Data are shown as mean ± s.e.m. of three independent experiments. (b) Reporter gene assay in HeLa cells: 5-LO promoter construct pN10 was co-transfected with specific siRNAs for HDACs 1, 2 or 3 and combinations of these siRNAs. Sixteen hours after transfection, a change of medium was performed. After 48 h, luciferase activity was determined and normalized to transfection efficiencies (given as relative light units (RLUs)). Data are shown as mean ± s.e.m. of at least three independent experiments. (c) Medium was changed 16 h after transfection and TSA (330 nM) was added. After 48 h, luciferase activity was determined and results were calculated as x-fold induction by TSA. Data are shown as mean ± s.e.m. of at least three independent experiments.
were able to inhibit SEM cell growth but also compromised cell viability, whereas the HDAC1, 2 inhibitor Mocetinostat (300 nM) as well as MC1568 (1 μM) and PCI-34051 (5 μM) were less active. The data suggest that inhibition of HDAC1–3 activities and subsequent blockage of the MLL-AF4 oncofusion protein results in reduced cell viability and inhibition of cell proliferation.

Knockdown of endogenous MLL enhances functions of MLL-AF4

In order to validate our hypothesis that activation of endogenous MLL by HDAC class I inhibitors neutralizes the oncogenic properties of MLL-AF4, we performed two additional experiments. First, we recorded dose–response curves for the class I HDACi Apicidin, MS-275 and Mocetinostat in the presence of transfected MLL and MLL-AF4. As shown in Figure 7a, Apicidin, MS-275 and Mocetinostat were all able to reduce the strong transcriptional activation function deriving from MLL-AF4 to the level that is also reached either by endogenous or transfected MLL.

Second, we used an short hairpin RNA-mediated knockdown of endogenous MLL to demonstrate that endogenous MLL is responsible for the observed HDACi effects on MLL-AF4. As shown in Figure 7b, an MLL knockdown of about 30% could be reached. This was sufficient to increase the MLL-AF4-mediated activation of the 5-LO promoter, indicating that endogenous MLL antagonizes the transcriptional activity of MLL-AF4. Furthermore, reduction of MLL-AF4 transcriptional activity by the class I HDACi was impaired by the MLL knockdown.

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Figure 6. (a) Dose–response curve of Apicidin, MS-275 and Mocetinostat for 5-LO promoter activation (pTarget, pT-MLL) and inhibition of der11-dependent 5-LO promoter activity (pT-der11). HeLa cells were transfected with the 5-LO promoter construct pN10 as well as pTarget or the expression vector for MLL or MLL-AF4 (der11). Sixteen hours after transfection, cells were cultured in the presence of the indicated HDACi for 24 h. Then, 5-LO promoter activity was determined by reporter gene assays. Values are given as percentage of the maximal 5-LO promoter activity. Data are shown as the mean ± s.e.m. of three independent experiments. (b) Cell counting kit-8 assay and cell growth data. Cells were grown in the presence of TSA (330 nM), MC1568 (1 μM), Apicidin (300 nM), MS-275 (3 μM), PCI-34051 (5 μM), Mocetinostat (300 nM) or Droxinostat (100 μM) for the indicated times. Then, cell viability was determined by the cell counting kit-8 assay, a non-toxic form of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cell growth was measured independently by counting living cells in a cell counter after trypan blue addition. Results are presented as mean of three independent experiments. Each experiment was performed in triplicate.
investigated the chromatin signatures at the endogenous promoter in order to understand transcript initiation in more detail, we actin as housekeeping gene. One sample purpose of our studies, we used a promoter fragment that was fused to a luciferase reporter gene. In our experiments, the cells were treated with lentiviral particles, generated by co-transfection of HEK293T cells with the short hairpin RNA plasmid for MLL, psPAX2 and pMD2.G. Knockdown efficiency of MLL was checked by quantitative PCR, using β-actin as housekeeping gene. One sample t-test was performed to determine the significance of MLL knockdown (left graph). HeLa wild-type and HeLa MLL knockdown cells were co-transfected with pTarget, MLL or MLL-AF4. In cells transfected with MLL, HDAC inhibition by Apicidin slightly increases the expression construct for MLL or MLL/MLL-AF4. In cells transfected with MLL-AF4, there is a prominent binding of endogenous MLL to the ALOX5 promoter as detected by the MLL antibody directed against the MLL C-terminus, which recognizes MLL but not MLL-AF4. Interestingly, Apicidin does not seem to alter the abundance of MLL at the 5-LO promoter but the compound strongly downregulates binding of MLL-AF4 to the ALOX5 promoter (Figure 8c vs d), which is in accordance to our observation that Apicidin downregulates the ability of MLL-AF4 to bind to the 5-LO promoter, while MLL does.

DISCUSSION

Expression of the ALOX5 gene is upregulated during myeloid cell maturation. The gene is regulated at the level of transcript initiation and transcriptional elongation. In the present study, we focused only on the regulation of transcriptional initiation. For the purpose of our studies, we used a promoter fragment (~778 to +78) of ALOX5 that was fused to a luciferase reporter gene. In order to understand transcript initiation in more detail, we investigated the chromatin signatures at the endogenous promoter and observed that TSA treatment leads to a profound upregulation of the canonical H3K4me3 signature. Therefore, we started to investigate the function of the H3K4 histone methyltransferase MLL and its role in the control of ALOX5 transcription. As the ALOX5 gene expression has previously been linked to the onset of leukemia development, we also investigated two fusion genes of MLL, MLL-AF4 and AF4-MLL, which produce the most common fusion proteins found in MLL-rearranged leukemia patients. From earlier studies, it was known that MLL-AF4 acts on promoters and enhances gene transcription, while AF4-MLL has an important role for transcriptional elongation. We were able to demonstrate that either selective inhibition of class I HDACs or the siRNA-mediated knockdown of HDAC1–3 has significant effects on the activation of the ALOX5 promoter in an MLL-dependent manner. As the MLL protein complex is known to bear an intrinsic regulatory mechanism, enabling MLL to act either as transcriptional activator or repressor, our findings about HDACi and the MLL functions are quite intriguing. The activation and inhibitory functions are mediated by distinct domains of the MLL protein. The N-terminal portion of MLL exhibits the inhibitory MT domain that binds BMI-1 and HDAC1 and 2 whereas the C-terminal activation domain can recruit CBP and potentially other members of the coactivator complex. The MLL PHD3 domain binds either to H3K4me3 signatures or to the prolyl isomerase CYP33 (mutually exclusive). CYP33 regulates the conformation of MLL by proline isomerization, which facilitates an interaction to the complex of BMI-1 and HDAC1/2. Therefore, CYP33 modulates the function of MLL and allows it to act as a transcriptional repressor.
Interestingly, the MLL-AF4 fusion protein still retains the MT domain, but lacks the PHD domain. According to our data, MLL-AF4 acts similar to a constitutively active version of the MLL protein on the tested ALOX5 promoter. Addition of different HDACi that specifically block HDAC1–3 activities allows efficient activation of the reporter construct by endogenous MLL, while binding to and activation of the ALOX5 promoter by MLL-AF4 is concomitantly reduced. This mode of action is displayed in Figure 9, where class I HDACs (presumably HDAC1 and 2) seem to act on the activator/repressor switch of MLL, which significantly reduce binding of MLL-AF4 to the ALOX5 promoter. Interestingly, the presence of MLL-AF4 seems to dramatically enhance the binding of MLL. This may indicate an additional and novel function of MLL-AF4, which could be ‘chromatin opening’, for example, by recruiting the SWI/SNF complex. This needs to be investigated in future experiments.

However, our current data suggest that the application of class I HDACi should have a therapeutic benefit in MLL-rearranged leukemias, as they abolish activities deriving from MLL-AF4 product by activation of endogenous MLL. This assumption was validated by the experiments displayed in Figure 6b, where proliferation and viability of t(4;11) cells could be strongly inhibited by the class I HDACi.

Our data also confirm earlier findings that the AF4-MLL fusion protein has effects mainly on transcriptional elongation and acts less on transcriptional initiation. AF4-MLL lacks the DNA-binding domain of MLL. Instead, the AF4-MLL fusion protein links the N-terminal part of AF4, which mediates binding to the transcription elongation factor p-TEFb, to the C-terminus of MLL, which can interact with the transcription factors such as CBP or E2F2, and can stimulate transcript elongation, if a given transcript is elongation controlled. When we tested the p-TEFb inhibitor Flavopiridol (30 nM), we could not inhibit reporter gene activity, indicating that transcription from the ALOX5 promoter reporter plasmid is not elongation controlled (data not shown).

By contrast, VDR/RXR as well as SMAD3/SMAD4 support the induction of ALOX5 promoter activity by MLL, AF4-MLL and MLL-AF4. Interestingly, their respective ligands 1,25(OH)2D3, TGFβ are strong inducers of 5-LO mRNA expression in myeloid cells and it was shown that the 5-LO promoter contains vitamin D-responsive elements and binds the VDR, but that these ligand-dependent effects are not mediated by the ALOX5 promoter and are due to stimulation of transcriptional elongation, which seems to be controlled by regulatory elements outside of the ALOX5 promoter. These data are in line with our observation here.
that VDR/RXR as well as SMAD3/SMAD4 support MLL induction of ALOX5 promoter in a ligand-independent manner. Our results suggest that there is an interaction between VDR/RXR as well as SMADs and MLL, which supports the model of an MLL associated transcription-regulating multiprotein complex.37

In conclusion, our data show that both MLL and MLL-AF4 are able to activate the ALOX5 promoter. Of note, we found that wild-type MLL has low activating activity in the absence of HDAC inhibition and that HDAC class I inhibitors stimulate 5-LO promoter activity in an MLL-dependent manner. Our findings are in line with recent findings where gene expression profiling data of t(4;11) leukemia cells were related to connectivity maps. This type of analyses revealed that MLL leukemia cells could profit from HDACi.38 Here we demonstrate for the first time by using a bona fide target gene of the MLL complex that the high constitutive oncogenic activity of MLL-AF4 can be diminished by class I HDACi. This observation might be of clinical importance and it will be of interest to elucidate the benefit of class I HDACi as an add-on treatment for t(4;11) leukemia patients.

**MATERIALS AND METHODS**

**Cells and cell culture**

Mono Mac 6 (MM6) cells (DSMZ number: ACC-124) were grown at 37 °C in a humidified atmosphere with 5% CO2 in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, l-glutamine (2 mM), L-glutamine (2 mM), oxalacetate (1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and insulin (10 μg/ml). HL-60 cells (DSMZ number: ACC-3) and SEM K2 cells (DSMZ number: ACC-546) were grown under same conditions in RPMI 1640 medium, 10% (v/v) fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). HeLa cells (DSMZ number: ACC-57) were grown in Dulbecco’s modified Eagle medium supplemented with 10% (v/v) fetal calf serum, l-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultivation of the HeLa cells was carried out in a humidified atmosphere of 5% CO2 at 37 °C.

**Reagents**

TSA (Sigma-Aldrich, Schnelldorf, Germany: T1952), MC-1568 (Sigma-Aldrich: M1824), Apicidin (Sigma-Aldrich: A8851), MS-275 (Enzo Life Sciences, Lörrach, Germany: ALX-270-378-M005), PCI-34051 (Cayman Chemical, Ann Arbor, MI, USA: 10444), Mocetinostat (Selleckchem,
München, Germany; S1122) and Droxistatin (Selleckchem; S1422) were dissolved in dimethyl sulfoxide (AppliChem, Darmstadt, Germany; A3006). Calcirol was purchased from Sigma-Aldrich. Human TGFβ1 was purified from platelets according to Weiz et al.\(^9\)

**RNA extraction and cDNA synthesis**

Two micrograms of total RNA were extracted from HL-60, MM6 or HeLa cells by the Total RNA Kit from Omega Bio-Tek (Norcross, GA, USA; R6834-02). RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, München, Germany: 170–8897) or the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Darmstadt, Germany: 4368814) 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 (Bio-Rad). The sequences of the 5-LO primers were 5′-GTCTCTGATAGGCTTGAAC-3′ (forward) and 5′-GGCAGGAGGACATCTGG-3′ (reverse). Results were normalized to riboprotein P0 Ct values. Sequences of the riboprotein P0 primers were as follows: 5′-AGATGCAGCAGATGCCTGATC-3′ (forward) and 5′-GTGCTGATACCTAAGCGTCG-3′ (reverse). Each sample was set up in triplicates. The expression was quantified by comparative ΔΔCT method.

**ChIP assay for H2K4me3**

ChIP assays were performed as described previously, with some modifications.\(^11\) 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 pH 8, 1 mM EDTA pH 8) before harvest. The antibodies used for immunoprecipitation were anti-histone H3 lysine 4 trimethyl (Upstate, Merck Millipore, Schwalbach, Germany: sc-2028) and normal rabbit IgG (Santa Cruz Biotechnology, Heidelberg, Germany: sc-2027). The DNA was analysed by PCR (32 cycles) using the following primers: 5′-AGGAGACACAGCCTGCTGAGGAG-3′ (forward) and 5′-GGGCTGATAGGAGTAATCTGAGC-3′ (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 (Bio-Rad).

**ChIP assay on the recruitment of MLL and MLL-AF4 to the 5-LO promoter region**

Twenty-four hours before transfection, HeLa cells were seeded at a density of 2×10⁵ cells in a 145-mm cell culture plate with the cell line-specific medium. Transient transfection of the cells with 13.75 μg 5-LO promoter reporter gene construct pN10 and 6.875 μg expression vector pTarget (pT-MLL, pT-der4 or pT-der11), 50 ng nuclear receptor expression vector (pSGS-VDR and pSGS-RXR or pcGN-SMAD3 and pCGN-SMAD4) and 20 ng Renilla luciferase control vector (pRL-SV40) per well was performed by calcium phosphate precipitation. The medium was changed 16 h after transfection and the cells were incubated without or with 50 μM calcirol and 1 ng/ml TGFβ or inhibitors, as indicated in the text. Forty hours after transfection, firefly and Renilla luciferase activity was determined with the Dual-Glo Luciferase Assay System (Promega) and a TECAN infinite M200 luminometer. Data were calculated as relative light units by normalization of transfection efficiency with the values of Renilla luciferase activity.

**Stable knockdown of MLL in HeLa cells by lentiviral transduction**

MISSION short hairpin RNA plasmid for MLL knockdown (NM_005933.1-2990s1c1: TRCN0000005954) was obtained from Sigma-Aldrich. Hek293T cells were transfected with 10 μg short hairpin RNA plasmid, 6.5 μg lentiviral packaging vector psPAX2 (Addgene, Cambridge, MA, USA: 12260) and 2.5 μg envelope plasmid pMD2.G (Addgene: 12299) by calcium phosphate precipitation method. The medium was changed 4 h after transfection and supernatant was collected after 72 h. For transduction, HeLa cells were prepared in 24-well plates and were treated after 24 h with 4 μg/ml protamine sulfate and 10, 50, 100 or 500 μl supernatant including lentiviral particles. The cells were centrifuged (90 min, 2500 rpm, 32°C) and cultured for 72 h. Selection of transduced cells was performed by puromycin (0.75 μg/ml) and knockdown efficiency was checked by quantitative PCR analysis with a StepOnePlus System (Life Technologies). The sequences of the MLL primers were 5′-AGGAGAATTGCAGGACATTGAA-3′ (forward) and 5′-TTTCTGGAAGCTGATTTGCGG-3′ (reverse). Results were normalized to β-actin Ct values. Sequences of the β-actin primers as: 5′-CGGAGCTGTCAGCCTATGCTTTG-5′ (forward) and 5′-CTCTCTTCCATAGCTCACCGACG-3′ (reverse). Each sample was set up in triplicates. The expression was quantified by comparative ΔΔCT method.

**Calcium phosphate transfection and reporter gene assay**

Twenty-four hours before transfection, HeLa cells were seeded at a density of 4×10⁵ cells per well in 24-well plates by using the cell line-specific medium without phenol red. Transient transfection of the cells with 225 or 275 ng 5-LO promoter firefly luciferase reporter gene construct pN10, 225 or 275 ng expression vector (pTarget, pT-MLL, pT-der4 or pT-der11), 50 ng nuclear receptor expression vector (pSGS-VDR and pSGS-RXR or pcGN-SMAD3 and pCGN-SMAD4) and 20 ng Renilla luciferase control vector (pRL-SV40) per well was performed by calcium phosphate precipitation method.\(^11\) The medium was changed 16 h after transfection and the cells were incubated without or with 50 μM calcirol and 1 ng/ml TGFβ or inhibitors, as indicated in the text. Forty hours after transfection, firefly and Renilla luciferase activity was determined with the Dual-Glo Luciferase Assay System (Promega) and a TECAN infinite M200 luminometer. Data were calculated as relative light units by normalization of transfection efficiency with the values of Renilla luciferase activity.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.
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