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Class I lysine deacetylases promote glucocorticoid-induced transcriptional repression through functional interaction with LSD1

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Abstract

Small molecule inhibitors of lysine deacetylases (KDACs) are approved for clinical use in treatment of several diseases. Nuclear receptors, such as the glucocorticoid receptor (GR) use lysine acetyltransferases (KATs or HATs) and KDACs to regulate transcription through acetylation and deacetylation of protein targets such as histones. Previously we have shown that KDAC1 activity facilitates GR-activated transcription at about half of all cellular target genes. In the current study we examine the role of Class I KDACs in glucocorticoid-mediated repression of gene expression. Inhibition of KDACs through two structurally distinct Class I-selective inhibitors prevented dexamethasone (Dex)-mediated transcriptional repression in a gene-selective fashion. In addition, KDAC activity is also necessary to maintain repression. Steroid receptor coactivator 2 (SRC2), which is known to play a vital role in GR-mediated repression of pro-inflammatory genes, was found to be dispensable for repression of glucocorticoid target genes sensitive to KDAC inhibition. At the promoters of these genes, KDAC inhibition did not result in altered nucleosome occupancy or histone H3 acetylation. Surprisingly, KDAC inhibition rapidly induced a significant decrease in H3K4Me2 at promoter nucleosomes with no corresponding change in H3K4Me3, suggesting the activation of the lysine demethylase, LSD1/KDM1A. Depletion of LSD1 expression via siRNA restored Dex-mediated repression in the presence of KDAC

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Conflict of interest statement

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

CLS conceived and, along with NMP, coordinated the study. CLS, NMP, and CAG wrote the paper. VK, CAG, MMG, and ALI designed, performed and analyzed the experiments shown in Figs. 1 and 2. NMP designed, performed and analyzed the experiments shown in Figs. 3 and 4. CAG and NMP designed, performed and analyzed the experiments shown in Fig. 5. VK, CAG, MMG, and ALI designed, performed and analyzed the experiments shown in Fig. 6. RJF provided technical assistance for all experiments shown. All authors reviewed the results and approved the final version of the manuscript.

inhibitors, suggesting that LSD1 activation at these gene promoters is incompatible with transcriptional repression. Treatment with KDAC inhibitors does not alter cellular levels of LSD1 or its association with Dex-repressed gene promoters. Therefore, we conclude that Class I KDACs facilitate Dex-induced transcriptional repression by suppressing LSD1 complex activity at selected target gene promoters. Rather than facilitating repression of transcription, LSD1 opposes it in these gene contexts.

Keywords

Lysine deacetylase; Glucocorticoid; Transcription; Repression; Lysine demethylase

1. Introduction

Glucocorticoids influence a variety of important physiological processes, including anti-inflammatory responses, tissue differentiation during organismal development, programming of neuroendocrine function in adult animals, and diurnal regulation of metabolism. The most common clinical use of glucocorticoids is for immunosuppression, including treatment of inflammation, autoimmune diseases, and asthma [1,2]. Glucocorticoid receptor (GR, gene designation: NR3C1) acts as a transcription factor to bring about in vivo responses to glucocorticoids by activating and repressing expression of target genes. Activation of transcription by GR involves its interaction with lysine acetyltransferases (KATs, also known as histone acetyltransferases or HATs) that acetylate nucleosomal histones at enhancers and promoters of target genes. In some cases GR has been shown to recruit lysine deacetylases (KDACs, also known as histone deacetylases or HDACs) to gene promoters to repress transcription, in part via effects on the local chromatin environment [3–7]. According to this model, KDACs facilitate GR-mediated transcriptional repression through histone deacetylation.

Mammalian KDACs comprise a family of proteins that are divided into four classes based on structural homologies to yeast KDACs. Because they remove acetyl groups from histones and were found in complexes known to be involved in transcriptional repression, KDACs were originally cast as transcriptional corepressors. However, more recent studies have shown that KDACs also facilitate transcription, indicating that these enzymes are versatile modulators of transcription [reviewed in [8–11]]. The development of small molecule lysine deacetylase inhibitors (KDACi, also known as HDACi) against the zinc-dependent KDACs has deepened interest in their roles in development, physiology, cell biology, and disease. The KDACi, valproic acid (VPA), is approved for treatment of epilepsy, bipolar disorder, and migraines, while vorinostat, romidepsin, belinostat, and panobinostat are approved for treatment of specific hematological malignancies. VPA is considered a Class-I selective inhibitor because of its higher potency for Class I KDACs [12]. After more than 30 years of clinical use, it is clear that VPA causes metabolic and reproductive side effects in about 50% of users [13–15]. Nuclear receptors (NR) are important regulators of reproduction and metabolism, and they are known to use KDACs and KATs to regulate transcription. Thus, it is possible that these side effects are caused in part by a negative impact of KDAC inhibition on NR signaling. KDACi are being evaluated for treatment of other diseases, including

inflammatory and neurological disorders [16–19], so it is essential to clearly understand the roles of KDACs in regulating endocrine signaling pathways.

Mammalian Class I KDACs are subunits of multiple complexes. KDACs 1 and 2 (also known as HDAC1 and HDAC2) are subunits of the Sin3, NURD, and CoREST complexes while KDAC3 (also known as HDAC3) associates with the NCoR/SMRT complex [16]. Recent studies have revealed that all of these complexes can associate with specific lysine demethylases, suggesting that deacetylation and demethylation are coregulated [20–23]. Lysine-specific demethylase 1 (LSD1, gene designation: KDM1A), the first identified lysine demethylase (KDM), was initially identified as a component of the CoREST-REST transcriptional repressor complex that is important for repression of neuronal genes [24]. More recently it has also been reported to associate with a subset of NURD complexes [22]. LSD1 specifically removes mono- and di-methylation of histone H3 lysine 4 [25], modifications that are associated with active promoters and enhancers [26]. In addition to its repressive functions, LSD1 was shown to facilitate transcription activated by the androgen and estrogen receptors through demethylation of H3 lysine 9 (H3K9) [27–31].

We recently reported that Class I KDACs facilitate GR-activated transcription [32], a finding which contradicts the traditional model that portrays KDACs as transcriptional corepressors. Furthermore, we demonstrated that KDAC1 is a major player in this process. In the current study, we investigated the involvement of KDACs in glucocorticoid-induced repression of gene expression in the same hepatoma cell line that was used to demonstrate a positive role for KDACs in GR transactivation. We show that Class I KDAC activity facilitates both onset and maintenance of glucocorticoid-induced transcriptional repression in a gene-selective fashion. Steroid receptor coactivator 2 (SRC2, also known as GRIP1 and TIF2, gene designation: NCOA2), which mediates GR transrepression in immune cells [33], is not required for repression of the KDACi-sensitive genes tested. Finally, we show that while KDACs do not deacetylate histones in the proximal promoter regions of these repressed target genes, they do regulate H3K4 methylation through suppression of LSD1 activity to promote glucocorticoid-induced repression. Our study shows that Class I KDACs are versatile coregulators of glucocorticoid-regulated transcription and reveals a novel functional interplay between KDACs and LSD1 that impacts glucocorticoid-repressed transcription.

2. Materials and methods

2.1. Cell lines, antibodies, and reagents

Murine hepatoma cells (Hepa-1c1c7) were maintained in Minimum Essential Media α (MEM α) (Invitrogen) containing 10% fetal bovine serum (FBS) (Gemini Bio-Products) and 0.1% gentamicin (Invitrogen). Anti-acetylated histone H3 [06-599], anti-H3K4Me2 [07-030], anti-H3K4Me3 [2591878] and anti-LSD1 [17-10531] antibodies were purchased from Millipore. Antibodies against SRC1 (sc-8995), KDAC1 (sc-6298), KDAC2 (sc-9959), KDAC3 (sc-11417), and glyceraldehyde phosphate dehydrogenase (GAPDH) (sc-25778) were purchased from Santa Cruz Biotechnology. Anti-SRC2 [610984] was purchased from BD Labs. Anti-SRC3 (5E11), anti-H3K9Me2 [97535] and anti- α -tubulin were obtained from Cell Signaling technology. Histone H3 (ab1791) antibody was obtained from Abcam. Glucocorticoid receptor (Ab-2) mouse monoclonal antibody (BUGR2, GR32L) was

obtained from Calbiochem. Anti-KDAC1 (PA1-860) for the co-immunoprecipitation studies was from Thermo while immunoprecipitation antibodies for KDAC2 (PA1-861 and A300-705A) were purchased from Thermo and Bethyl, respectively. The secondary anti-mouse (115-035-146) and antirabbit (111-035-144) antibodies were purchased from Jackson ImmunoResearch and anti-goat (sc-2056) was purchased from Santa Cruz Biotechnology. Dexamethasone, VPA and apicidin were obtained from Sigma-Aldrich.

2.2. RNA analysis

Cells were seeded in 6-well dishes at 2×10^5 cells per well. After 24 h, cells were treated with VPA (5 mM), apicidin (0.25 $\mu\text{g/ml}$) for 5 h and Dex (100 nM) for 4 h followed by lysis in Trizol (Invitrogen). Total RNA was isolated according to the manufacturer's protocol using the Nucleospin RNA II kit (Clontech). cDNA was generated using the qScript cDNA synthesis kit (Quanta). qPCR was performed using a Stepone Plus instrument (Applied Biosystems) with SYBR Green mastermix (Quanta) according to the manufacturer's specifications. Table 1 lists the gene-specific exon-exon and exon-intron primer pairs used to measure levels of mRNA and nascent transcripts, respectively. In each experiment the test gene Ct values were normalized against the geometric mean of the corresponding GAPDH and hypoxanthine guanine phosphoribosyltransferase (HPRT) Ct values to obtain Ct values for each sample. To determine changes in expression between treated and untreated samples (Ct), the Ct values for treated samples were normalized against those from the untreated controls for each test gene. In each experiment, primer efficiency was calculated from standard curves and used to convert the Ct values into fold change. The fold change values were then used to graph results and calculate SEM. The Ct values of two different treatments (e.g. Dex alone vs. Dex+ Drug or Dex+non-targeting siRNA vs. Dex + targeting siRNA) were compared using the paired *t*-test to determine whether changes were statistically significant ($p < 0.05$).

2.3. siRNA-mediated depletions

Hepa-lclc7 cells were plated in 12 or 24 well dishes at a density of 5×10^5 cells/well or 2×10^4 cells/well, respectively, in antibiotic-free MEM α . DharmaFECT reagent 1 (Dharmacon) was used at a concentration of 0.75 $\mu\text{l/well}$ according to manufacturer's specifications to transfect cells with siRNA. KDACs, SRCs and LSD1 were knocked down using the corresponding ON-TARGET plus SMART-pool ORF siRNA (Dharmacon) targeting KDAC1, KDAC2, KDAC3, SRC1, SRC2, SRC3 and LSD1. Successful knockdown was confirmed using western blotting. Non-targeting (NT) siRNA was used as a control.

2.4. Western blotting

Cell lysates for analysis of KDAC, Steroid Receptor Coactivator (SRC), or LSD1 expression after siRNA transfection were prepared by adding 2X SDS-PAGE Buffer to treated cells. Western blotting conditions for KDACs are described in our previous study [32]. For analysis of SRC proteins, cellular proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Biorad) at 400 mA for 4 h. Membranes were blocked with 2% non-fat dry milk for 1 h, followed by exposure to primary antibodies at 4°C overnight. After subsequent exposure to appropriate secondary antibodies, the membrane was washed 3 times with 1X TBS/0.1% Tween-20 solution. The proteins were visualized using a 1:1 ratio

of hydrogen peroxide and luminol (Pierce) with the Chemi Doc XRS + molecular imager (Bio-Rad).

2.5. Co-immunoprecipitation

Hepa1c1c7 cells were seeded in 150 mm plates at a density of 2.2×10^6 cells per plate. After two days cells were harvested, washed with D-PBS and pelleted by centrifugation at 1500 rpm for 4 min at 4°C. After washing with D-PBS cell pellets were resuspended in Buffer A [(10 mM HEPES pH8.0, 1mM EDTA pH 8.0, 50 mM NaCl, 0.5 M sucrose, 10 mM sodium phosphate pH 7.3, 5 mM DTT, 0.1% NP-40, phosphatase inhibitors (10 nM NaF, 0.1 mM Na_3VO_4 , 25 mM β -glycerophosphate), and protease inhibitor cocktail (Roche)]. Cells were lysed using a Dounce homogenizer and cell nuclei were pelleted by centrifugation (1500 rpm for 5 min at 4°C). Nuclei were resuspended thoroughly in Buffer B at half the desired volume (10 mM HEPES pH8.0, 0.1 mM EDTA pH 8.0, 100 mM NaCl, 25% glycerol, 10 mM sodium phosphate pH 7.3, 5 mM DTT, 0.1% NP-40, phosphatase and protease inhibitors). While vortexing, an equal volume of Buffer B containing 0.6 M NaCl was added to obtain a final concentration of 0.3 M NaCl. After centrifugation ($30,000 \times g$, 15 min), the supernatant was aliquoted and flash frozen for storage at -80°C . Protein A-agarose and protein G-agarose slurry was used to pre-clear 500 μg of nuclear protein diluted in IP-buffer (1 M HEPES, 0.5 M EDTA, 1 M DTT, 10% Triton-X, with phosphatase and protease inhibitors). To this supernatant 3 μg of either KDAC1, KDAC2 or 5 μg anti-GFP antibody and a mixture of protein A-agarose and protein G-agarose beads (Pierce) was added and rotated overnight at 4°C. The beads were pelleted and washed three times with IP-buffer. The bound proteins were eluted using 2X SDS-PAGE Buffer (20% glycerol, 4% sodium dodecyl sulfate, 0.0025% bromo phenol blue, 0.125 M Tris HCl, pH 6.8) followed by incubation at 95°C for 5 min prior to separation by SDS-PAGE.

2.6. Chromatin immunoprecipitation

Hepa-1c1c7 cells were seeded at a density of 2.2×10^6 cells/15-cm plate. After drug treatment cells were fixed with 1% formaldehyde at room temperature for 10 min and neutralized using 0.125 M glycine. Fixed cells were harvested and sonicated as described previously [32]. Five percent of the sonicated chromatin was removed for use as input. The remaining sonicated chromatin was precleared with protein A/G-agarose (pretreated with 1 mg/ml salmon sperm DNA and 1 mg/ml Bovine Serum Albumin) for 45 min. The chromatin equivalent of 1×10^7 cells was then rotated overnight at 4°C with no antibody or primary antibodies followed by 45 min. incubation with Protein A/G-agarose beads (Pierce). Washing of the bound fraction and DNA extraction was performed as described previously [32]. Input and bound DNA was amplified in 2X SYBR buffer (Quanta) using real time qPCR (ABI StepOnePlus). Primer sequences are listed in Table 1. All amplicon sizes are less than 130 bp for ChIP primer sets. Ct values for the bound DNAs were normalized against the input Ct values (Ct), and the various treatments were normalized against control (Ct). Primer efficiency was calculated using standard curves and used to convert the Ct value into fold change. The Ct values from the various treatments were compared with those of untreated control within each experiment and were used in a paired *t*-test to determine whether changes were statistically significant. Values of $p \leq 0.05$ were deemed significant.

3. Results

3.1. Inhibition of KDACs prevents glucocorticoid-induced repression of gene expression

Previously, we carried out expression profiling in the mouse hepatoma cell line, Hepa-1c1c7, and determined that GR transactivation was impaired at approximately half of the Dexamethasone (Dex)-activated genes in the presence of the Class I-selective KDACi, VPA [32]. The expression profiling data also revealed that at certain genes, VPA attenuated Dex-induced repression of gene expression (not shown). To verify this, we examined the effect of VPA on mRNA levels from selected Dex-repressed genes through RT-qPCR. Fig. 1A, B shows seven genes at which repression by Dex was significantly impaired in the presence of VPA. At three of these genes, VPA alone has no significant effect on mRNA levels (Fig. 1A), while at four others, VPA treatment increases mRNA levels very modestly, about 1.5- to 2-fold (Fig. 1B). The effect of VPA on Dex-induced repression is, however, gene-selective. Fig. 1C shows two genes, *Ikbke* and *Mllt11*, at which VPA has no effect on Dex-induced down-regulation.

VPA is known to induce cellular effects through targets other than KDACs [34]. To confirm that impaired repression was due to KDAC inhibition rather than an off-target effect, we repeated the experiment with apicidin, another class I-selective KDACi that, as a cyclic tetrapeptide, has a very different chemical structure when compared to VPA, a simple aliphatic acid. The major difference in structure makes it very unlikely that these drugs have the same off-target effects. As shown in Fig. 1, apicidin and VPA have very similar effects at all nine genes tested both in the presence and absence of Dex, strongly indicating that the impairment of glucocorticoid-repressed gene expression is due to inhibition of the shared targets of these drugs, the class I KDACs.

3.2. Class I KDAC activity is required for onset and maintenance of Dex-repressed transcription

The microarray and RT-qPCR results explicitly show that Dex-induced decreases in mRNA levels can be alleviated by KDAC inhibition in a gene-selective fashion. To determine whether these effects are transcriptional, we measured levels of nascent, unspliced transcripts from six GR target genes using exon-intron primer sets in RT-qPCR (Table 1). Hepa-1c1c7 cells were treated with Dex or a combination of Dex plus VPA for up to 4 h as shown in Fig. 2A–F. Transcription from six genes analyzed was significantly decreased in the presence of Dex relative to no treatment (Control). Transcription of the *Igsf9*, *Hes1* and *Hbegf* genes was significantly repressed within 1 h of Dex treatment, while transcription of the *Mex3a*, *Fam20c* and *Cdon* genes was repressed more slowly. In the presence of VPA, all of the genes tested showed significant impairment of Dex-induced gene repression at multiple time points. At several genes (*Mex3a*, *Igsf9*, *Cdon*, and *Hbegf*) the combination of Dex and VPA significantly increases transcription over the basal level, although the increases are modest, with the exception of *Cdon*.

In the experiments described above, cells were pre-treated with VPA for 1 h prior to Dex treatment. It is possible that Class I KDAC activity is required to initiate transcriptional repression but may be dispensable for its maintenance. To address this issue, we exposed

cells to Dex for 1 h prior to addition of VPA and then measured nascent transcript levels from three genes that are significantly repressed by Dex within 1 h, *Igsf9*, *Hes1*, and *Hbegf* (see Fig. 2D–F). As shown in Fig. 2G–I, within 30 min of VPA exposure, there is a dramatic alleviation of transcriptional repression at all three genes. Altogether the results show that KDAC activity is crucial for both the onset and maintenance of glucocorticoid-induced transcriptional repression.

3.3. SRC2 is dispensable for dex-induced transcriptional repression at genes sensitive to KDAC activity

In the immune system GR represses transcription of pro-inflammatory genes by mechanisms that are dependent on the coregulator, SRC2 [reviewed in [33]]. The repressive activity of SRC2 is mediated through a repression domain, just C-terminal to its NR boxes, through which it interacts with GR and other nuclear receptors. In a proteomic study of cellular protein acetylation, SRC2 was found to be acetylated at lysine residues within the repression domain [35]. This raises the possibility that increased acetylation of SRC2 upon inhibition or depletion of KDACs may inactivate its repressive function. To investigate the role of SRC2 on Dex-induced transcriptional repression we used siRNA to deplete the SRC2 protein individually or to selectively reduce the expression of SRCs 1 and 3 (Fig. 3A). This would result in cells that express only SRCs 1 and 3 or that express only SRC2, respectively.

As shown in Fig. 3B–D, loss of SRC2 did not impair transcriptional repression induced by Dex alone at any of the genes tested, nor did expression of only SRC2 (through combined SRC1 and SRC3 depletion) increase the magnitude of Dex-induced repression. Thus, at these genes SRC2 is dispensable for Dex-induced repression. Expression of SRC2 alone had no effect on the ability of VPA to alleviate Dex-induced repression at any of the genes. Likewise, loss of SRC2 did not significantly impact the alleviation of Dex-induced repression at the *Hbegf* (Fig. 3C) or *Hes1* genes (Fig. 3D). Although the difference in expression of the *Igsf9* gene between the Dex and VPA/Dex treatment conditions did not achieve significance by the paired *t*-test upon SRC2 depletion, it is clear that Dex-induced repression is impaired in the presence of VPA (Fig. 3B).

3.4. Transcriptional regulation of Dex-repressed genes does not involve significant changes in nucleosome density or histone H3 acetylation

Class I KDACs have been found to be enriched in transcription-ally-active regions of the genome [36–38], but their role in the transcription cycle of active genes is not well-understood. Since they are established to target histones and, through reducing histone acetylation, contribute to chromatin remodeling, we investigated the impact of glucocorticoid signaling and/or KDAC inhibition on nucleosome density and histone H3 acetylation through chromatin immunoprecipitation (ChIP). The rapid repression of transcription by Dex at the *Hes1*, *Igsf9*, and *Hbegf* genes (Fig. 2D–F) indicates that it is not mediated through secondary effects requiring altered expression of other GR target genes. Thus, we focused the ChIP analysis on these genes. GR ChIP-sequencing performed after 1 h Dex treatment in several cell lines [39], and mouse liver, shows that the closest GR peaks are at least 50–100 kb away from the transcription start sites (TSS) of these genes (G.L. Hager, unpublished data) making it unclear whether GR binding to these very distant sites

actually influences their transcription. Recently, Guertin et al. reported that estrogen-repressed genes in MCF-7 cells show early transient interactions (10 min post treatment) with estrogen receptor at enhancer regions that do not contain canonical estrogen response elements [40]. Since GR ChIP-seq data at early time points of Dex treatment in Hepa1c1c7 cells is unavailable to identify such enhancer regions, we focused our ChIP analysis on the proximal promoter/TSS regions of these genes in an effort to determine how KDACs contribute to glucocorticoid-mediated gene repression.

The results of ChIP assays performed with antibody against total histone H3 show that none of the treatments caused a significant change in nucleosome density at any of the gene promoters (Fig. 4A). Thus, Dex-induced transcriptional repression is not caused by increased nucleosome density, and inhibition of KDAC activity does not lead to decreased nucleosome density in blocking Dex-induced repression of these promoters.

KDACs are required for transcriptional repression by GR at several model target genes [8,41]. In some studies this corresponded with a decrease in histone acetylation. Therefore, we tested whether histone H3 acetylation (H3K9/K14Ac) is altered by any of the treatments (Fig. 4B). Dex treatment does not alter histone H3 acetylation at any of the promoters. Contrary to what would be expected, VPA treatment does not increase H3 acetylation. In fact there is a significant decrease in H3 acetylation at the Hbegf promoter upon VPA treatment. This is reminiscent of decreased histone acetylation at the mouse mammary tumor virus promoter in response to treatment with the pan-KDACi, trichostatin A [42]. Finally, Dex and VPA co-treatment also does not alter histone H3 acetylation. These results show that the level of promoter histone H3 acetylation does not reflect the transcriptional status of these genes and suggest that the key targets of KDACs that facilitate Dex-induced repression may be non-histone proteins.

3.5. Class I KDACs regulate H3K4 methylation at Dex-repressed promoters

Because H3K4 and H3K9 methylation have been associated with transcriptional activation and repression, respectively, we determined the effects of glucocorticoid signaling and/or KDAC inhibition on H3 methylation at these glucocorticoid-repressed genes. Dex treatment does not cause any significant changes in H3K4 dimethylation (H3K4Me2) or trimethylation (H3K4Me3) at the three gene promoters (Fig. 4C, D). At the Hes1 promoter, Dex treatment causes a small, but significant increase in H3K9 dimethylation (H3K9Me2) but no change at the Igsf9 promoter (Fig. 4E). At the Hbegf promoter H3K9Me2 was not significantly detectable above background (no antibody), which was also true of H3K9Me3 at all the gene promoters for all treatment conditions (not shown).

While VPA treatment had no significant effect on H3K9Me2 at the Hes1 and Igsf9 promoters (Fig. 3E), it caused a significant decrease in H3K4Me2 at all three gene promoters in both the presence and absence of Dex (Fig. 4C). Since nucleosome occupancy was unaffected under these conditions (Fig. 4A), the decrease in H3K4Me2 cannot be explained by loss of nucleosomes from these promoters. An alternative explanation is that H3K4Me2 is reduced due to an increase in lysine methyltransferase (KMT) activity that converts H3K4Me2 to H3K4Me3. We measured H3K4Me3 levels at the three gene promoters and found that they do not significantly change under any of the treatment

conditions tested (Fig. 4D). Thus, the loss of H3K4Me2 at these promoters may be caused by a VPA-induced activation of a lysine demethylase (KDM). This effect is likely to be gene-specific because VPA treatment does not increase in global H3K4 dimethylation or trimethylation (Fig. 4F).

To determine whether the VPA-induced loss of H3K4Me2 was due to its KDAC-inhibiting activity rather than an off-target effect, we repeated the H3K4 methylation ChIP experiments substituting apicidin for VPA. We first examined histone H3 occupancy (Suppl. Fig. 1A). In contrast to VPA, apicidin causes a moderate but significant loss of nucleosome occupancy at the Hes1 promoter (~40%). While the changes did not reach significance, H3 levels at the Igsf9 and Hbegf promoters also trend downward in response to apicidin treatment. This may be due to the fact that apicidin is more efficient than VPA in inhibiting KDAC3 complexes [43]. However, like VPA, apicidin causes significant decreases in H3K4Me2 levels at all three promoters in the presence or absence of Dex (Suppl. Fig. 1B). In addition, the magnitude of these decreases is larger than those observed for the change in total H3 levels at these promoters, indicating that they cannot be entirely explained by loss of nucleosome occupancy. Finally, like VPA, apicidin treatment does not increase levels of H3K4Me3 at promoter nucleosomes (Suppl. Fig. 1C). Altogether the results show that KDAC inhibition results in increased activity of a KDM that targets H3K4Me2 at the promoters of genes where KDACs facilitate glucocorticoid-induced transcriptional repression. Furthermore, activation of this KDM occurs even in the presence of glucocorticoid signaling.

3.6. LSD1 depletion restores Dex-induced repression in the absence of KDAC activity

The unique chemistry of the KDM, LSD1, allows it to demethylate mono- and di-methylated H3K4 but not tri-methylated H3K4 [25]. LSD1 was originally identified as a transcriptional corepressor in neuronal cells as a subunit of the CoREST-REST complex, which also includes KDACs 1 and 2 [24]. However, recent studies have identified it as a coactivator of estrogen-, androgen-, and retinoic acid-regulated transcription [27,29,44].

Due to this more recent association with transcriptional activity, we hypothesized that activation of LSD1 present at our target genes through KDAC inhibition may work against Dex-induced transcriptional repression. To address this issue, we depleted LSD1 using siRNA and show that it does not affect expression of KDACs 1 and 2, which associate with LSD1 in the CoREST complex (Fig. 5A). We then measured the effect of LSD1 depletion on transcription at the KDACi-sensitive, Dex-repressed genes as shown in Fig. 5B–E. In the presence of non-targeting siRNA, KDAC inhibition by VPA treatment significantly impairs Dex-induced repression at the three genes tested, as expected. In the presence of LSD1 siRNA, Dex-induced transcriptional repression is unaffected (Dex treatment alone). However, LSD1 depletion largely restores Dex-mediated transcriptional repression at these genes in the absence of KDAC activity (Dex plus VPA treatment). Thus, when activation of LSD1 at these promoters is prevented, transcriptional repression mediated by glucocorticoid signaling is no longer impeded by KDAC inhibition.

One mechanism by which KDAC inhibition could lead to increased activity of LSD1 at target promoters is through increased expression. However, as shown in Fig. 5F, up to 5 h of VPA treatment does not increase cellular LSD1 levels. It is also possible that KDAC

inhibition increases association of LSD1 with promoter chromatin. Through ChIP with LSD1 antibody, we determined that LSD1 is present at the *Hes1*, *Igsf9*, and *Hbegf* promoters (Suppl. Fig. 2A–C), but its association with promoter chromatin relative to untreated cells is unchanged under any of the treatment conditions (Fig. 5G, Suppl. Fig. 2). Altogether our results suggest that KDACs regulate the demethylase activity of LSD1 at the three promoters tested.

Both KDACs 1 and 2 have been found associated with LSD1 in the CoREST complex, which we confirmed in our cell line by co-immunoprecipitation (Suppl. Fig. 3A,B). To determine whether these KDACs facilitate glucocorticoid-mediated repression, we depleted their expression individually or in combination using siRNA (Fig. 6A) and measured Dex-mediated repression at a variety of Dex-repressed genes sensitive to KDACi. As shown in Fig. 6B,C, these genes can be divided into two groups based on their responses to the KDAC1 and KDAC2 depletions. In the first group (*Igsf9*, *Mex3a*, *Cdon*), Dex-induced repression was significantly alleviated in response to co-depletion of KDACs 1 and 2, as shown in Fig. 6B. However, the magnitude of the alleviation was modest compared to the effect of VPA or apicidin (see Fig. 1). The second group of Dex-repressed genes (*Fam20c*, *Hbegf*, and *Rgs16*) was unaffected by any of the KDAC1 and KDAC2 depletions (Fig. 6B). We depleted KDAC3 to determine whether it contributes to Dex-mediated repression but its loss did not alleviate repression at any of the genes tested (Fig. 6A–C). Because neither VPA nor apicidin are efficient inhibitors of KDAC8 [43], the effects of its depletion were not tested. It is possible that KDACs 1–3 all contribute of Dex-mediated repression, but simultaneous depletion of all three was not feasible due to cytotoxicity.

Several studies have shown that LSD1 and CoREST (gene designation: *RCOR1*) expression/stability is dependent on the integrity of the entire CoREST complex [45,46]. If expression of LSD1 is depleted, CoREST expression also decreases and vice versa. This is coupled with a decrease in cellular levels of the CoREST complex. One possible explanation for the absence of any strong effects of KDAC1/KDAC2 depletion on Dex-mediated transcriptional repression is that loss of both KDAC1 and 2, which are also core components of the CoREST complex, results in reduced expression of LSD1 due to complex destabilization. Thus, we simultaneously depleted KDACs 1 and 2 using siRNA and show that LSD1 levels are reproducibly reduced 25–30% (Fig. 6D). This result suggests that loss of the physical presence of KDACs 1 and 2 destabilizes LSD1. Thus, KDAC1 and 2 depletion partially phenocopies LSD1 depletion and results in only moderate impairment of Dex-mediated repression compared to that observed with KDACi, which do not effect LSD1 expression (Fig. 5F).

4. Discussion

KDACs are generally considered to function as corepressors of transcription. However, our previous studies show that Class I KDACs facilitate GR transactivation. Here we show that, in the same cell context, Class I KDAC activity is also required for the onset and maintenance of glucocorticoid-induced transcriptional repression. Two structurally-distinct Class I-selective KDACi had highly similar effects on nine Dex-repressed genes, indicating that their effects are mediated through their common targets rather than by off-target effects.

Surprisingly, the mechanism of KDAC-facilitated repression is independent of the GR coregulator, SRC2, which plays a major role in GR transrepression in immune cells. Our study has revealed a novel mechanism by which KDACs function in transcriptional repression through glucocorticoid signaling. We show that Class I KDACs facilitate glucocorticoid-induced transcriptional repression through an LSD1-dependent mechanism (model shown in Fig. 7). Rather than promoting transcriptional repression, LSD1 impairs it in these gene contexts.

Known mechanisms by which glucocorticoids repress transcription include binding of GR to DNA in gene promoters/enhancers directly or indirectly through tethering to other bound proteins or protein complexes [reviewed in [41]]. In fact, GR recruitment to several glucocorticoid-repressed genes that require KDACs, such as POMC and CSF-1, has been demonstrated [3,7]. However, recent ChIP-sequencing studies suggest that this is not the predominant mechanism of glucocorticoid-mediated gene repression. GR binding sites are found much farther from glucocorticoid-repressed genes relative to activated genes [47–49]. While GR expression was necessary for both activation and repression of target genes, Reddy et al. observed that the average median distance between GR binding sites and the TSS of activated target genes was 11 kb, while for repressed genes it was 146kb [48]. Consistent with this, ChIP-sequencing data from mouse liver [50] and GL Hager, National Cancer Institute, unpublished data] show that the closest GR binding sites are at least 50–100 kb from the TSS of the Hbegf, Hes1, and Igsf9 genes. These findings raise questions about whether GR binding to these very distant regions, either directly or indirectly, is actually required for repression of these genes.

Reddy et al. [48] suggested two possible mechanisms for glucocorticoid-induced repression at genes far from GR binding sites: 1.) secondary effects dependent on protein synthesis or 2.) protein-protein interactions that occur away from the target gene, consistent with a squelching mechanism, which posits that activated steroid receptors compete for limiting amounts of coregulators and thereby sequester them at target genes to which the receptors bind. Recently, Guertin et al. provided strong evidence for squelching by the estrogen receptor. Genes rapidly repressed by estrogen in MCF-7 cells showed early (<10 min post treatment), transient interactions with estrogen receptor at enhancer regions that do not contain canonical estrogen response elements, and this correlated with a redistribution of p300 away from repressed genes to ER-activated genes [40]. Transcription of the Hbegf, Igsf9, Rgs16, and Hes1 genes is significantly repressed within 1 h of Dex treatment making it highly unlikely that their repression is due to a secondary effect that requires protein synthesis. GR ChIP-seq data for <1 h of Dex treatment in Hepa1c1c7 cells or mouse liver is unavailable but the rapid repression of Hbegf, Igsf9, and Hes1 transcription suggests that they may be in this category.

Analysis of the proximal promoter regions of the most rapidly repressed genes in our study showed that Dex down-regulates transcription without changes in nucleosome density, H3K4 di- or tri-methylation, H3K9/K14 acetylation or H3K9 di-methylation, consistent with the idea that repression could be caused, at least in part, by events that are removed from the gene promoters. Surprisingly, KDAC inhibition did not increase histone acetylation at the promoters of the genes tested, suggesting that KDACs targeted by VPA are not present at

these promoters or that their primary substrates are not histones. While VPA did not cause significant changes in H3K9Me2 or H3K4Me3, it induced a significant loss of H3K4Me2 at all three gene promoters that was not due to decreased nucleosome density or conversion to H3K4Me3 through increased methyltransferase activity. The structurally-distinct KDACi, apicidin, had the same effect, indicating that their common targets, class I KDACs, mediate this change. The lack of effect on H3K4Me3 indicates that KDAC inhibition activates a demethylase that can act on H3K4Me2 but not H3K4Me3. LSD1 is unique among KDMs for its inability to remove trimethylation and it specifically targets H3K4 and sometimes H3K9 [25,27]. It is a core component of the CoREST complex and can associate with NURD complexes; both CoREST and NURD complexes also contain KDACs 1 and 2 [22,51]. Interestingly, Bantscheff et al. showed that apicidin and VPA were both able to efficiently inhibit KDAC activity in the CoREST complex [43].

Our results show that VPA treatment modestly increases transcription at many of the genes assayed (Figs. 2 and 5). However, it is unlikely that the activation of LSD1 contributes to that increase because LSD1 depletion does not prevent it at the *Hbegf*, *Igsf9*, and *Rgs16* genes (Fig. 5). Dex treatment alone does not change H3K4 di-methylation at our promoters of interest, indicating that it does not alter LSD1 activity. Accordingly, the loss of H3K4Me2 induced by KDACi treatment at the promoters of interest was not reversed by the presence of Dex. However, this raised the possibility that activation of LSD1 through KDAC inhibition makes these genes resistant to Dex-mediated transcriptional repression. To prevent activation of LSD1, we depleted its expression through siRNA and found that loss of LSD1 largely restored the repressive transcriptional effect of Dex treatment in the presence of KDACi. This is analogous to an intergenic suppressor mutation in yeast genetics, where the effects of a mutation in one gene are negated by a mutation in a second gene. In our case, the first “mutation” is loss of KDAC activity and the second is loss of LSD1 expression. Identification of suppressor mutations is useful because they reveal unrecognized functional interactions between the proteins encoded by the mutated/inactivated genes. Altogether our results strongly suggest that the level of LSD1 activity at these genes becomes functionally relevant to transcription upon activation of glucocorticoid signaling. Although we demonstrate LSD1 activation by a change in H3K4 dimethylation, it is not clear whether H3 demethylation is the key target of LSD1 that contributes to glucocorticoid response. Nonhistone transcriptional regulatory proteins are also LSD1 substrates [52,53].

To explain the function of Class I KDACs and LSD1 in Dex-mediated transcriptional repression at the genes we have examined in this study, we propose the models shown in Fig. 7. When KDACs are active (Fig. 7A) they suppress the activity of LSD1 that is present at the promoters of the genes examined in our study and makes them susceptible to Dex-mediated transcriptional repression. When KDAC activity is inhibited (Fig. 7B), LSD1 is activated and disrupts the mechanism by which glucocorticoids repress transcription, thereby making the genes resistant to Dex-mediated repression. Upon LSD1 depletion (Fig. 7C), the enzyme is no longer present at the promoters and cannot be activated upon KDAC inhibition, thereby allowing the repression of transcription through glucocorticoid signaling. Although LSD1 is generally considered a transcriptional corepressor, global ChIP analyses showed that LSD1 is enriched at distal elements and promoters of active genes with surprisingly little localization in repressed chromatin [29,38,54]. This is consistent with the results of our

ChIP assays showing the constitutive presence of LSD1 at the Hes1, Igsf9, and Hbegf promoters. In addition, LSD1 associated with AR-activated genes is required for AR-activated transcription in a gene-selective fashion [28,55]. Our study shows that LSD1 activity blocks transcriptional repression rather than promoting it, further supporting the contention that LSD1 can be a corepressor or a coactivator, depending on the gene context.

Like LSD1, KDACs 1 and 2 are core components of the CoREST complex. This suggests that these Class I KDACs may regulate LSD1 activity at our genes of interest and facilitate Dex-mediated repression. Unlike the strong effects of the KDACi, depletion of KDACs 1 and 2 only modestly alleviated Dex-mediated repression at three genes. The partial effect is likely due to down-regulation of LSD1 expression by simultaneous depletion of KDACs 1 and 2. LSD1 expression has been shown to be dependent on the integrity of the CoREST complex [45,46]. Thus, the loss of LSD1 upon depletion of KDACs 1 and 2 may reflect a decrease in complex stability. Reduced CoREST complex levels may partially mimic LSD1 depletion and thereby blunt the effect of KDAC 1/2 depletion on the Dex-induced repression. At four additional genes KDAC depletions had no effect on Dex-induced repression. KDACs are highly likely to be involved in repression of these genes because both structurally-distinct KDACi strongly alleviated it. These genes may be highly sensitive to reduced LSD1 levels or expression/activity of only one Class I KDAC is sufficient to facilitate Dex-induced repression. Due to cytotoxicity, simultaneous depletion of KDACs 1–3 is not feasible.

Histone H3 acetylation at our promoters of interest was unchanged by VPA treatment, suggesting that Class I KDACs facilitate Dex-induced repression by targeting non-histone proteins, either at or away from these gene promoters, as depicted in Fig. 7A. Many transcription factors and coregulators are now known to be acetylated [35,56,57]. Since most transcriptional regulatory proteins have very dynamic interactions with their target promoters [58], it is certainly possible that their acetylation status may be rapidly changed upon KDAC inhibition in the time they are not engaged at target promoters. Acetylation of regulatory factors may render them inactive when they return to these promoters or may cause them to be directed to other regions of the genome or to other cellular compartments. The mechanism by which KDAC activity regulates LSD1 does not involve altered LSD1 expression or association with promoter chromatin. It is possible that KDAC activity may regulate LSD1 activity through deacetylation of core complex subunits or of proteins that can associate with the complex in a regulated fashion. A very recent study shows that acetylation of LSD1 disrupts its ability to interact with nucleosomes in vitro and cellular chromatin [59]. However, we do not observe a decrease in LSD1 association with promoter chromatin upon KDAC inhibition. Future studies will be focused on investigating the mechanistic details of the functional interplay between KDACs and LSD1 in glucocorticoid-repressed transcription.

Overall, our studies show that Class I KDACs are versatile coregulators of glucocorticoid-modulated transcription, facilitating both activation and repression of transcription depending on the gene context. In addition, we have uncovered a functional interaction between Class I KDACs and LSD1 in the repression of gene expression by glucocorticoid signaling, in which LSD1 activity opposes transcriptional repression rather than promoting

it. Thus, our work contributes to the emerging idea that gene context dictates the transcriptional function of coregulators such as LSD1 and Class I KDACs. KDACi are approved for clinical use in humans and there is growing interest in developing LSD1 inhibitors for cancer treatment [60]. Our studies indicate that pharmaceutical modulation of KDACs or LSD1 could have a significant impact on glucocorticoid signaling in vivo. A more comprehensive understanding of the role of these modifying enzymes in signaling-induced transcription is essential for developing therapeutic regimens involving their inhibitors that minimize serious side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jsbmb.2016.09.014>.

Abbreviations

KDAC	lysine deacetylase
KDACi	lysine deacetylase inhibitor
KAT	lysine acetyltransferase
GR	glucocorticoid receptor
VPA	valproic acid
NR	nuclear receptor
Dex	dexamethasone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HDAC	histone deacetylase
HDACi	HDAC inhibitor
HAT	histone acetyltransferase
RT-qPCR	real time quantitative polymerase chain reaction

Ct	threshold cycle
siRNA	small interfering RNA
LSD1	lysine-specific demethylase 1
AR	androgen receptor
CoREST	co-repressor of RE1 silencing transcription factor
REST	RE1 silencing transcription factor
NURD	nucleosome remodeling and histone deacetylation
SRC	steroid receptor co-activator
ER	estrogen receptor
ChIP	chromatin immunoprecipitation

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Fig. 1.

Class I-selective KDAC inhibitors alleviate Dex-induced repression of gene expression. Hepa-1c1c7 cells were treated with Dex (100nM), VPA (5mM) or apicidin (0.25 µg/ml) alone, and in combination with Dex and either KDACi. Cells were exposed to the KDACi for 5 h and Dex for 4 h. For the combination treatments, cells were dosed with the KDACi 1 h prior to the 4 h Dex treatment. RNA was isolated and subjected to RT-qPCR with exon-exon primers to measure levels of mRNA. The results are represented as fold change relative to Control (untreated). (A,B) Genes at which KDACi attenuates Dex-induced gene repression. (C) Genes at which Dex-induced gene repression is unaffected by KDACi. All graphs represent the results of 3–6 independent replicates. Error bars represent standard

error of the mean (SEM). *,# – $p \leq 0.05$, **,## – $p \leq 0.01$, ***,### – $p \leq 0.001$. Pound signs (#) represent significant changes between untreated cells and cells treated with Dex or KDACi alone. Asterisks (*) represent significant changes between cells treated with Dex and cells treated with the combination of Dex and KDACi.

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Fig. 2.

VPA prevents onset and maintenance of Dex-induced repression of transcription. Hepa-lclc7 cells were treated with either Dex (100 nM) or in combination with Dex and VPA (5 mM). (A–F) For the combination treatment cells were exposed to VPA 1 h *prior to* the addition of Dex. Cells were harvested at 30, 60, 120, and 240 min after Dex addition. RNA was isolated and subjected to RT-qPCR using intron-exon primer sets to measure levels of nascent transcripts. Fold changes in nascent transcripts for each treatment time relative to levels in untreated cells for *Mex3a* (A), *Cdon* (B), *Rgs16* (C), *Igsf9* (D), *Hes1* (E), and *Hbegf* (F). (G–I) For the combination treatment cells were exposed to VPA 1 h *after* the addition of Dex. Fold changes in nascent transcripts for each treatment time relative to levels in untreated cells for *Igsf9* (G), *Hes1* (H), and *Hbegf* (I). The results shown were derived from 3 to 5 independent experiments. Error bars represent SEM. *, # – $p < 0.05$, **, ### – $p < 0.01$, ***, #### – $p < 0.001$. Pound signs (#) represent significant changes between untreated cells and Dex treated cells. Asterisks (*) represent significant changes between cells treated with Dex and cells treated with the combination of Dex and VPA.

Fig. 3.

SRC2 is dispensable for transcriptional repression induced by Dex at KDACi-sensitive genes. Hepa-lclc7 cells were transfected with no siRNA (Mock), nontargeting (NT) siRNA, or siRNAs targeted to either SRC2 or to a combination of SRC1 and SRC3 as described in Experimental Procedures. Forty-eight hours after transfection, cells were left untreated (Control) or treated with Dex (100nM) for 2 h, or with a combination of VPA (5 mM) plus Dex (3 h VPA, 2 h Dex). (A) Protein was isolated from transfected cells and subjected to Western blotting with antibodies against SRCs 1, 2, or 3. Alpha-Tubulin (α-Tub) was used as

a loading control. (B–D) RNA was isolated from cells and subjected to RT-qPCR with intron-exon primer sets specific for nascent transcripts from the *Igsf9* (B), *Hbegf*(C), and *Hes1* (D) genes. The graphs are a summary of 3–7 independent experiments and represent the data as fold change in the presence of Dex relative to the corresponding untreated control for the cells transfected with NT, SRC2, or SRC1 + SRC3 siRNAs. Asterisks denote significant changes between Dex-treated and Dex + VPA-treated cells for each transfection. *, $p=0.05$; **, $p=0.01$. Error bars represent S.E.M.

Fig. 4.

Effects of Dex and VPA on total Histone H3, and Histone H3 modifications at the transcription start site regions of rapidly-repressed genes. (A–E) Hepa-lclc7 cells were treated with Dex (100nM) for 1 h, VPA (5mM) for 2 h, or a combination of VPA (5mM) plus Dex. In the latter, cells were exposed to VPA for 1 h prior to the addition of Dex for 1 h. Chromatin immunoprecipitation was performed with an antibodies against total histone H3 (A), acetylated histone H3 (B), H3K4Me2 (C), H3K4Me3 (D), and H3K9Me2 (E). Primer sets specific for the TSS/proximal promoter regions of the Hes1, Igsf9, and Hbegf gene were

used in qPCR. The graphs show fold changes in the level of histone modification for each of the treatments relative to untreated and are a summary of 4–7 independent experiments. Pound signs (#) represent significant changes between untreated cells and drug-treated cells. Asterisks (*) represent significant changes between cells treated with Dex and cells treated with VPA or the combination of Dex and VPA. *,# – $p < 0.05$, **,## – $p < 0.01$, ***,### – $p < 0.001$ *Error bars* represent S.E.M. (F) Hepa-1c1c7 cells were treated with VPA (5 mM) for 0, 2, or 5 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with antibodies against H3K4Me2, H3K4Me3, and total histone H3.

Fig. 5.

LSD1 depletion overcomes the requirement for KDAC activity in Dex-induced gene repression. Hepa-1c1c7 cells were transfected with non-targeting (NT) control siRNAs or siRNAs targeted to LSD1. (A) Forty-eight hours after transfection cell lysates were generated and subjected to SDS-PAGE. Western blotting was performed with antibodies against LSD1, KDAC1, or KDAC2. GAPDH was used as a loading control. (B–D) Forty-eight hours after transfection cells were not treated or treated with Dex (100 nM) for 1 h, VPA (5 mM) for 2h, or a combination of VPA (5 mM) plus Dex. In the latter, cells were

exposed to VPA for 1 h prior to the addition of Dex for 1 h. RNA was isolated from transfected cells and subjected to RT-qPCR. The graphs represent the fold changes for nascent transcript levels for Hbegf (B), Igsf9 (C), Hes1 (D), and Rgs16 (E) genes. The graphs are a summary of 4–5 independent experiments. Error bars represent SEM. *Asterisks* denote significant changes between Dex-treated cells and the combination (VPA/Dex) treatment for each siRNA. * – $p < 0.05$, *** – $p < 0.001$, **** – $p < 0.0001$. (F) Hepa-1c1c7 cells were treated with VPA (5 mM) for 0, 2, or 5 h. Whole cell lysates were subjected to SDS-PAGE and immunoblotting with antibodies against LSD1 and GAPDH. (G) Hepa-1c1c7 cells were treated with Dex (100nM) for 1 h, VPA (5 mM) for 2h, or a combination of VPA(5 mM) plus Dex. In the latter, cells were exposed to VPA for 1 h prior to the addition of Dex for 1 h. Chromatin immunoprecipitation was performed with antibody against LSD1. The graph shows fold changes in the level of LSD1 association with the promoters of the genes tested for each of the treatments relative to untreated and are a summary of 4–5 independent experiments. Statistical analysis (paired *t*-test) did not show any significant changes.

Fig. 6.

KDAC1 depletion mimics the effect of KDACi on Dex-repressed gene expression in a gene-specific manner. Hepa-lclc7 cells were transfected with non-targeting (NT) control siRNA or siRNAs targeted to various Class I KDACs as shown. (A) Forty-eight hours after transfection cell lysates were generated and subjected to SDS-PAGE. Western blotting was performed with antibodies against KDAC1, KDAC2, or KDAC3. GAPDH was used as a loading control. (B,C) Forty-eight hours after transfection cells were treated with or without Dex (100 nM) for 4 h. RNA was isolated from cells and subjected to RT-qPCR with exon-exon primers to measure levels of mRNA. The graphs represent the fold change in mRNA levels for genes at which repression is sensitive to KDACi. The graphs are a summary of 3–5 independent experiments. Error bars represent SEM. *Asterisks* denote significant changes between Dex-treated cells transfected with KDAC siRNA relative to control (NT) siRNA. *, $p < 0.05$. (D) Forty-eight hours after transfection with the siRNAs indicated cell lysates were generated and subjected to SDS-PAGE. Western blotting was performed with antibodies against LSD1 and GAPDH. Levels of LSD1 protein upon simultaneous KDAC1 and KDAC2 depletion are graphed relative to LSD1 protein levels in the presence of NT siRNA. The graph is a summary of 4 independent experiments.

Fig. 7.

Proposed model for Class I KDAC and LSD1 action in glucocorticoid-mediated repression. A.) Class I KDACs facilitate glucocorticoid-mediated transcriptional repression through inhibition of LSD1 activity at the promoters of the model genes used in this study, namely Hbegf, Igsf9, Hes1, and Rgs16. At these genes GR action is likely to be indirect. Class I KDACs may suppress LSD1 activity at or away from the gene. B.) Loss of KDAC activity causes LSD1 activation, which blocks glucocorticoid-mediated repression by an as yet undetermined mechanism. C.) Depletion of LSD1 removes it from the gene promoters and may destabilize the CoREST complex. The lack of activated LSD1 removes the block to glucocorticoid-mediated repression even if KDACs are inhibited.

Table 1

PCR Primers used in the study.

Gene	Forward primer	Reverse primer
Exon primers		
<i>Fam20c</i>	GATGTGACGCGGGATAAGAAG	GCTCGGTGGAACAGTAGTAGG
<i>Mex3a</i>	CAAGGCTGCAAGATTAAGGCT	GCGTGAGGCTCTTATCATGGAG
<i>Igsf9</i>	TGACCGTCCTCAGCCTGAT	GGACTCGTCCCACGTAATCG
<i>Hbegf</i>	ATGCCTCCCTGGTTACCAC	AGTCAGCCCATGACACCTGT
<i>Cdon</i>	CAAAGCTGAGGTGCGCTATAA	CCATTGAGGAAGGACGACTC
<i>Ikbke</i>	GCGGAGGCTGAATCACCAG	GAAAGCCCGAACGTGTTCTCA
<i>Mllt11</i>	CCTACCTACGAGAGCAAGGAT	AAGGTGCTGTACTCAAGGAGG
<i>Hes1</i>	ATAGCTCCCGGCATTCCAAG	GCGCGGTATTTCCCAACA
Exon-intron primers		
<i>Rgs16</i>	TCTTGGGACTGAACTCTGGGTGG	AAAGATTCCCAGCCGCGT
<i>Mex3a</i>	TCTCCTCTTCTCCCTTCCACCACC	CCCCCGAAACATCCTAGTTCTCCA
<i>Igsf9</i>	CAGAGCCCCTTCCTTCATTCAACA	GGCACTCGTACCAACCCTGGTCTT
<i>Hbegf</i>	CAGATGCTGGGGTGGGTGAAAT	CATTCCTTTCTTTGCTTGGGGTG
<i>Cdon</i>	CAAACATGGCAGCACACAGTCAGA	ATGCCCCCAAACACAGTCC
<i>Hes1</i>	TGCTACCCCAGCCAGTGTC AAC	CCACGGGATCTGCCATTTCAC
ChIP primers		
<i>Hes1</i>	AGACCTTGTCCTAGCGGCCAAT	CGAACGGCTCGTGTGAAACTTC
<i>Igsf9</i>	CCCCTATTGTGTGGCTGCGA	CCCGCTGGCACCCCATTT
<i>Hbegf</i>	GGCGGGGCTCCCACTCCTT	CAGCCGCGTGCAGTCCC