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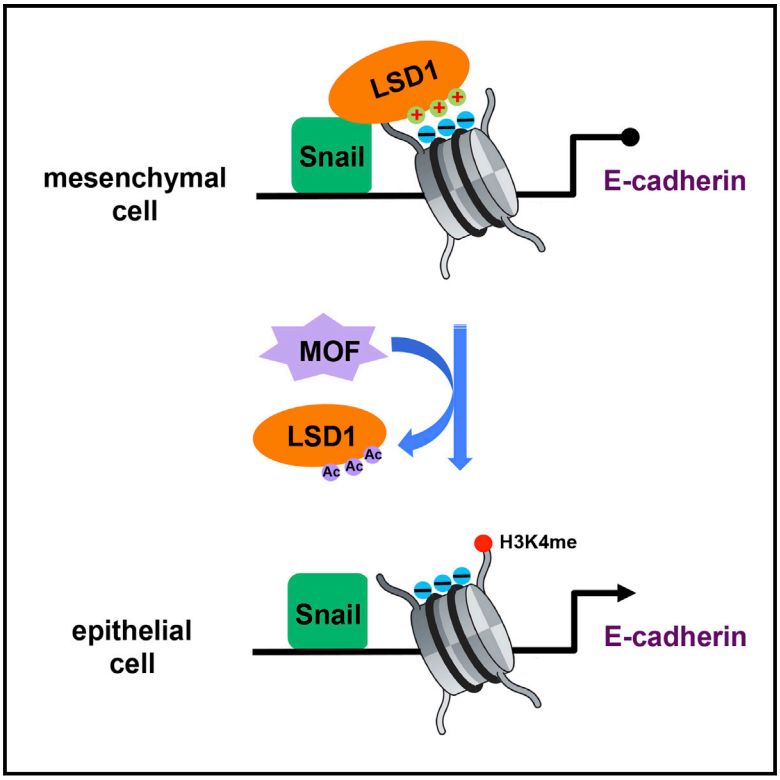
MOF Acetylates the Histone Demethylase LSD1 to Suppress Epithelial-to-Mesenchymal Transition

Graphical Abstract



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In Brief

The histone demethylase LSD1 is critical for epithelial-to-mesenchymal transition (EMT) and tumor progression. Luo et al. report that LSD1 is acetylated by the MOF acetyltransferase. This modification reduces association of LSD1 with nucleosomes and decreases histone demethylation, thus suppressing EMT.

Highlights

- LSD1 is acetylated in epithelial but not mesenchymal cells
- MOF directly acetylates LSD1 and is required for LSD1 acetylation in epithelial cells
- Ectopic MOF expression reduces LSD1 at epithelial gene promoters and blocks EMT
- MOF inhibits tumor invasion and predicts positive clinical outcomes in human cancer

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MOF Acetylates the Histone Demethylase LSD1 to Suppress Epithelial-to-Mesenchymal Transition

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SUMMARY

The histone demethylase LSD1 facilitates epithelial-to-mesenchymal transition (EMT) and tumor progression by repressing epithelial marker expression. However, little is known about how its function may be modulated. Here, we report that LSD1 is acetylated in epithelial but not mesenchymal cells. Acetylation of LSD1 reduces its association with nucleosomes, thus increasing histone H3K4 methylation at its target genes and activating transcription. The MOF acetyltransferase interacts with LSD1 and is responsible for its acetylation. MOF is preferentially expressed in epithelial cells and is downregulated by EMT-inducing signals. Expression of exogenous MOF impedes LSD1 binding to epithelial gene promoters and histone demethylation, thereby suppressing EMT and tumor invasion. Conversely, MOF depletion enhances EMT and tumor metastasis. In human cancer, high MOF expression correlates with epithelial markers and a favorable prognosis. These findings provide insight into the regulation of LSD1 and EMT and identify MOF as a critical suppressor of EMT and tumor progression.

INTRODUCTION

Dynamic posttranslational modifications of histones profoundly impact chromatin accessibility and gene transcription. Aberrant histone modifications deregulate gene expression and actively contribute to cancer initiation and progression (Baylin and Jones, 2011; Chi et al., 2010). Lysine-specific demethylase 1 (LSD1 or KDM1A) was the first histone demethylase discovered (Shi et al., 2004). LSD1 can repress gene transcription by demethylating mono- and di-methyl lysine 4 on histone H3 (H3K4me1/2) (Shi et al., 2004), which are histone marks associated with enhancers and active promoters, respectively (Kooistra and Helin, 2012; Martin and Zhang, 2005). Interaction with CoREST enables LSD1 to bind to nucleosomes and demethylate H3K4 on nucleosomal substrates (Lee et al., 2005; Shi et al., 2005; Yang et al., 2006). LSD1-containing repressive protein complexes also include histone reader subunits BHC80 and SFMBT1 that help anchor LSD1 to chromatin (Lan et al., 2007; Tang et al., 2013; Zhang et al., 2013). Moreover, through associations with various sequence-specific DNA-binding transcription factors, LSD1 is recruited to specific genomic loci to regulate target genes involved in a broad spectrum of biological processes including embryonic development and cancer (Amente et al., 2013; Mosammaparast and Shi, 2010).

Overexpression of LSD1 has been observed in a wide range of human solid tumors, and high levels of LSD1 are associated with tumor aggressiveness, recurrence, and adverse prognosis (Højfeldt et al., 2013; Lim et al., 2010; Shi, 2007). LSD1 physically associates with the Snail family of zinc-finger transcription factors (Lin et al., 2010a, 2010b; Tang et al., 2013; Wu et al., 2012), which are central drivers of epithelial-to-mesenchymal transition (EMT) (Peinado et al., 2007). EMT is a cellular reprogramming process that is defined by the loss of epithelial characteristics, including cell-cell adhesion and expression of epithelial cell markers, and the acquisition of a more migratory and invasive mesenchymal phenotype (Kalluri and Weinberg, 2009; Lamouille et al., 2014). It has been proposed that reversible EMT may contribute to tumor metastasis (Thiery, 2002; Tsai and Yang, 2013). Snail directly represses epithelial gene expression through the recruitment of LSD1 and subsequent LSD1-mediated H3K4 demethylation (Lin et al., 2010a, 2010b; Tang et al., 2013). LSD1 is indispensable for Snail-mediated EMT and transcriptional repression of epithelial genes in mesenchymal cancer cells. LSD1 is ubiquitously expressed; however, very little is known about whether and how its activities might be modulated to facilitate EMT and tumor progression.

MOF (also known as KAT8) is a member of the MYST family of lysine acetyltransferases (Sapountzi and Côté, 2011) and is a major enzyme that catalyzes histone H4K16 acetylation in

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mammalian cells (Smith et al., 2005; Taipale et al., 2005). MOF exists in two distinct and evolutionarily conserved multiprotein complexes: MSL and NSL (Cai et al., 2010; Li et al., 2009; Mendjan et al., 2006; Prestel et al., 2010; Raja et al., 2010; Smith et al., 2005). Both complexes are capable of acetylating H4K16, but the NSL complex has a broader substrate specificity and can acetylate non-histone proteins such as p53 (Cai et al., 2010; Li et al., 2009). MOF plays important roles in transcription activation, DNA damage response, and maintenance of embryonic stem cell pluripotency (Gupta et al., 2005, 2014; Li et al., 2010, 2012; Sharma et al., 2010). Compared with normal tissues, expression of MOF is frequently downregulated in several types of solid tumors (Cao et al., 2014; Liu et al., 2013; Pfister et al., 2008; Zhang et al., 2014). However, there are also reports that MOF expression is elevated in certain cancers and MOF may stimulate cell proliferation and transformation (Gupta et al., 2008; Zhao et al., 2013a). Overall, the role of MOF in cancer remains poorly defined.

In this study, we found that LSD1 was acetylated specifically in epithelial cells. This modification impairs LSD1's association with nucleosomes and prevents transcriptional repression resulting from the demethylation of nucleosomal H3K4. MOF was identified as the only acetyltransferase responsible for LSD1 acetylation. The NSL complex interacted with and acetylated LSD1. MOF expression was highly enriched in epithelial cells but was downregulated upon induction of EMT. Depletion of MOF in epithelial cells accelerated EMT and enhanced tumor metastasis. Expression of exogenous MOF dissociated LSD1 from epithelial gene promoters, activated epithelial marker expression, and decreased tumor invasion. Collectively, the results suggest that acetylation of LSD1 by MOF functions as a key switch to suppress EMT and tumor progression.

RESULTS

LSD1 Is Preferentially Acetylated in Epithelial Cells

LSD1 is a crucial regulator of EMT and is expressed in both epithelial and mesenchymal cells. We wondered whether LSD1's activity might be differentially regulated in the two cell types. Lysine acetylation of non-histone proteins has proven a critical covalent modification that regulates protein functions (Choudhary et al., 2014; Yang and Seto, 2008). Therefore, we sought to determine whether LSD1 might undergo acetylation. We transduced MCF7 epithelial breast cancer cells with lentiviruses expressing Flag-tagged LSD1. Flag-LSD1 proteins were immunoprecipitated from infected cells with anti-Flag antibodies, followed by immunoblotting with pan-acetyl-lysine antibodies. LSD1 acetylation was observed in MCF7 cells (Figure 1A). Treatment of MCF7 cells with the HDAC inhibitor Trichostatin A or SIRT1 inhibitor nicotinamide enhanced LSD1 acetylation, and a combination of both inhibitors additively increased LSD1 acetylation levels (Figure 1A). The results suggest that LSD1 is acetylated in MCF7 epithelial cells and multiple classes of deacetylases actively deacetylate LSD1.

To determine whether LSD1 might be differentially acetylated in epithelial versus mesenchymal cells, lentiviral Flag-LSD1 was similarly expressed in MDA-MB-231 mesenchymal breast cancer cells. However, LSD1 acetylation was undetectable in such cells even after treatment with deacetylase inhibitors (Figure 1A). To verify that LSD1 might be acetylated preferentially in epithelial cells, we compared LSD1 acetylation in a panel of epithelial and mesenchymal cancer cell lines (Figure 1B). These cells were infected with lentiviral Flag-LSD1, and acetylation of LSD1 was subsequently monitored. LSD1 acetylation was readily detected in all epithelial cells examined, including T47D, MDA-MB-468, BT474, and MCF7, but was virtually absent in mesenchymal cells, including Hs578T, MDA-MB-435, BT549, and MDA-MB-231 (Figure 1B). This observation supports the notion that LSD1 is acetylated specifically in epithelial cells.

MOF Induces LSD1 Acetylation and Is Required for Acetylation of Endogenous LSD1 in Epithelial Cells

To identify the enzyme(s) that might acetylate LSD1, we screened a panel of candidates from multiple lysine acetyltransferase families (Kahali et al., 2014; Yang, 2004). When Flag-LSD1 alone was expressed in HEK293 cells, it was not acetylated (Figure 1C). We thus co-expressed various candidate acetyltransferases together with Flag-LSD1 and examined their ability to induce LSD1 acetylation. Among a total of 18 acetyltransferases examined, only the MYST family member MOF (Sapountzi and Côté, 2011) strongly promoted LSD1 acetylation (Figure 1C). Moreover, MOF did not affect LSD1 protein abundance. Other acetyltransferases, including several related members of the MYST family (e.g., Tip60), did not cause notable acetylation of LSD1 (Figure 1C).

Because LSD1 acetylation was primarily detected in epithelial cells (Figure 1B), we examined the expression of endogenous MOF in epithelial and mesenchymal cancer cell lines. Unlike the ubiquitous expression of LSD1, MOF was largely confined to epithelial cells and was weakly expressed or undetectable in mesenchymal cells, which correlated with the epithelial marker E-cadherin (Figure 1D). The epithelial-enriched expression of MOF is consistent with the LSD1 acetylation pattern.

To verify whether endogenous LSD1 was acetylated in epithelial cells and MOF was the responsible enzyme, we depleted MOF in MCF7 epithelial cells and MDA-MB-231 mesenchymal cells with lentiviral short hairpin RNAs (shRNAs) (Figure 1E). MOF RNA levels in MCF7 were indeed higher than those in MDA-MB-231 (Figure 1E). Endogenous LSD1 proteins were immunoprecipitated from the cells for evaluation of lysine acetylation. In vector-infected control MCF7 and MDA-MB-231 cells, the abundance of endogenous LSD1 proteins was comparable between the two cell lines, but acetylation of endogenous LSD1 was only observed in MCF7 cells (Figure 1E). Furthermore, when MOF was depleted in MCF7 cells, LSD1 protein abundance was not affected but its acetylation was markedly reduced (Figure 1E). Taken together, the results suggest that MOF can induce LSD1 acetylation and is required for the acetylation of endogenous LSD1.

LSD1 Interacts with the NSL Complex

As MOF was responsible for LSD1 acetylation, we tested whether the two proteins might interact with each other. In a co-immunoprecipitation assay, when Myc-tagged LSD1 was co-expressed with Flag-tagged MOF in HEK293 cells,

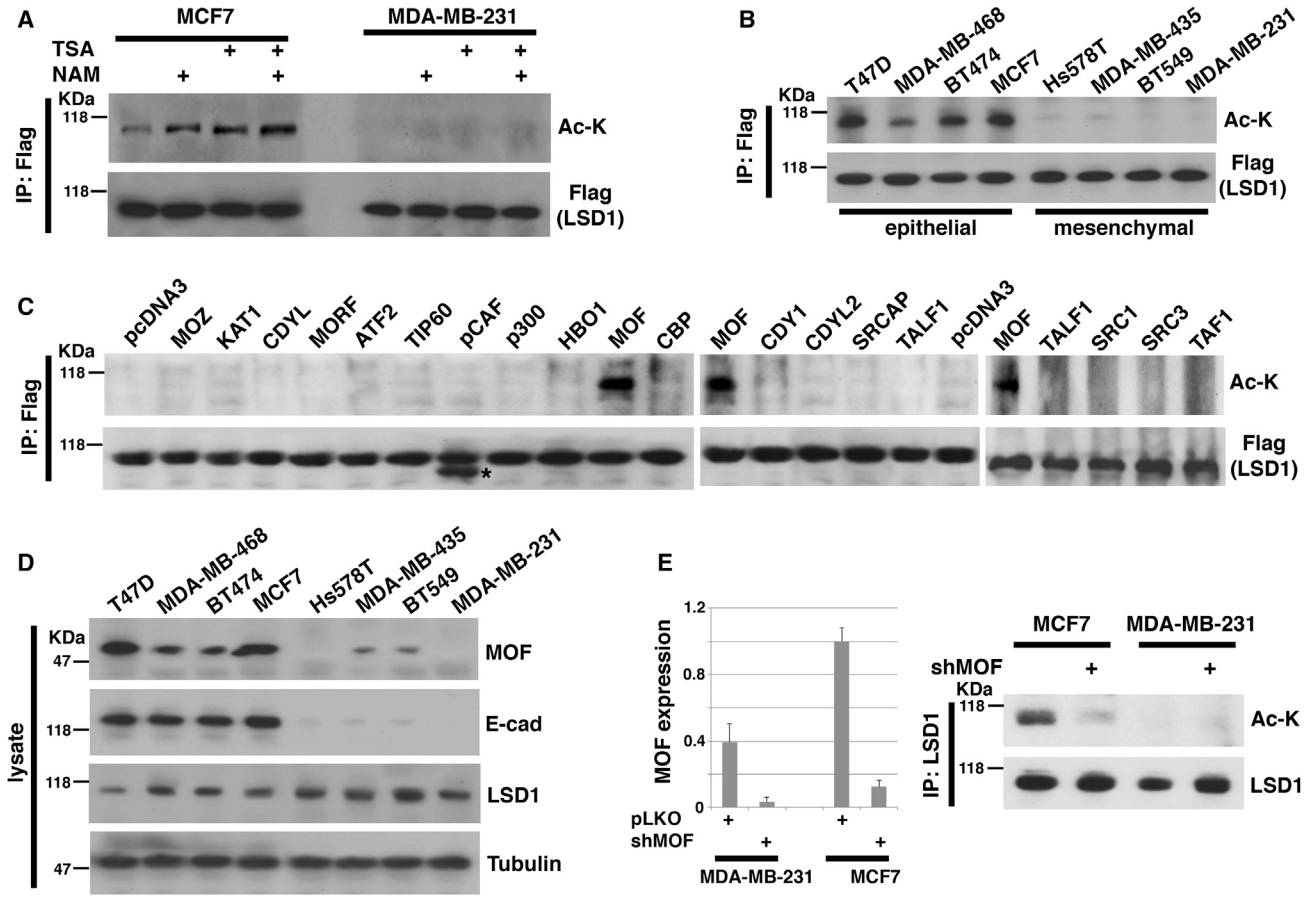


Figure 1. LSD1 Is Acetylated by MOF Specifically in Epithelial Cells

(A) LSD1 is acetylated in MCF7 epithelial but not MDA-MB-231 mesenchymal breast cancer cells, and deacetylase inhibitors enhance its acetylation. MCF7 and MDA-MB-231 cells were transduced with lentiviral Flag-LSD1, treated with Trichostatin A (TSA) and/or Nicotinamide (NAM) for 24 hr, and subjected to immunoprecipitation with anti-Flag antibodies, followed by immunoblotting with anti-acetyl-lysine (Ac-K) and anti-Flag antibodies.

(B) LSD1 is preferentially acetylated in epithelial cells. Indicated epithelial and mesenchymal cancer cells were transduced with lentiviral Flag-LSD1, treated with TSA and NAM for 24 hr, and examined for LSD1 acetylation by immunoprecipitation and immunoblotting as in (A).

(C) MOF induces LSD1 acetylation. HEK293 cells were transfected with Flag-LSD1 in combination with various acetyltransferases as indicated. Flag-LSD1 was immunoprecipitated with anti-Flag antibodies and the precipitate was analyzed by immunoblotting for lysine acetylation. Asterisk denotes the pCAF protein, which is also Flag-tagged.

(D) Expression of MOF is enriched in epithelial cells. Indicated cancer cells were lysed and immunoblotted with antibodies as indicated. E-cad, E-cadherin.

(E) Endogenous LSD1 is acetylated in MCF7 cells in a MOF-dependent manner. MCF7 and MDA-MB-231 cells were infected with lentiviral control vector pLKO or vector expressing shRNAs targeting MOF (shMOF). Depletion of MOF was verified by quantitative RT-PCR (left). The RNA levels of MOF in MCF7 cells were set as 1. Endogenous LSD1 proteins were immunoprecipitated with anti-LSD1 antibodies, followed by immunoblotting with anti-Ac-K antibodies (right).

immunoprecipitation of MOF with anti-Flag antibodies failed to pull down LSD1 (Figure 2A). Since the NSL complex is able to acetylate non-histone proteins and the MSL1v1 subunit (also known as KANSL1) recognizes substrates (Li et al., 2009), we investigated whether MSL1v1 might bind to LSD1. MSL1v1 was unstable when expressed alone in HEK293 cells but could be stabilized by co-expression of MOF (data not shown). When Myc-LSD1 was co-expressed with both Flag-MSL1v1 and untagged MOF, immunoprecipitation of MSL1v1 with anti-Flag antibodies indeed co-precipitated LSD1 (Figure 2A). In a similar assay, in HEK293 cells expressing Myc-LSD1 and Myc-MOF as well as Flag-MSL1v1, immunoprecipitation of MSL1v1 with anti-Flag antibodies pulled down both LSD1 and MOF (Figure S1A). The results suggest that the NSL complex associates with LSD1 in cells.

To further verify the MSL1v1-LSD1 interaction, we isolated Flag-LSD1 proteins from transfected HEK293 cells by immunoprecipitation with anti-Flag antibodies, followed by elution with Flag peptide. We also purified recombinant His-tagged MOF and MSL1v1 proteins from bacteria and incubated them with purified Flag-LSD1 proteins. MOF was unable to bind to LSD1 (Figure 2B). By contrast, both the full-length and carboxyl terminus of MSL1v1 displayed association with LSD1 (Figure 2B). The in vitro binding result suggests that LSD1 associates with the NSL complex via direct interaction with MSL1v1. Such association may facilitate LSD1 acetylation by MOF.

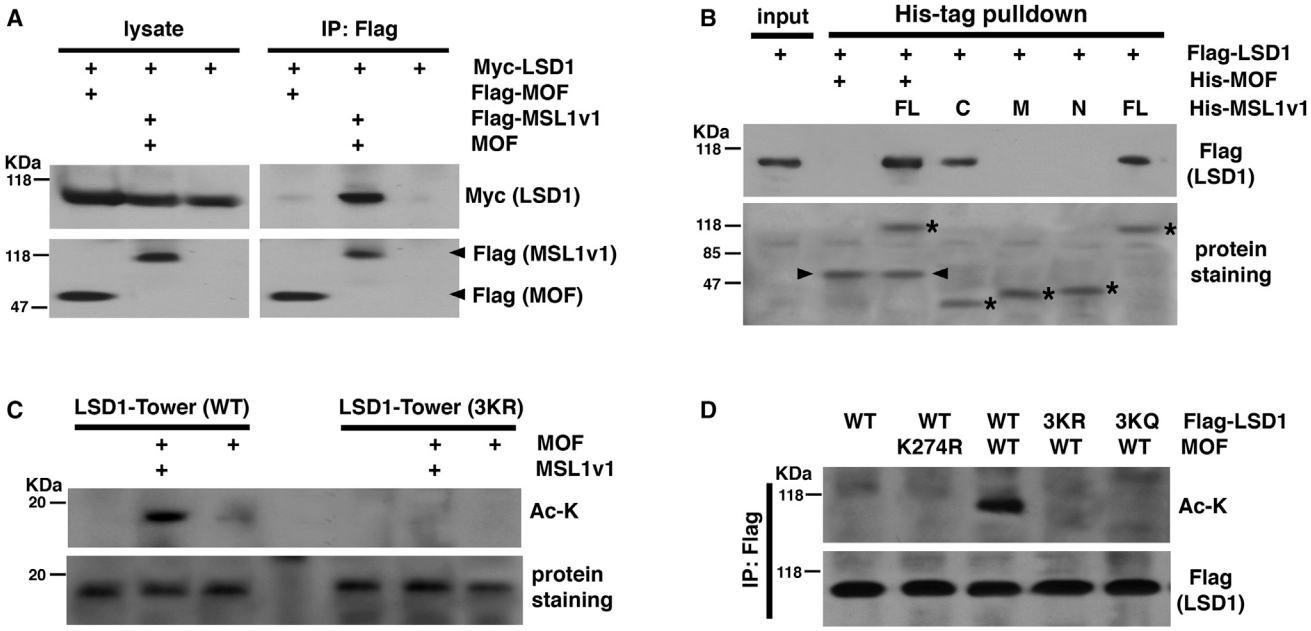


Figure 2. LSD1 Interacts with MOF-MSL1v1 and Is Acetylated by MOF at K432, K433, and K436

(A) LSD1 interacts with MOF-MSL1v1 in cells. HEK293 cells were transfected with vectors expressing Myc-tagged LSD1 alone or in combination with Flag-MOF or Flag-MSL1v1 and untagged MOF. Whole-cell lysates were subjected to immunoprecipitation with anti-Flag antibodies, followed by immunoblotting with Myc or Flag antibodies. See also Figure S1A.

(B) LSD1 binds to MSL1v1 in vitro. Flag-LSD1 proteins were immunoprecipitated from transfected HEK293 cells with anti-Flag antibodies and eluted with Flag peptide. His-tagged MOF and MSL1v1 (FL, full-length; N, aa 1–429; M, aa 429–800; C, aa 800–1105) were purified from bacteria and incubated with purified Flag-LSD1 proteins. His-tagged proteins were precipitated with Ni-NTA agarose beads, and bound proteins were examined for the presence of LSD1 by immunoblotting with anti-Flag antibodies. Coomassie blue protein staining is shown in the lower panel. Arrowheads denote the MOF protein. Asterisks indicate full-length and truncated MSL1v1 proteins.

(C) Acetylation of the LSD1 Tower domain by MOF-MSL1v1 in vitro. HEK293 cells were transfected with vectors expressing Flag-MOF alone or in combination with MSL1v1, followed by immunoprecipitation with anti-Flag antibodies. The precipitates were incubated with recombinant LSD1 Tower domain (WT or 3KR). Acetylation of LSD1 was determined by immunoblotting with anti-Ac-K antibodies. See also Figure S1C.

(D) Lysine 432, 433 and 436 of LSD1 are the MOF acetylation sites. Flag-tagged WT and mutants (3KR and 3KQ) of LSD1 were co-expressed with MOF (WT or K274R mutant) in HEK293 cells. Acetylation of LSD1 was evaluated by immunoprecipitation with anti-Flag antibodies and immunoblotting with anti-Ac-K antibodies. See also Figure S1D.

MOF Directly Acetylates LSD1 at Lysine Residues 432, 433, and 436

We sought to identify the MOF acetylation sites in LSD1. Two in silico online programs, LAceP and BRABSB-PHKA, both predicted potential lysine (K) acetylation sites in human LSD1, including K432 and K436 (http://www.scbit.org/iPTM) (Hou et al., 2014; Shao et al., 2012). Global proteomics also uncovered acetylation of LSD1 K432, K433, and K436 (http://www.phosphosite.org). These three residues are located in the Tower domain of LSD1.

To test whether MOF might acetylate the LSD1 Tower domain, we purified recombinant proteins consisting of this region from bacteria. We also substituted K432, K433, and K436 with arginine (R) to make an acetylation-resistant mimic (the 3KR mutant). To prepare the MOF enzyme, we expressed Flag-MOF in HEK293 cells together with or without MSL1v1 and immunoprecipitated the MOF proteins with anti-Flag antibodies. The LSD1 Tower domain was weakly acetylated when incubated with the MOF-only immunoprecipitates in vitro and became strongly acetylated when incubated with the MOF-MSL1v1 immunoprecipitates (Figure 2C). By contrast, the 3KR mutant Tower domain was resistant to MOF-mediated acetylation (Figure 2C). Similarly, we purified recombinant full-length wild-type (WT) and 3KR mutant LSD1 proteins (Figure S1B) and incubated them with the MOF-containing immunoprecipitates. Robust acetylation of WT LSD1 by MOF-MSL1v1 was observed, and there was no detectable acetylation of the 3KR mutant LSD1 (Figure S1C). The results suggest that LSD1 K432, K433, and K436 are the only sites acetylated by MOF in vitro.

MOF induced LSD1 acetylation when both were expressed in HEK293 cells (Figures 1C and 2D). However, a catalytically inactive MOF mutant (K274R) (Sun et al., 2011) failed to acetylate LSD1 in these cells (Figure 2D). This result is consistent with direct acetylation of LSD1 by MOF. When LSD1 K432, K433, and K436 were individually replaced by R, the single mutants were still efficiently acetylated by MOF in the transfection assay (Figure S1D). Only when all three Ks were mutated (i.e., 3KR), LSD1 acetylation by MOF was completely abolished (Figures 2D and S1D). Similarly, when the three Ks of LSD1 were simultaneously replaced with glutamine (Q), the resulting 3KQ mutant

LSD1 also resisted MOF-mediated acetylation (Figure 2D). These results confirm that K432, K433, and K436 of LSD1 are the MOF acetylation sites.

Acetylation of LSD1 Disrupts Its Association with Nucleosomes and Demethylation of Nucleosomal Substrates In Vitro

We next investigated the effect of lysine acetylation on the function of LSD1. The Tower domain of LSD1 protrudes from the catalytic core and provides the binding platform for CoREST (Figure S2A) (Chen et al., 2006; Stavropoulos et al., 2006; Yang et al., 2006). However, the acetylation sites K432, K433, and K436 are not located at the binding interface between LSD1 and CoREST. Indeed, WT, 3KR, and 3KQ mutant LSD1 proteins all associated with CoREST in co-immunoprecipitation assays (Figure S2B), suggesting that LSD1 acetylation does not affect its interaction with CoREST.

To demethylate histone tails in chromatin, LSD1 must associate with nucleosomes (Lee et al., 2005; Shi et al., 2005; Yang et al., 2006). Based on the crystal structure of the LSD1-CoREST complex (Yang et al., 2006), K432, K433, and K436 of LSD1 may directly contact nucleosomal DNA (Figure S2A). Acetylation of these residues neutralizes their positive charge and thus abrogates their electrostatic interaction with the negatively charged phosphate backbone of DNA. Therefore, acetylation of LSD1 may impair its association with nucleosomes and subsequent demethylation of nucleosomal substrates.

We tested whether the acetylation-mimicking 3KQ LSD1 mutant was able to bind nucleosomes in vitro. Mononucleosomes were reconstituted with recombinant core histones and biotin-tagged DNA fragments (Li et al., 2014). The resultant nucleosome particles were subsequently incubated with recombinant WT or 3KQ LSD1 proteins in the absence or presence of recombinant CoREST (Figure S1B). The nucleosomes were isolated with streptavidin-conjugated beads, and associated proteins were examined by immunoblotting. Consistent with previous reports showing that LSD1 requires CoREST for nucleosome binding (Lee et al., 2005; Shi et al., 2005; Yang et al., 2006), LSD1 alone (WT or 3KQ) did not bind to nucleosomes (Figure 3A). In the presence of CoREST, the association of WT LSD1 with nucleosomes became readily detected (Figure 3A). By contrast, the binding of acetylation-mimicking 3KQ mutant to nucleosomes was substantially reduced (Figure 3A). LSD1 and CoREST did not bind to nucleosome-free DNA in vitro (Figure S2C). The results suggest that acetylation of LSD1 decreases its association with nucleosomes.

LSD1 alone is capable of demethylating H3K4me1/2 in peptides or bulk histones, but its demethylation of nucleosomal H3K4 depends on CoREST-assisted nucleosome binding (Lee et al., 2005; Shi et al., 2005; Yang et al., 2006). Acetylation of LSD1 impairs its association with nucleosomes and hence may compromise its ability to demethylate nucleosomal substrates. When incubated with bulk histones, recombinant WT, 3KR, and 3KQ LSD1 proteins all strongly decreased H3K4me2 levels (Figure 3B), suggesting that the acetylation status of K432, K433, and K436 in LSD1 does not impact its enzymatic activity on free histones. However, when incubated with mononucleosomes, WT LSD1 alone was unable to demethylate H3K4me2 (Figure 3C). With the addition of recombinant CoREST, WT LSD1 efficiently demethylated H3K4me2 in nucleosomes, and the 3KR mutant LSD1 also actively demethylated nucleosomal substrates (Figure 3C). By contrast, the acetylation-mimicking 3KQ mutant failed to demethylate nucleosomal H3K4me2 even in the presence of CoREST (Figure 3C). These results suggest that acetylation of LSD1 diminishes its demethylation activity toward nucleosomal substrates.

Acetylation-Mimicking Mutant LSD1 Is Compromised for Chromatin Association, H3K4 Demethylation, and Repression of Target Genes in Cells

Based on the above in vitro assays, we asked whether LSD1 acetylation might negatively impact its association with chromatin, H3K4me2 demethylation, and transcriptional repression in intact cells. In HCT116 colon cancer cells, multiple direct target genes of LSD1 were identified, including AIM1 and VAT1L, and their expression was upregulated when the LSD1 gene was deleted through homologous recombination (Jin et al., 2013). We reconstituted the LSD1-null HCT116 cells with Flag-tagged WT, 3KR, or 3KQ LSD1 through lentiviral infection. Because LSD1 aspartic acid (D) 555 and 556 are essential for its enzymatic activity (Stavropoulos et al., 2006), we substituted both residues with asparagine (N) to generate an inactive mutant (2DN) as control. All forms of exogenous LSD1 were expressed at comparable levels in the LSD1-null cells (Figure S2D).

To evaluate the binding of WT and mutant exogenous LSD1 proteins to genomic targets, we performed chromatin immunoprecipitation (ChIP) analysis with anti-Flag antibodies. While WT LSD1 bound to the AIM1 and VAT1L promoters, the 3KQ acetylation-mimetic mutant LSD1 demonstrated significantly decreased association with these genomic loci (Figure 3D), suggesting that acetylation of LSD1 attenuates its recruitment to chromatin in cells. Chromatin binding of the enzymatically inactive 2DN mutant LSD1 was comparable to that of WT LSD1. Acetylation-resistant 3KR mutant LSD1 exhibited stronger chromatin binding than WT LSD1 (Figure 3D), which might be attributed to acetylation of WT LSD1 by endogenous MOF in these cells.

As expected, the H3K4me2 levels at the LSD1 target gene promoters were high in LSD1-null cells reconstituted with empty vector or the inactive 2DN mutant (Figure 3E). Introduction of WT or 3KR LSD1 potently reduced H3K4me2 levels at these loci (Figure 3E). By contrast, the 3KQ mutant LSD1 failed to decrease H3K4me2 (Figure 3E), which is consistent with its reduced association with chromatin. Accordingly, expression of LSD1 target genes was strongly repressed by reconstitution with WT and 3KR LSD1, but not with 3KQ and 2DN mutant LSD1 (Figure 3F). These observations suggest that acetylation of LSD1 inhibits its ability to bind to chromatin, demethylate H3K4, and repress transcription.

MOF Dissociates LSD1 from Chromatin and Activates E-Cadherin Expression at Least in Part via Suppression of LSD1 Function

We further explored the biological significance of MOF-mediated regulation of LSD1. LSD1 is indispensable for repression of

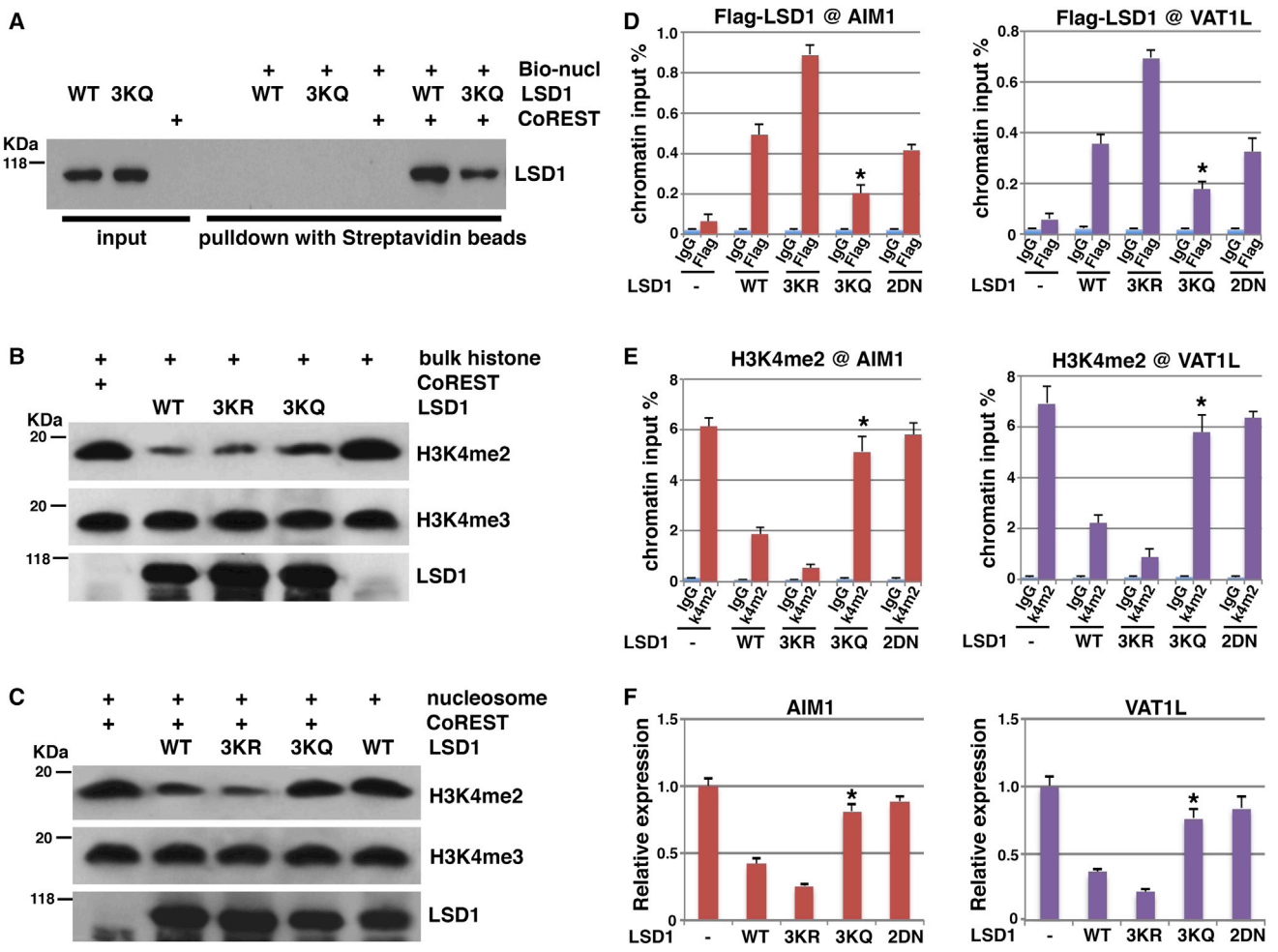


Figure 3. Acetylation of LSD1 Impairs Its Ability to Associate with Nucleosomes, Demethylate Nucleosomal Substrates, and Repress Target Gene Transcription

(A) LSD1 acetylation attenuates its affinity for nucleosomes in vitro. Nucleosomes were reconstituted with recombinant core histones and biotin-labeled DNA (Bio-nucl). Reconstituted nucleosomes were then incubated with recombinant WT or 3KQ mutant LSD1 proteins in the absence or presence of CoREST. Nucleosomes were isolated with streptavidin beads, and associated proteins were examined by immunoblotting with anti-LSD1 antibodies. See also Figure S2C.

(B) LSD1 acetylation does not affect its ability to demethylate H3K4 in bulk histones in vitro. Equal amounts of bulk histones were incubated with recombinant WT or mutant (3KR, 3KQ) LSD1, followed by immunoblotting with anti-H3K4me2, -H3K4me3, and -LSD1 antibodies. H3K4me3 levels were used as loading controls.

(C) Acetylation of LSD1 abolishes its demethylation activity on nucleosomal substrates. Mononucleosomes were prepared from cells and incubated with recombinant LSD1 (WT, 3KR, 3KQ) and/or CoREST proteins. Histone demethylation was verified by immunoblotting with anti-H3K4me2/3 antibodies.

LSD1-null HCT116 cells were reconstituted with Flag-tagged WT or mutant (3KR, 3KQ, 2DN) LSD1 through lentiviral infection (see also Figure S2D).

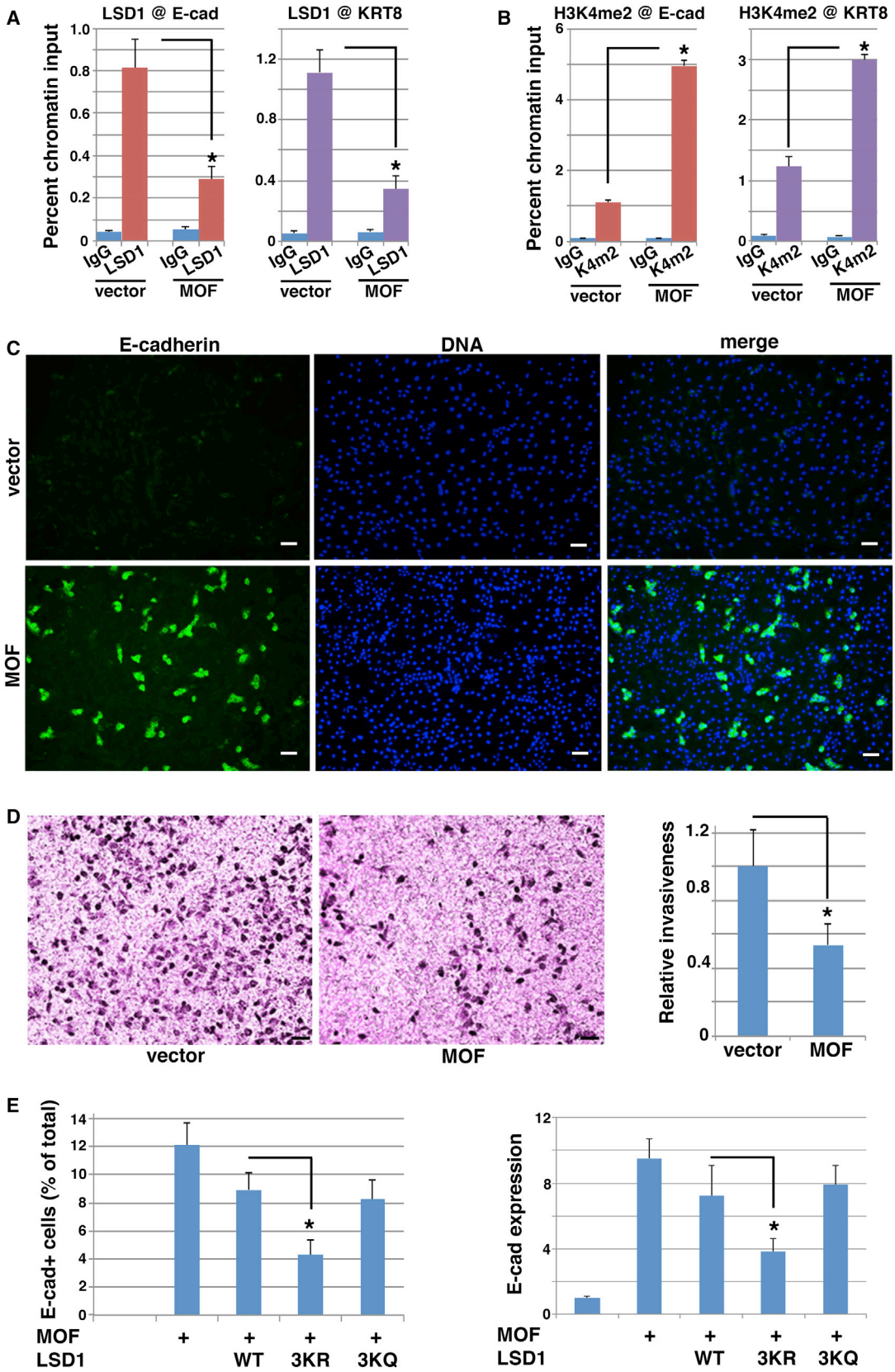
(D) Binding of WT and mutant Flag-LSD1 proteins to the AIM1 and VAT1L promoters was evaluated by ChIP analysis with anti-Flag antibodies. Normal immunoglobulin G (IgG) was used as a non-specific antibody control.

(E) H3K4me2 (K4m2) levels at the AIM1 and VAT1L promoters in the reconstituted cells were measured by ChIP analysis.

(F) Expression of indicated LSD1 target genes was determined by real-time RT-PCR.

Error bars represent SD from triplicate experiments. *$p < 0.05$ (3KQ versus WT LSD1).

epithelial genes in mesenchymal cells (Lin et al., 2010a, 2010b; Tang et al., 2013). Since MOF was able to induce LSD1 acetylation that impaired LSD1's nucleosomal binding and H3K4 demethylation, we expected that ectopic expression of MOF in mesenchymal cells might dissociate LSD1 from chromatin and thus de-repress/activate epithelial markers. We transduced mouse embryonic fibroblasts (MEFs) with a lentiviral vector expressing MOF or a control vector. In MOF-transduced MEFs, the binding of LSD1 at the epithelial genes E-cadherin and KRT8 was indeed decreased (Figure 4A), and, consequently, H3K4me2 levels were increased (Figure 4B). MEFs were negative for E-cadherin expression. However, E-cadherin protein expression became detectable in many MOF-transduced cells (Figure 4C). This increase in E-cadherin expression was accompanied by a reduction of cellular invasiveness in vitro (Figure 4D). These results suggest that MOF is able to dissociate LSD1 from its chromatin targets, increase H3K4me2 levels at these sites, and activate epithelial marker gene expression.



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MOF acetylates H4K16 and plays a critical role in transcriptional activation (Sapountzi and Côté, 2011). To verify whether activation of E-cadherin by MOF in MEFs was attributed to LSD1 acetylation, we tested whether acetylation-resistant LSD1 mutant might suppress MOF's activity. We transduced MEFs with MOF in combination with WT and mutant forms of LSD1. WT LSD1 slightly attenuated MOF-activated E-cadherin expression (Figure 4E). Interestingly, the acetylation-resistant 3KR mutant LSD1 strongly suppressed MOF's capability to induce E-cadherin (Figure 4E). Meanwhile, the acetylation-mimetic 3KQ LSD1 was similar to WT LSD1 (Figure 4E), implying that WT LSD1 might be prevalently acetylated by overexpressed MOF in this assay. These results suggest that MOF activates E-cadherin expression at least in part through acetylation of LSD1.

MOF Expression Is Downregulated and LSD1 Is Deacetylated upon Induction of EMT

LSD1 critically represses epithelial gene expression during EMT (Lin et al., 2010a, 2010b; Tang et al., 2013). In response to EMT-inducing signals (e.g., transforming growth factor β [TGF-β], nickel), A549 epithelial lung cancer cells undergo robust EMT, and the recruitment of LSD1 to epithelial gene promoters was crucial for the induction of EMT (Tang et al., 2013). Because MOF is highly expressed in epithelial cells (Figure 1D) and MOF can suppress LSD1's function through acetylation, it is puzzling why endogenous MOF cannot prevent EMT. We examined the expression of endogenous MOF during EMT. In untreated A549 cells, MOF was expressed at high levels (Figure 5A). However, both RNA and protein levels of MOF were strongly downregulated by nickel treatment (Figure 5A). Consistent with reduced MOF expression, LSD1 proteins were deacetylated in A549 cells following nickel treatment (Figure 5B). The LSD1 protein abundance remained unchanged (Figure 5B). Downregulation of MOF by EMT-inducing signals may pave the way for EMT.

MOF Suppresses LSD1's Chromatin Association, H3K4 Demethylation, EMT, and Cell Invasion

If downregulation of MOF was a prerequisite for EMT, persistent MOF expression might prevent EMT. We transduced A549 cells with lentiviral Flag-MOF (driven by the CMV enhancer/promoter) or control vector, followed by nickel treatment. Expression of exogenous MOF was confirmed by immunoblotting with anti-Flag antibodies (Figure 5C). Because A549 cells expressed high levels of endogenous MOF, the total amount of MOF proteins was not increased by exogenous MOF expression (Figure 5C). After nickel treatment, MOF protein abundance in control A549 cells was strongly decreased; however, MOF protein expression persisted (albeit slightly reduced) in the MOF-transduced cells (Figure 5C). We further examined epithelial marker expression. In nickel-treated control A549 cells, E-cadherin and KRT8 were markedly downregulated (Figures 5C and S3). By contrast, their expression in MOF-transduced cells was only partially decreased by nickel (Figures 5C and S3). The results suggest that MOF critically maintains epithelial gene expression.

Nickel treatment of control A549 cells markedly increased the binding of endogenous LSD1 proteins to the epithelial genes E-cadherin and KRT8 (Figure 5D). Consequently, H3K4me2 levels at these loci were significantly reduced (Figure 5E). However, in MOF-transduced cells, nickel-induced recruitment of LSD1 to these epithelial genes was blunted (Figure 5D), and, correspondingly, nickel treatment failed to reduce the H3K4me2 levels at these genes (Figure 5E). Because MOF is a major H4K16 acetyltransferase, we also examined this histone mark and found that its presence at epithelial gene promoters was markedly decreased by nickel treatment in control cells, which is consistent with downregulation of MOF expression but remained high in MOF-transduced cells (Figure 5F). These results suggest that persistent MOF expression blocks nickel-induced recruitment of LSD1 to epithelial gene promoters and LSD1-mediated H3K4 demethylation at these loci.

A549 cells attached to each other and exhibited an epithelial morphology in culture (Figure 5G). Following nickel treatment, these cells became elongated and dispersed (Figure 5G), which was characteristic of EMT. However, many MOF-transduced cells resisted nickel-induced EMT: they maintained cell-cell adhesion and remained clustered (Figure 5G). EMT endows cells with increased invasiveness. In response to nickel treatment, control A549 cells showed an enhanced ability to invade extracellular matrix in the Transwell in vitro invasion assay (Figure 5H). This nickel-stimulated invasiveness was significantly suppressed by MOF overexpression (Figure 5H). Together, these data suggest that MOF can counteract nickel-induced EMT.

Snail physically recruits LSD1 to epithelial genes and represses their expression in an LSD1-dependent manner (Lin et al., 2010a, 2010b; Tang et al., 2013). Cells expressing an inducible form of Snail, Snail-ER, exhibited increased LSD1

Figure 4. MOF Dissociates LSD1 from Chromatin and Activates E-Cadherin Expression in MEFs at Least in Part through Acetylation of LSD1

(A) Overexpression of MOF in MEFs causes dissociation of LSD1 from epithelial gene promoters. MEFs were transduced with lentiviral control vector or vector expressing MOF, followed by ChIP analysis to determine the binding of LSD1 at the epithelial genes E-cadherin and KRT8.

(B) MOF increases H3K4me2 levels at epithelial genes in MEFs. Control and MOF-transduced MEFs were subjected to ChIP with anti-H3K4me2 (K4m2) antibodies.

(C) MOF induces E-cadherin expression in MEFs. Control and MOF-transduced MEFs were subjected to immunofluorescence staining with anti-E-cadherin antibodies. DNA was stained with Hoechst.

(D) MOF inhibits cell invasion in vitro. Control and MOF-transduced MEFs were assayed for Transwell invasion. Cells that invaded through the matrix membrane were imaged and counted. Representative images are shown (left). The histogram shows the quantification of relative number of cells migrated through matrix (from five random fields). Error bars indicate SD. *$p < 0.05$.

(E) Acetylation-resistant 3KR LSD1 mutant suppresses MOF's ability to activate E-cadherin. MEFs were transduced with lentiviruses expressing MOF in combination with WT or mutant LSD1. E-cadherin expression was assessed by quantification of cells positive for immunostaining with anti-E-cadherin antibodies (left) or real-time RT-PCR (right). Error bars indicate SD from triplicate experiments. *$p < 0.05$.

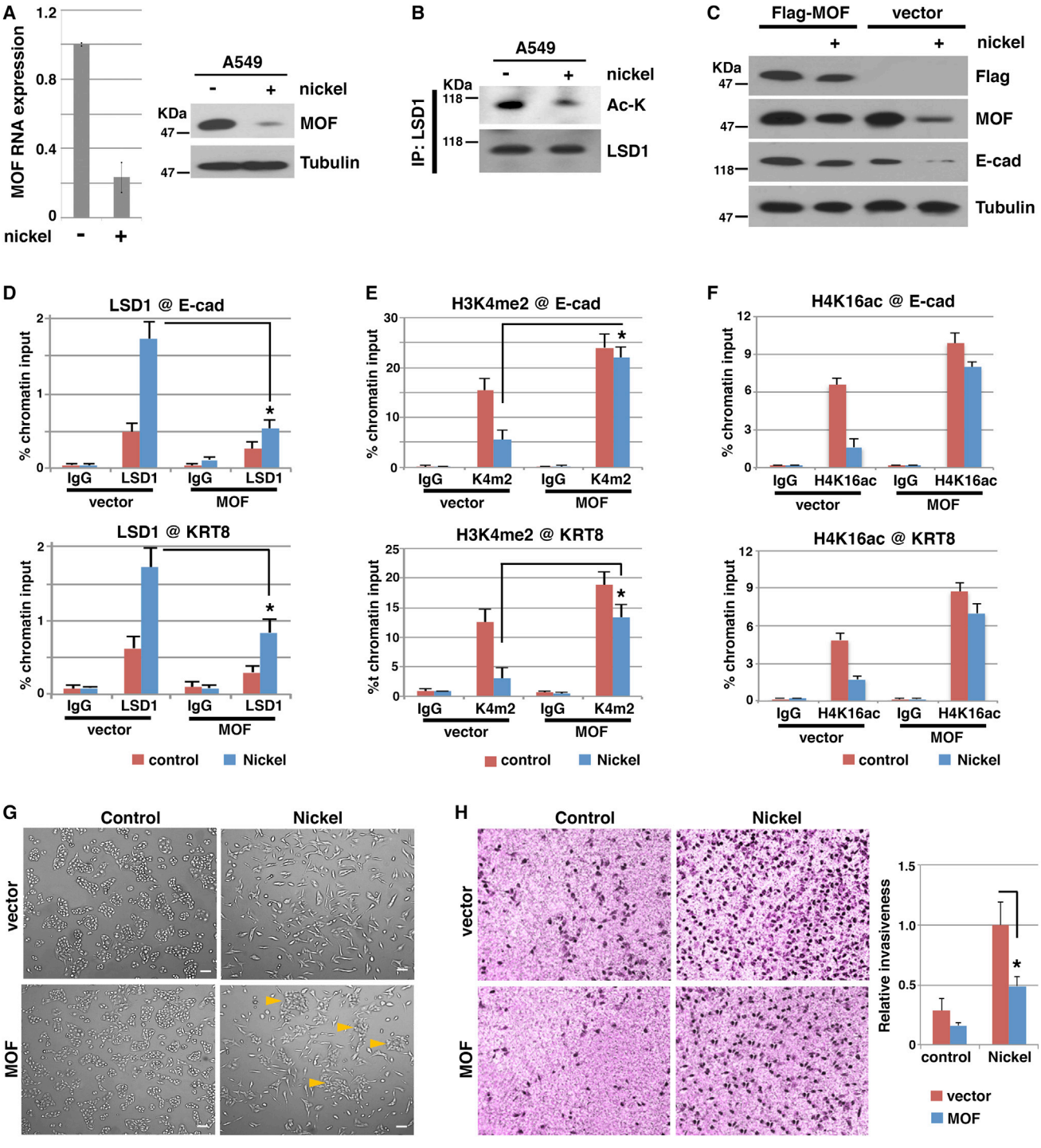

Figure 5. MOF Is Downregulated by Nickel, and Persistent MOF Expression Prevents the Recruitment of LSD1 to Chromatin, H3K4me2 Demethylation, and EMT

(A) MOF expression is reduced during nickel-induced EMT. A549 cells were treated with nickel for 2 days. RNA and protein levels of MOF were determined by real-time RT-PCR and immunoblotting, respectively.

(B) Endogenous LSD1 is deacetylated during nickel-induced EMT. Endogenous LSD1 proteins were immunoprecipitated from A549 cells (with or without nickel treatment), followed by immunoblotting for lysine acetylation.

(C) Persistent expression of MOF in A549 cells maintains E-cadherin expression. A549 cells were transduced with lentiviral Flag-MOF or empty vector, followed by treatment with nickel or control (H_2O) for 2 days. Cells were lysed for immunoblotting with indicated antibodies. See also Figure S3.

(D) Recruitment of endogenous LSD1 to the promoters of epithelial genes, E-cadherin and KRT8, was determined by ChIP analysis with anti-LSD1 antibodies.

(legend continued on next page)

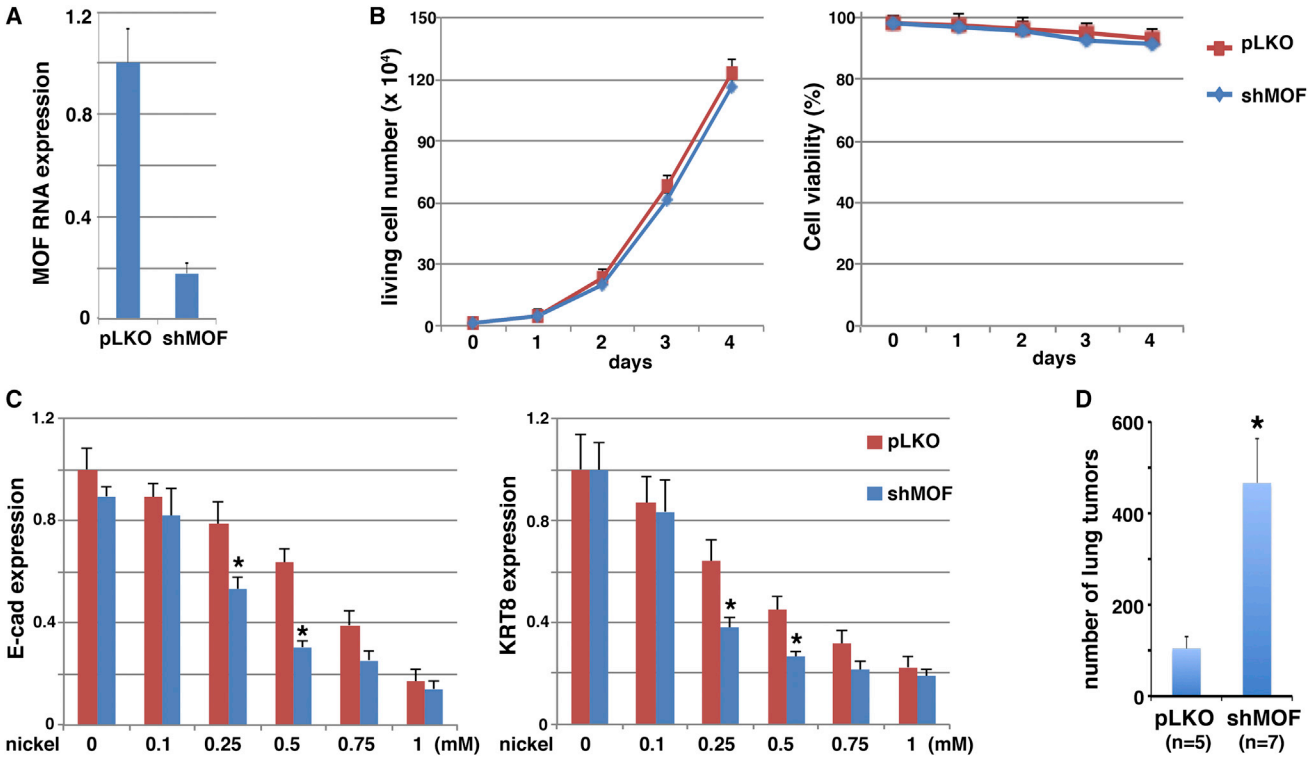

Figure 6. Depletion of MOF Accelerates EMT and Increases Tumor Metastatic Potential

(A) Depletion of MOF in A549 cells with lentiviral shRNAs was verified by real-time RT-PCR.

(B) Depletion of MOF does not affect cell growth and survival. Control and MOF-depleted A549 cells were plated, and cell viability was determined by Trypan blue staining. Living cells were counted.

(C) Depletion of MOF facilitates the induction of EMT by nickel. Control and MOF-depleted A549 cells were treated with varying concentrations of nickel for 2 days. Expression of epithelial cell markers E-cadherin and KRT8 was quantified by RT-PCR.

(D) Depletion of MOF enhances metastasis. Control and MOF-depleted A549 cells were injected into tail vein of immunodeficient mice. Lung tumors were counted. The number of mice in each group is shown.

Error bars indicate SD. *p < 0.05 (comparing shMOF with pLKO).

recruitment to epithelial genes following Snail induction (Tang et al., 2013). We infected DLD1 epithelial colon cancer cells with lentiviral Snail-ER. As expected, induction of Snail boosted LSD1 binding and reduced H3K4me2 levels at the E-cadherin promoter (Figure S4A). However, when exogenous MOF was co-expressed in the Snail-ER cells via lentiviral transduction, Snail-induced LSD1 chromatin recruitment and H3K4me2 demethylation were substantially blocked (Figure S4A). Morphologically, while control Snail-ER cells exhibited complete EMT upon induction of Snail, overexpression of MOF in such cells enabled many cells to resist Snail-induced EMT and largely retain the epithelial status (Figure S4B). Consistently, repression of E-cadherin by Snail was also partially blocked by MOF (Figure S4B). Collectively, the results suggest that MOF is able to suppress nickel- and Snail-induced EMT.

To validate the anti-EMT role of endogenous MOF, we depleted MOF in A549 cells with lentiviral shRNAs (Figure 6A). Depletion of MOF did not alter cell growth or survival (Figure 6B). Without EMT-inducing signals, MOF-depleted A549 cells did not show evident morphological changes compared with control cells, and expression of epithelial markers E-cadherin and keratin KRT8 was not significantly affected by MOF depletion (Figure 6C). When treated with increasing concentrations of nickel, expression of epithelial markers became gradually decreased in control cells, but MOF-depleted cells downregulated these markers more robustly than control cells (Figure 6C), suggesting

(E) H3K4me2 (K4m2) levels at the epithelial gene promoters were measured by ChIP analysis.

(F) Acetyl H4K16 levels at the epithelial genes were determined by ChIP.

(G) Overexpression of MOF blocks nickel-induced EMT in A549 cells. Phase-contrast images of control and MOF-overexpressing A549 cells treated with nickel or control (H_2O) are shown. Arrowheads indicate cells that maintain the epithelial morphology.

(H) MOF expression attenuates nickel-induced cell invasion in vitro. Control and MOF-overexpressing A549 cells were treated with or without nickel for 2 days, and were subjected to the Transwell invasion assay. The histogram shows the quantification of relative number of cells that migrated through matrix (from five random fields).

Error bars indicate SD. *p < 0.05.

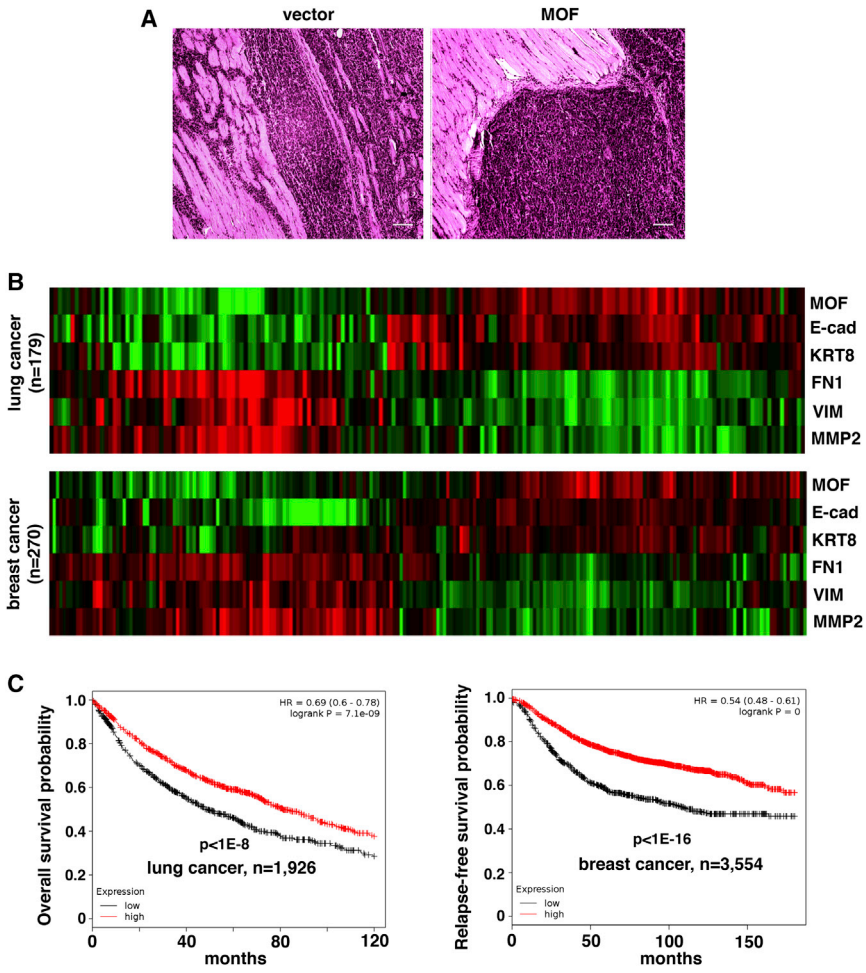

Figure 7. MOF Suppresses Tumor Progression and Is a Favorable Prognostic Factor in Human Cancer

(A) MOF inhibits tumor invasion in xenograft models. MDA-MB-231 cells were transduced with lentiviral MOF or control vector (see also Figure S5), and injected into immunodeficient mice. Tumors were sectioned and stained by H&E.

(B) Correlation of MOF with epithelial cell markers in human lung and breast cancers. Tumors were clustered based on expression levels of MOF along with epithelial markers E-cadherin (E-cad) and KRT8, and mesenchymal markers fibronectin (FN1), vimentin (Vim), and MMP2 in human lung and breast cancer cohorts (TCGA dataset GSE62944). Green and red colors indicate lower and higher expression, respectively. See also Figure S6.

(C) Higher levels of MOF expression predict better clinical outcomes in human lung and breast cancers. Kaplan-Meier survival was analyzed based on expression levels of MOF in large cohorts of lung (left) and breast (right) cancer patients using online software (http://kmplot.com/analysis/). Log-rank p values are shown.

that depletion of MOF facilitates EMT. EMT enhances lung metastasis in the experimental metastasis assay (Tsai and Yang, 2013). MOF-depleted A549 cells formed significantly more tumors than control cells in mouse lungs following tail vein injection (Figure 6D). These observations suggest that endogenous MOF functions against EMT.

MOF Suppresses Tumor Invasion and Is Associated with Epithelial Gene Expression and Favorable Prognosis in Human Cancer

The role of MOF in malignant progression remains poorly understood. Because MOF inhibited LSD1-dependent EMT, we asked whether MOF might suppress tumor progression. MDA-MB-231 triple-negative breast cancer cells are mesenchymal and aggressive. Similar to MEFs, infection of MDA-MB-231 cells with lentiviral MOF also decreased LSD1's binding (Figure S5A) and increased H3K4me2 levels (Figure S5B) at epithelial gene promoters, resulting in E-cadherin expression (Figure S5C). When injected into immunodeficient mice, control MDA-MB-231 cells were invasive and exhibited extensive infiltration into neighboring muscle fibers (Figure 7A). By contrast, tumors from MOF-overexpressing MDA-MB-231 cells showed generally clear boundaries (Figure 7A). The results suggest that MOF may inhibit LSD1 function, activate epithelial gene expression, and suppress tumor invasion in vivo.

The importance of MOF in suppressing EMT and tumor progression prompted us to investigate its expression in human cancer. Because MOF positively regulated epithelial genes and itself was downregulated upon EMT, we compared MOF expression with EMT markers in more than 500 cell lines derived from human solid tumors (Barretina et al., 2012). MOF was highly expressed in cancer cells that expressed epithelial markers E-cadherin and EPCAM and was absent or low in cells expressing mesenchymal markers Vimentin and Zeb1 (Figure S6). We next examined microarray data from human primary tumor samples. MOF expression was heterogeneous among tumor samples (Figure 7B). Some tumors expressed high levels of MOF, whereas others were negative for MOF expression. Interestingly, in both lung and breast cancer cohorts, MOF expression overlapped with epithelial markers, such as E-cadherin and KRT8, and inversely correlated with mesenchymal markers (Figure 7B).

Given the association of MOF expression with the epithelial phenotype, we further investigated whether MOF might be an independent prognostic indicator in human cancer. In a cohort of nearly 2,000 lung cancer patients (Győrffy et al., 2013), high MOF expression in tumors strongly predicted a better overall survival (Figure 7C). Similarly, in a large cohort of over 3,500 breast cancers (Győrffy et al., 2010), elevated MOF levels robustly correlated with relapse-free survival (Figure 7C). These data suggest that MOF is a potent indicator of favorable prognosis in human cancer, which is consistent with its ability to suppress LSD1 function and tumor progression.

DISCUSSION

LSD1-catalyzed demethylation of H3K4 is required for Snail-mediated transcriptional repression of epithelial markers in cells undergoing EMT. Demethylation of nucleosomal H3K4 by LSD1 depends on its association with nucleosomes. In this study, we found that LSD1 was acetylated preferentially in epithelial cells. This covalent modification impaired LSD1's ability to bind to nucleosomes, demethylate nucleosomal substrates, and repress target gene expression. Therefore, despite its ubiquitous expression, LSD1's functionality may differ between epithelial and mesenchymal cells due to its differential acetylation. The MYST family acetyltransferase MOF was identified as the only enzyme that was sufficient and necessary for LSD1 acetylation. Expression of MOF is enriched in epithelial cells, which is consistent with epithelial-specific acetylation of LSD1. MOF prevented the recruitment of LSD1 to epithelial gene promoters and LSD1-dependent H3K4 demethylation, thereby maintaining epithelial gene expression and suppressing EMT and tumor invasion (Figure S7). Taken together, this study uncovers MOF-mediated acetylation of LSD1 as a crucial regulatory switch controlling LSD1 function and provides a mechanistic link to EMT regulation. Furthermore, our data illustrate MOF as an important suppressor of EMT and tumor progression.

Recruitment of the LSD1 corepressor complex to chromatin involves multivalent interactions. Association with sequence-specific DNA-binding transcription factors may guide the complex to particular genomic loci, and local chromatin environment also plays an essential role in this process. SFMBT1, a putative reader subunit recognizing certain histone marks, is required for the chromatin recruitment of LSD1 (Tang et al., 2013). Association of LSD1 with chromatin also depends on CoREST, which not only interacts with LSD1, but also directly binds to nucleosomal DNA, thereby tethering LSD1 to nucleosomes (Lee et al., 2005; Shi et al., 2005; Yang et al., 2006). The present study suggests that the binding of LSD1 to nucleosomes critically involves a cluster of lysine residues in its Tower domain, which may directly contact the negatively charged DNA backbone. MOF-mediated acetylation neutralizes their positive charge and thus de-stabilizes the LSD1-nucleosome association. LSD1 interacts with many transcription factors and regulates diverse biological processes. It will be interesting to investigate whether MOF may impact LSD1-dependent regulation in general.

MOF is primarily responsible for H4K16 acetylation. Our study shows that MOF blocks H3K4 demethylation by LSD1. Moreover, the NSL complex shares common subunits with the MLL H3K4 methyltransferase complexes (Cai et al., 2010; Dias et al., 2014; Dou et al., 2005; Li et al., 2009; Mendjan et al., 2006; Sharma et al., 2010; Zhao et al., 2013b). Therefore, in addition to implementing H4K16 acetylation, MOF may promote H3K4 methylation by dissociating the LSD1 demethylase and engaging the MLL methyltransferase, thereby driving the transition from a transcription-repressed state to an active state.

The physiological role of MOF has been extensively characterized in mice. Targeted deletion of MOF in mice results in early embryonic lethality and cell death (Gupta et al., 2008; Thomas et al., 2008). Purkinje cell-specific deletion of mouse MOF causes loss of these cells (Kumar et al., 2011). Ablation of MOF specifically in mouse T cells leads to defective cell differentiation and reduces T cell number and thymus size (Gupta et al., 2013). In mouse podocytes, MOF is required for cell-cycle progression in proliferating cells but is dispensable for terminally differentiated, postmitotic cells. However, following injury, MOF is critical for podocyte maintenance in vivo (Horikoshi et al., 2015; Sheikh et al., 2015). Overall, a complete lack of MOF is deleterious to normal cell proliferation and/or survival. In the present study, RNA interference-mediated depletion of MOF in cancer cells influences EMT but does not impact their growth and viability. These observations imply that the biological significance of MOF is context dependent, and reduced MOF expression/activity, rather than a complete loss of MOF, may promote malignant progression.

The role of MOF in cancer has just begun to be understood. In this study, MOF was found to suppress EMT and tumor invasion. High MOF expression was strongly associated with the epithelial state and favorable clinical outcomes in cancer patients. Therefore, MOF functions as a critical suppressor of tumor progression. MOF expression was downregulated by EMT-inducing signals. Thus, increasing MOF expression and/or activity may represent a therapeutic approach for cancer treatment. The acetyltransferase activity of MOF depends on its autoacetylation at K274 (Sun et al., 2011; Yuan et al., 2012). SIRT1 deacetylates MOF and inhibits its enzymatic activity (Peng et al., 2012). Therefore, SIRT1 inhibitors may stimulate MOF activity and LSD1 acetylation and impede tumor progression.

EXPERIMENTAL PROCEDURES

Cell Culture and Chemical Reagents

The human cell lines HEK293, MCF7, and MDA-MB-231 cells were cultured in DMEM supplemented with 10% bovine calf serum. A549, DLD1, T47D, MDA-MB-468, BT474, Hs578T, MDA-MB-435, BT549, and MEF cells were cultured in DMEM with 10% fetal bovine serum (FBS). HCT116 LSD1 knockout cells were cultured in McCoy's 5A medium with 10% FBS. Where indicated, the following drugs were used: 4-hydroxy-tamoxifen (4HT) (100 nM), nickel chloride (1 mM), Trichostatin A (TSA) (100 nM), Nicotinamide (5 mM). Except nicotine, chemical reagents were added to culture media from 1,000-fold concentrated stock solutions, and equal volumes of vehicle (DMSO or H_2O) were added as controls.

For additional experimental descriptions see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.05.050.

AUTHOR CONTRIBUTIONS

H.L., A.K.S., X.L., Y.J., Q.C., M.T., Y.L., and H.C. performed experiments, L.J., D.R., L.W., E.S., Y.Q., Y.D., and R.A.C. provided important reagents and/or discussion, and J.L. designed the project and wrote the paper.

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