

# Modulations of hMOF autoacetylation by SIRT1 regulate hMOF recruitment and activities on the chromatin

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A wide variety of nuclear regulators and enzymes are subjected to acetylation of the lysine residue, which regulates different aspects of protein functions. The MYST family histone acetyltransferase, human ortholog of MOF (hMOF), plays critical roles in transcription activation by acetylating nucleosomal H4K16. In this study, we found that hMOF acetylates itself *in vitro* and *in vivo*, and the acetylation is restricted to the conserved MYST domain (C2HC zinc finger and HAT), of which the K274 residue is the major autoacetylation site. Furthermore, the class III histone deacetylase SIRT1 was found to interact with the MYST domain of hMOF through the deacetylase catalytic region and deacetylate autoacetylated hMOF. *In vitro* binding assays showed that non-acetylated hMOF robustly binds to nucleosomes while acetylation decreases the binding ability. In HeLa cells, the recruitment of hMOF to the chromatin increases in response to SIRT1 overexpression and decreases after knockdown of SIRT1. The acetylation mimic mutation K274Q apparently decreases the chromatin recruitment of hMOF as well as the global H4K16Ac level in HeLa cells. Finally, upon SIRT1 knockdown, hMOF recruitment to the gene body region of its target gene *HoxA9* decreases, accompanied with decrease of H4K16Ac at the same region and repression of *HoxA9* transcription. These results suggest a dynamic interplay between SIRT1 and hMOF in regulating H4K16 acetylation.

**Keywords:** hMOF; autoacetylation; SIRT1; nucleosome; deacetylation

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## Introduction

Posttranslational modifications (PTMs) are critical for modulating the functions of a variety of eukaryotic proteins. Among different PTMs, reversible acetylation of the lysine residue can neutralize the positive charge of this amino acid (aa) and regulate protein activities in different ways [1, 2]. First identified in histones, lysine acetylation has been extended to play regulatory roles on more than 80 transcriptional factors, and a lot of other nuclear factors and cytoplasmic proteins [3]. Acetylation of lysine residues can affect protein stability, enzymatic activity and protein-protein interactions, and these

changes then contribute to dynamic control of the cellular signaling under diverse conditions [4].

Lysine acetylation is catalyzed by lysine acetyltransferases (KATs), which mainly include Gcn5-related *N*-acetyltransferases, E1A-associated protein of 300 kDa (p300)/CREB-binding protein and MYST family proteins [3]. KATs transfer acetyl groups to their substrates, and conversely, they can also receive acetyl groups from other KATs or even their own catalytic activities. Both of the two well-known acetyltransferases p300 and PCAF can be acetylated and many sites are autoacetylated. Autoacetylation of PCAF increases its histone acetyltransferase activity [5] and autoacetylation of p300 induces the conformational change, which is important for assembly of different p300-containing complexes [6].

The MYST family is one of the largest but, so far, intensive studies are still needed to fully understand their functions, although some members have been shown to play crucial roles in diverse cellular processes [7, 8]. PTMs, specifically acetylation and phosphorylation, are emerging to show regulatory roles on proteins of this

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family. TIP60, a MYST family member, was previously shown to be phosphorylated, and phosphorylation modulates its histone acetyltransferase activity [9]. A recent study on TIP60 also showed that its autoacetylation leads to the dissociation of TIP60 oligomer and strengthens its interaction with the substrates [10].

MOF (also called MYST1 or KAT8) is another well-studied MYST family member, which is the major enzyme acetylating histone H4 lysine 16 and required for dosage compensation in *Drosophila* [11-14]. The human ortholog of MOF (hMOF) has the same substrate specificity for nucleosomal H4K16 and is involved in regulating the chromatin structure in mammals [15, 16].

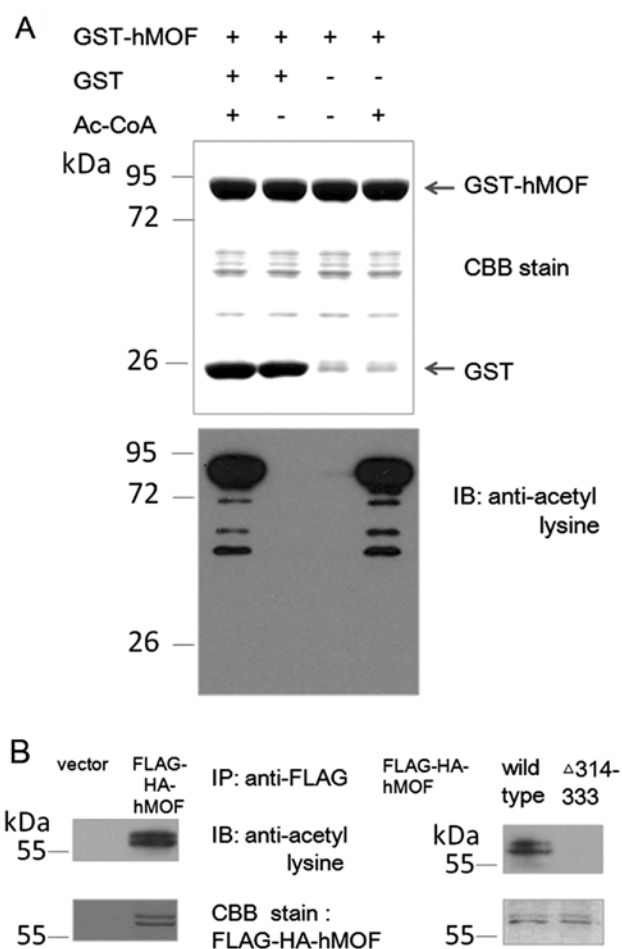
Until now, however, PTMs of hMOF and their potential effects still remain elusive. In this study, we found that hMOF is autoacetylated *in vitro* and *in vivo*, and SIRT1, the deacetylase for H4K16Ac, is responsible for deacetylating acetylated hMOF. *In vitro* binding assays showed that autoacetylation of hMOF diminishes its binding activity to nucleosomes. Furthermore, *in vivo* studies showed that modulations of hMOF autoacetylation by SIRT1 regulate hMOF recruitment and activities on the chromatin.

## Results

### *hMOF acetylates itself in vitro and in vivo*

Many acetyltransferases involved in transcriptional regulation and chromatin modifications, such as p300 and TIP60, were found to be autoacetylated [6, 10]. As a member of the MYST family acetyltransferases, it is quite possible that hMOF can also be autoacetylated. To test this, *in vitro* acetylation assay was carried out using purified GST-hMOF, and acetylation was detected by an acetylated lysine-specific antibody (Figure 1A). GST-hMOF displayed strong acetylation signals when supplemented with acetyl CoA and showed no acetylation signals without the cofactor. However, this is not sufficient to verify hMOF as autoacetylated, as GST could also be responsible for the detected acetylation signals. To exclude this, GST was incubated with GST-hMOF in the presence or absence of acetyl CoA. In both situations, GST showed no acetylation signal. In addition, we used thrombin to cleave the GST tag from GST-hMOF, and the pure hMOF proteins also showed comparable acetylation signals (Supplementary information, Figure S1). These data indicate that hMOF can autoacetylate itself *in vitro*.

*In vivo* acetylation analysis of hMOF was also performed to detect autoacetylation. Immunoprecipitated FLAG-HA-hMOF (referred to as FH-hMOF hereafter) showed acetylation signals, and moreover, the hMOF



**Figure 1** hMOF is self-acetylated *in vitro* and *in vivo*. **(A)** GST-hMOF was incubated in acetylation buffer with or without acetyl CoA at 30 °C for 1 h, and in parallel, GST-hMOF was mixed with GST under the same condition. The reaction product was then resolved in 10% SDS-PAGE followed by Commassie blue staining or immunoblotting with anti-acetyl lysine antibody (Cell signaling Technology). **(B)** hMOF lacking acetyl CoA-binding site was referred to as hMOF  $\Delta$ 314-333. 293T cells were transiently transfected with FLAG-HA-hMOF FL (full-length) or  $\Delta$ 314-333, and the glycine eluate of FLAG-IP was used to detect acetyl lysine by immunoblotting. The eluate of FLAG-IP from 293T cells transfected with vectors was used as a negative control.

mutant with deletion of the acetyl-CoA binding site showed significantly reduced acetylation signals compared with the wild type (Figure 1B). Disruption of the hMOF catalytic activity reduced its acetylation level, suggesting that hMOF is autoacetylated *in vivo*.

In a recent study using HDACI/II inhibitors SAHA- and MS275-treated human cell lines (Jurkat, MV4-11 and A549), two lysines (K113 and K238) within hMOF were identified as acetylated sites [17]. Hence, to test whether these two lysines were the self-acetylation sites,

we generated cognate mutants. Single or double mutants of K113R and K238R GST-hMOF proteins were purified to determine their acetyltransferase activities by *in vitro* acetylation assay using the recombinant histone H4 as substrates. All the three mutant forms demonstrated similar enzymatic activities on recombinant H4 and similar levels of autoacetylation compared with the wild-type proteins (Figure 2A). Point mutations of these two lysines did not change hMOF enzymatic activity, and their inability to be acetylated did not influence hMOF self-acetylation level either. Two possibilities for the observations exist: first, neither K113 nor K238 is the self-acetylation lysine; second, like p300, there are several self-acetylated lysines and mutations of one or two of them do not change the overall acetylation level.

Although no detailed studies are available, *Drosophila* MOF has been noticed to be autoacetylated [12, 13]; thus, autoacetylation is likely to be conserved in evolution. MYST domain, composed of C2HC zinc finger and HAT domain, is the signature of MYST family proteins and is conserved among even distant species. Sequence comparison of MYST domains among five different species using ClustalX reveals that, within hMOF catalytic domain, there are eight lysines conserved in *Caenorhabditis elegans*, *Drosophila*, Zebrafish, mouse and human, and seven conserved in the latter four species (Figure 2B). We speculated that these conserved lysines within MYST domain are more likely to be responsible for hMOF self-acetylation.

To test this, *in vitro* acetyltransferase assay was performed using recombinant GST-hMOF derivatives aa 1-130, 157-458 and 231-458 as substrates (Figure 2C). Equal amounts of full-length GST-hMOF were used to catalyze the separate reactions. Acetylation signals were observed on aa 157-458 and 231-458, while aa 1-130 showed no acetylated band. The aa 231-458 fragment contains the core HAT domain, while aa 157-458 includes an additional C2HC zinc finger, which is required for substrate recognition and efficient enzyme activity. So it can be inferred that self-acetylation also contributes to the robust acetylation signals of aa 157-458. The reactions were repeated with higher or lower doses of full-length hMOF, and all of them showed similar patterns of acetylation signals as above (data not shown). Therefore, autoacetylation of hMOF occurs mainly on the MYST domain containing several conserved lysine residues.

In a subsequent GST pull-down experiment, full-length GST-hMOF and N terminal GST-hMOF aa 1-130 were used to pull-down hMOF from lysates of 293T cells expressing FH-hMOF (Figure 2D). The results showed that GST-hMOF, but not the N terminal fragment GST-hMOF aa 1-130, can interact with FH-hMOF, indicating

that hMOF can form a dimer or oligomer mediated by the MYST domain. *In vitro* HAT assay also showed that the G327D mutant of GST-hMOF, which bears little enzyme activity [16], can be acetylated by the pure hMOF without GST tag (Supplementary information, Figure S2). These results indicate that the catalytic region could catalyze inter-molecular acetylation of hMOF itself, but an intra-molecular mechanism may act concomitantly.

To further map the autoacetylated sites, we adopted the serial point-mutation strategy. By using the predictor of acetylation at PHOSIDA (<http://www.phosida.com>), combined with our sequence alignment of MYST domains, we selected lysines 268, 274, 330, 407 and 410 as the most likely sites. We first converted all potential lysines (113, 238, 268, 274, 330, 407 and 410) to arginines to generate a 7KR mutant, and tested its autoacetylation and enzyme activity. The *in vitro* HAT assays showed that autoacetylation signals (Figure 3A) and enzyme activities on histone H4 (Figure 3B) dramatically decreased. Then we tested the effects of 3KR (113, 238 and 274), 4KR (113, 238, 274 and 268) and 6KR (113, 238, 274, 268, and 330), and all these mutants showed apparently decreased autoacetylation signals compared with the wild-type proteins (Figure 3C). As all the mutants contain the K274R mutation, it is most probable that K274 serves as the key point of autoacetylation. Thus, we generated the single K274R mutation and carried out HAT reactions using the wild-type hMOF without GST tag as the enzyme. The results showed that the single K274R mutation is sufficient to cause apparent decrease of acetylation signals (Figure 3D). To further confirm this, we generated K268R, K330R and K407-410R mutants, and these mutants showed efficient autoacetylation and catalytic activities on H4K16 (Figure 3E). Taken together, we conclude that K274 residue is the major site of hMOF autoacetylation, and K274R mutation impairs the enzyme activity on H4K16 of recombinant histone H4 *in vitro*.

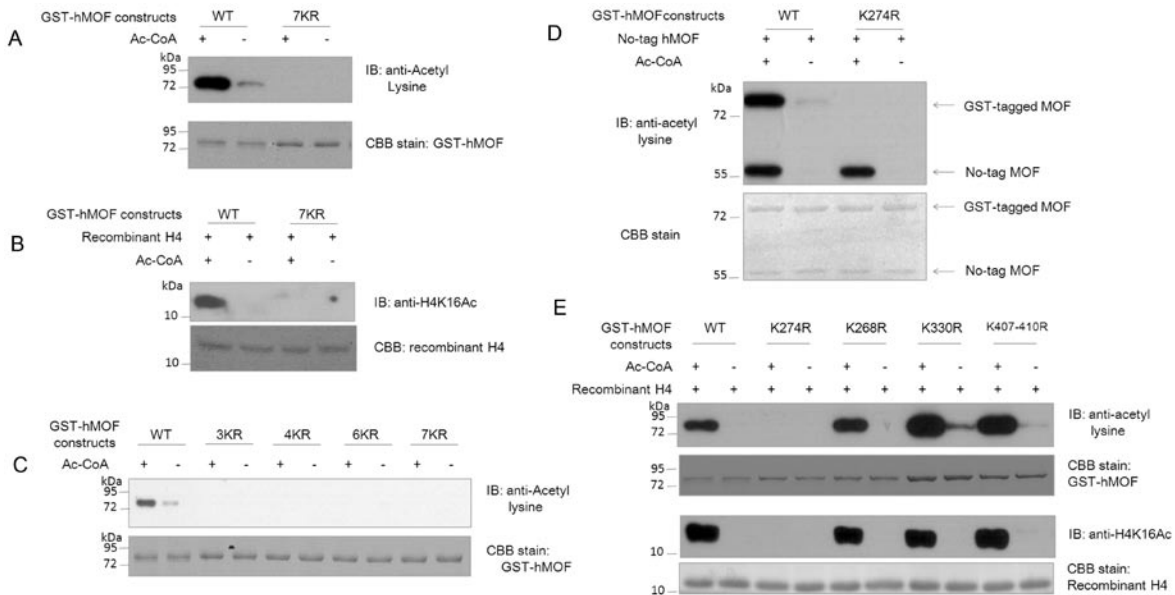
#### *SIRT1 physically interacts with hMOF*

Among the MYST family histone acetyltransferases, TIP60 and hMOF are more closely related due to their structural analogy and are divided into the same subgroup of MYST proteins. TIP60 is self-acetylated, and can interact with and be deacetylated by SIRT1 [10]. TIP60 and SIRT1 also share some common substrates, like p53 [18, 19]. Similarity between TIP60 and hMOF suggests that hMOF may also be deacetylated by SIRT1 and have mutual functional interactions with SIRT1.

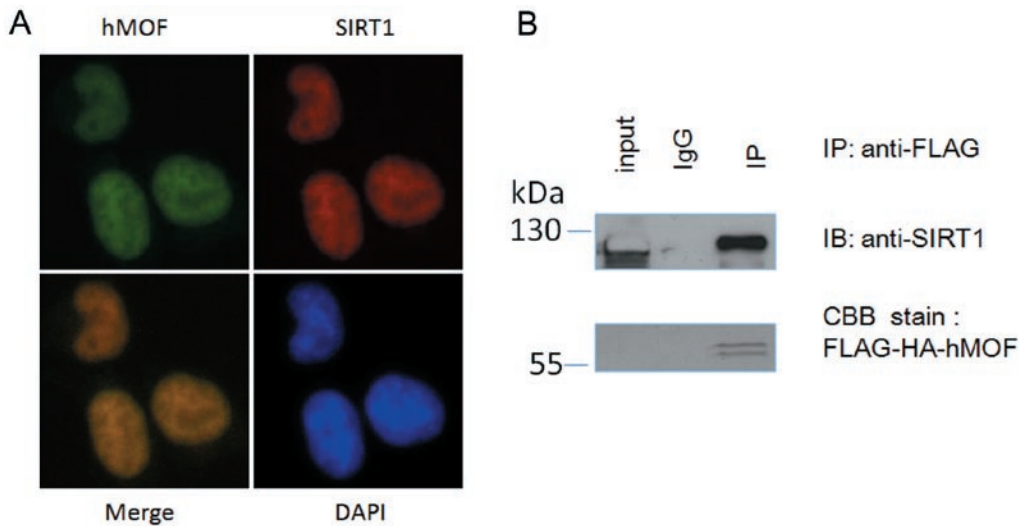
To test this hypothesis, localizations of hMOF and SIRT1 were studied in 293T cells stably expressing FH-hMOF. Immunofluorescence was performed to stain







**Figure 3** Autoacetylation of hMOF mainly occurs at lysine 274. **(A)** Wild-type GST-hMOF or 7KR (K113, K238, K274, K268, K330, K407 and K410 to R) proteins were used for *in vitro* HAT reactions. Acetylation signals were detected by anti-acetyl lysine antibody. **(B)** Wild-type GST-hMOF or 7KR proteins were incubated with recombinant histone H4 in HAT buffer at 30 °C for 1 h. H4K16Ac was detected by western blot using specific H4K16Ac antibody. **(C)** Wild-type GST-hMOF, 3KR (113, 238 and 274 to R), 4KR (113, 238, 274 and 268 to R), 6KR (113, 238, 274, 268 and 330 to R) and 7KR proteins were used for *in vitro* HAT reactions. Acetylation signals were detected by anti-acetyl lysine antibody. **(D)** Wild-type GST-hMOF or K274R-mutant proteins were used for *in vitro* HAT reactions catalyzed by pure hMOF proteins without GST tags. Acetylation signals were detected by anti-acetyl lysine antibody and the CBB staining showed equal loading. **(E)** Wild-type GST-hMOF, K274R, K268R, K330R or K407-410R proteins were incubated with recombinant histone H4 in HAT buffer at 30 °C for 1 h. Acetylation signals of MOF proteins were detected by anti-acetyl lysine antibody and acetylation of H4 was detected by the specific H4K16Ac antibody.

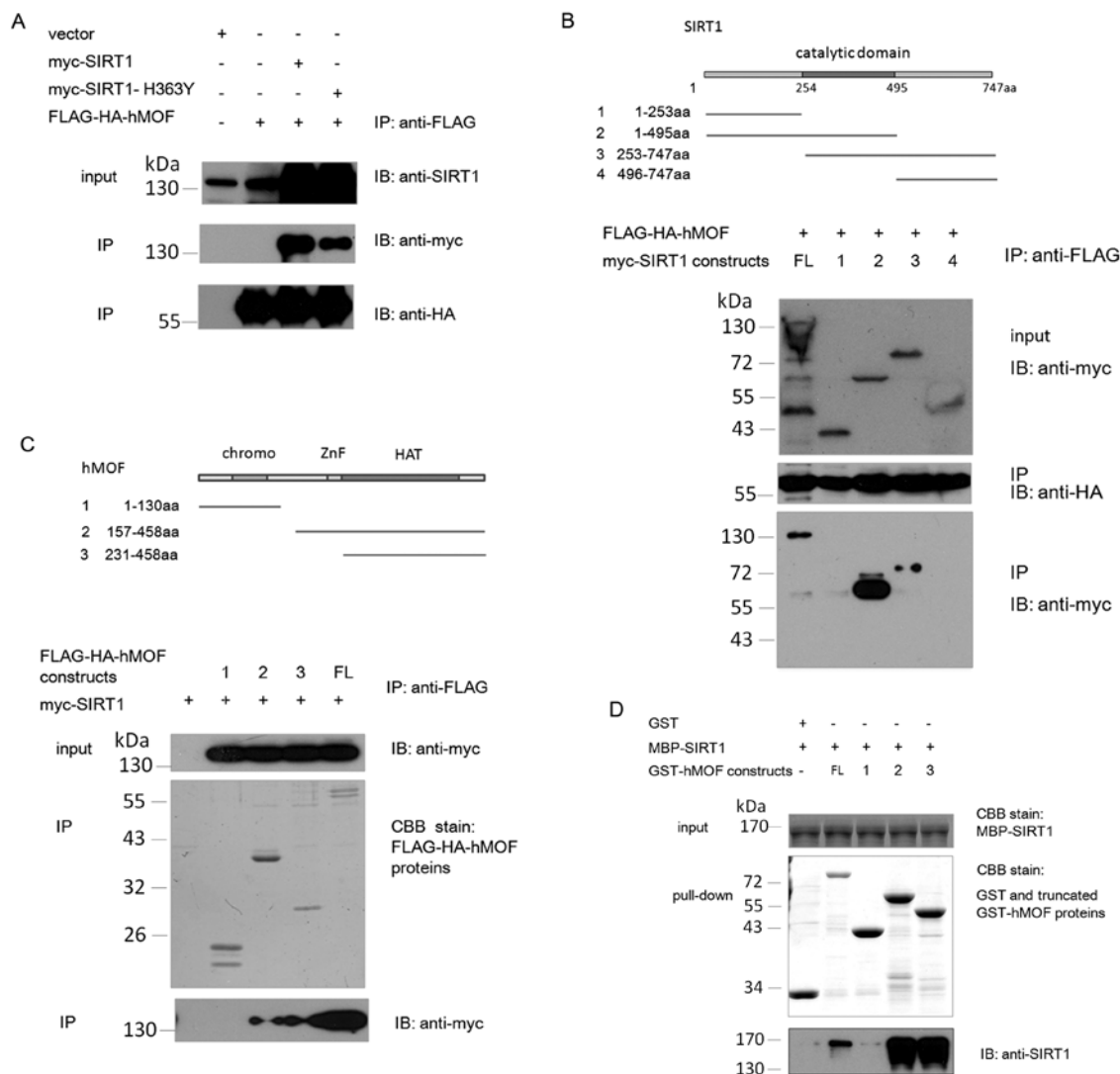


**Figure 4** hMOF interacts with SIRT1 *in vivo*. **(A)** The 293T cells stably expressing FLAG-HA-hMOF were fixed in 4% paraformaldehyde, and immunofluorescence was carried out using primary antibodies anti-FLAG (mouse IgG) and anti-SIRT1 (rabbit IgG) followed by secondary antibodies anti-mouse IgG/FITC (hMOF, green) and anti-rabbit IgG/TRITC (SIRT1, red). Nuclei were stained with DAPI. **(B)** The 293T cells were co-transfected with FLAG-HA-hMOF and myc-SIRT1. Cells were then lysed in IP buffer, and immunoprecipitation was performed using anti-FLAG antibodies with equal amounts of preimmune mouse IgG as the negative control. Immunoprecipitated FLAG-HA-hMOF was shown by using CBB stain, while co-IP product was detected with anti-myc antibody.

(Figure 4B). Both experiments verified the interaction between these two proteins.

Furthermore, the deacetylase-defective mutant SIRT1 (H363Y) showed less interaction signals compared with wild type (Figure 5A), and this observation suggested that the catalytic domain of SIRT1 may mediate the in-

teraction with hMOF. Therefore, we performed co-IP between truncated fragments of SIRT1 and full-length hMOF (Figure 5B). It turned out that the catalytic domain of SIRT1 mediates the interaction with hMOF. Co-IP between full-length SIRT1 and fragments of hMOF showed that both fragments aa 157-458 and 231-458 bind



**Figure 5** The MYST domain of hMOF interacts directly with SIRT1 catalytic domain. **(A)** The 293T cells stably expressing FH-hMOF were transfected with myc-tagged SIRT1 (wild type), SIRT1 H363Y mutant or empty vectors. IP was performed using anti-FLAG agarose, and eluted with glycine. hMOF was immunoblotted with anti-HA, and co-IP was detected with anti-myc. Overexpression of SIRT1 was detected with anti-SIRT1 antibody to compare with endogenous expression. **(B)** SIRT1 fragments were expressed with myc tag, as described, and the schematic diagram showed their length and relative location compared with full-length SIRT1. IP was done as described above. Co-IP and overexpression of SIRT1 fragments were immunoblotted with anti-myc, and immunoprecipitated hMOF was detected by HA antibody. **(C)** Truncated hMOF, as represented in the schematic picture, was expressed with N terminal FLAG-HA tag. Myc-SIRT1 full-length was co-expressed with these truncated hMOF, and IP was performed as above. Co-IP and overexpression of myc-SIRT1 were immunoblotted with anti-myc, while immunoprecipitated hMOF proteins were detected by CBB stain. **(D)** MBP-SIRT1 was purified from *E. coli* and was subjected to pull-down by GST-hMOF FL, aa 1-130, 157-458 and 231-458. GST served as the pull-down control. Equal amounts of input MBP-SIRT1 were measured by CBB stain, while the pull-down product was detected using anti-SIRT1 antibody.

to SIRT1, while aa 1-130 does not (Figure 5C). To further investigate whether their interaction is direct, GST pull-down was performed between purified GST-hMOF of different length and purified MBP-SIRT1 (Figure 5D). Using western blot, we detected binding of SIRT1 in hMOF aa 157-458, 231-458 and full-length groups. This is in agreement with the co-IP experiment. Thus, hMOF interacts directly with SIRT1, and the catalytic domains of these two enzymes mediate the interaction.

*SIRT1 deacetylates hMOF*

Interaction between SIRT1 and hMOF brought up the possibility that SIRT1 deacetylates acetylated hMOF. *In vitro* deacetylation assay was performed, and self-acetylated hMOF was used as the substrate (Figure 6A). SIRT1 erased the lysine acetylation signals from hMOF only when NAD<sup>+</sup> was supplemented. Treatment of the FH-hMOF-stable cell line with nicotinamide (NAM), a specific sirtuins inhibitor, led to elevated acetylation signals. SIRT1 overexpression in FH-hMOF 293T cells reduced the acetylation level of immunoprecipitated FH-hMOF, and on the contrary, knockdown of SIRT1 increased hMOF acetylation level slightly (Figure 6B).

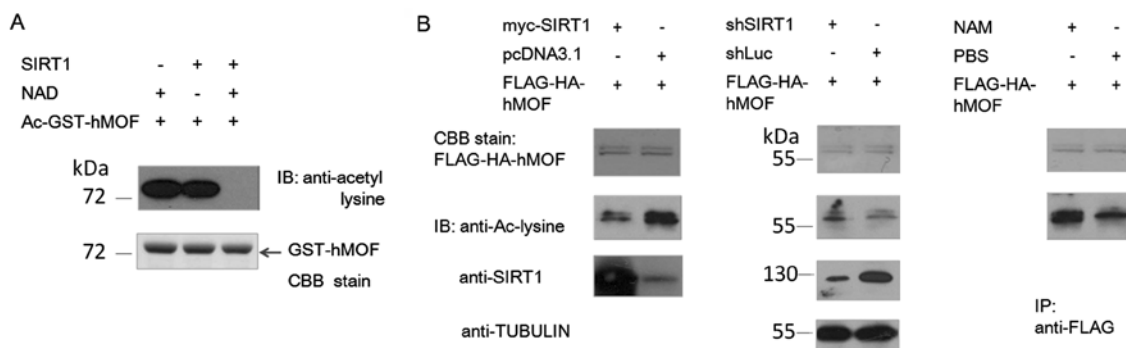
*Binding ability of hMOF to nucleosomes is regulated by its reversible acetylation*

Although hMOF manifests its acetyltransferase activity upon all four histones *in vitro*, immunoprecipitated hMOF catalyzes only H4K16 acetylation on nucleosomes. Depletion of hMOF in HeLa cells leads to specific decrease in H4K16Ac [16]. Therefore, regulation of binding of hMOF to nucleosomal substrates is critical

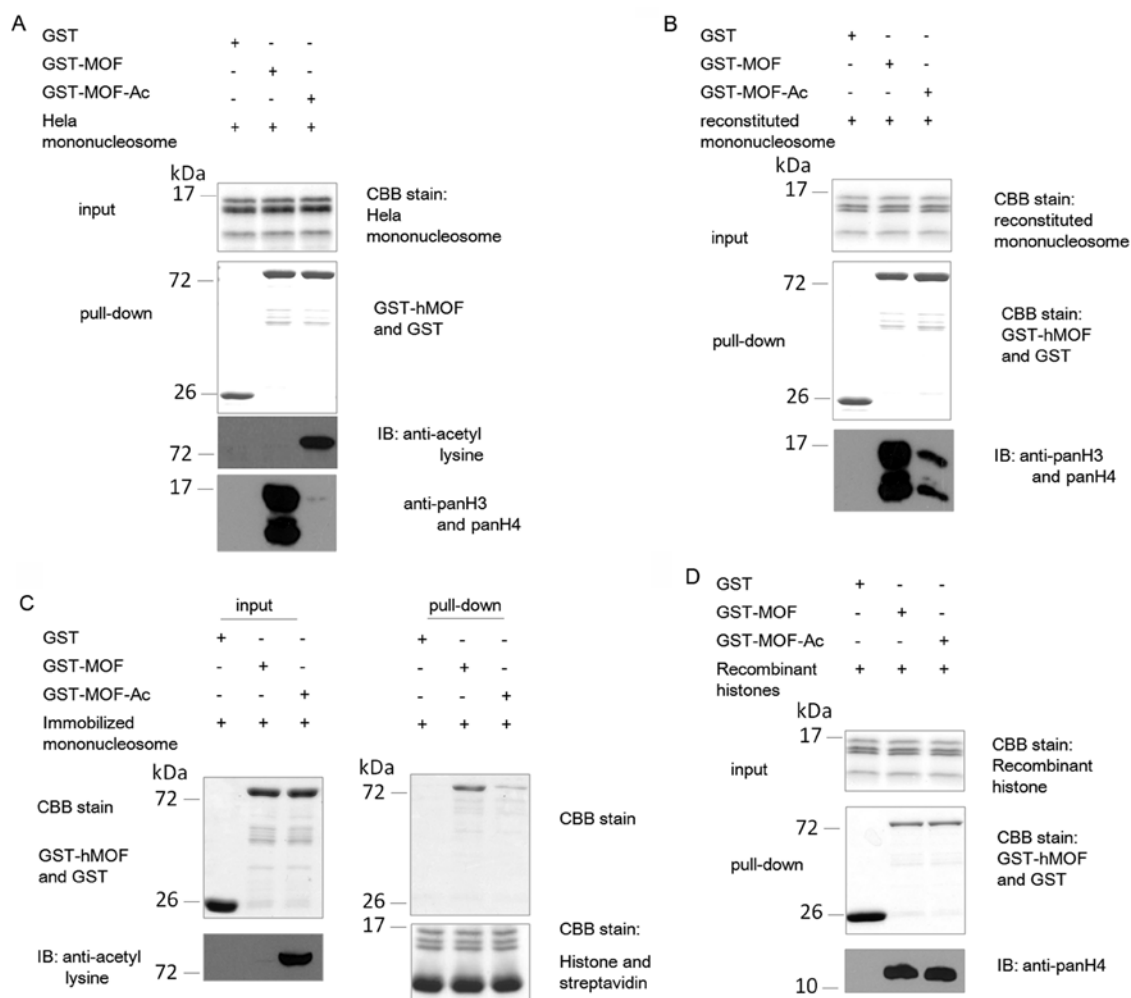
for its proper physiological functions.

As acetylation changes surface charges of proteins, we proposed that acetylation of hMOF may influence its conformation and its interaction with substrates. To test this, we measured the *in vitro* binding of HeLa nucleosomes, reconstituted mononucleosomes and recombinant histones by acetylated GST-hMOF in comparison with non-acetylated GST-hMOF and included GST as a negative control (Figure 7A, 7B and 7D). HeLa nucleosomes were measured for their length, and > 80% were mononucleosomes. Western blot shows that ac-GST-hMOF obviously binds less to HeLa nucleosomes or reconstituted mononucleosomes. In a reverse pull-down experiment, reconstituted mononucleosomes immobilized on streptavidin beads also bind less to ac-GST-hMOF (Figure 7C). However, western blot analysis showed that ac-GST-hMOF and GST-hMOF have similar binding ability to recombinant histone octamers (Figure 7D).

Theoretically, the isoelectric point of full-length hMOF is 8.48, while that of the MYST domain is 9.2 (calculated using ExPASy pI computation tool). Under physiological conditions, hMOF and its MYST domain are both positively charged. Positive charges on surface of hMOF (especially the catalytic domain) likely facilitate its binding to the negatively charged nucleosomes and hence are of great importance to hMOF function in chromatin modifications. Acetylation of the hMOF catalytic domain neutralizes its positive charges, which would disturb hMOF binding to nucleosomes. Also, acetyl groups in hMOF catalytic domain may lead to insufficient space in the catalytic pocket for substrate bind-



**Figure 6** Ac-hMOF is deacetylated by SIRT1 *in vitro* and *in vivo*. **(A)** GST-hMOF was first acetylated *in vitro*, and then coupled to GST beads followed by four washes to remove traces of acetyl CoA. GST beads were then incubated with purified SIRT1 in the presence of NAD<sup>+</sup> or not at 30 °C for 1 h, and then boiled in 2× sample buffer. Acetylation level was determined by anti-acetyl lysine antibody. **(B)** 293T cells stably expressing FLAG-HA-hMOF were treated with 2 mM nicotinamide for 16 h. 293T cells expressing FLAG-HA-hMOF were infected with SIRT1 RNAi and luciferase RNAi retroviruses separately. Myc-SIRT1 was transfected transiently into 293T cells expressing FLAG-HA-hMOF. FLAG-HA-hMOF was immunoprecipitated with anti-FLAG agarose and eluted with glycine for detection of acetylated lysines.



**Figure 7** Acetylation of hMOF reduces its affinity with nucleosomes. Equal amounts of acetylated or non-acetylated GST-hMOF were employed to pull-down HeLa nucleosomes (**A**), reconstituted nucleosomes (**B**) or recombinant histone octamers (**D**), and GST served as the negative control. Acetylation of GST-hMOF was shown by western blot in panel **A**. The pull-down product was detected with mixed anti-H3 and H4 antibodies. Biotin-labeled DNA was reconstituted into mononucleosomes and further coupled to streptavidin beads (**C**). After several washes, streptavidin beads were used to pull-down GST-hMOF (acetylated or not, shown by immunoblotting), and the pull-down product was detected by CBB stain. GST served as the negative control.

ing.

Nucleosomes and recombinant histone octamers differ in some ways, such as surface charges and histone accessibility, and this may explain the different performance of acetylated hMOF in binding nucleosomes and recombinant histone octamers compared with non-acetylated hMOF.

#### *Modulations of hMOF autoacetylation by SIRT1 regulate hMOF recruitment on the chromatin*

To understand how modulations of hMOF autoacetylation by SIRT1 affect hMOF affinity to the nucleosomes in cells, we performed chromatin retention assays to

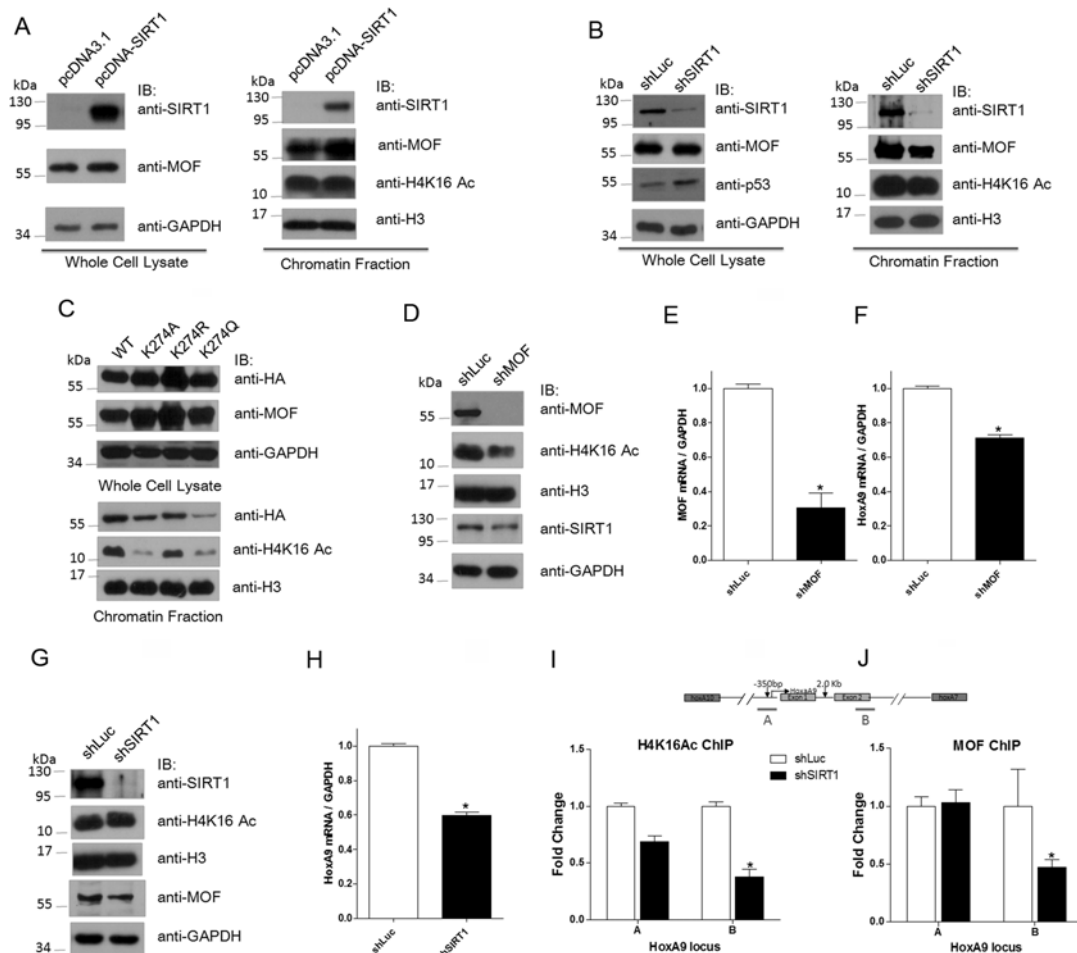
detect hMOF recruitment to the chromatin in response to changes of SIRT1 level. After overexpression of SIRT1 in HeLa cells, the cells were crosslinked with formaldehyde followed by fractionation into whole-cell soluble part and chromatin part. The results showed that upon SIRT1 overexpression, recruitment of hMOF to chromatin increases, while its expression levels in the whole-cell lysate fractions showed no apparent difference (Figure 8A). In SIRT1 knockdown HeLa cells, the recruitment of hMOF to chromatin decreased compared with the control group (Figure 8B), while the expression level of hMOF in the whole-cell lysate remained unchanged. It should be noted that the level of p53, which is suppressed by



SIRT1, apparently increased upon SIRT1 knockdown as expected, but the global H4K16Ac level did not increase. Two previous studies observed similar results for the global H4K16Ac level upon depletion of SIRT1 [20, 21], suggesting that deacetylation of H4K16Ac by SIRT1 is restricted to specific sites on the chromatin. Our results suggested a possibility that reduced hMOF recruitment

upon knockdown of SIRT1 may also be responsible for this observation.

To further characterize the effects of hMOF autoacetylation, we generated HeLa cell lines expressing FH-hMOF-wt, FH-hMOF-K274A, FH-hMOF-K274R and FH-hMOF-K274Q, and measured hMOF recruitment to the chromatin and levels of H4K16Ac in these cells.



**Figure 8** Modulations of hMOF autoacetylation by SIRT1 regulates hMOF recruitment to the chromatin. **(A)** HeLa cells were transfected with pcDNA3.1 or SIRT1 expression plasmids and 48 h later, the cells were harvested for chromatin retention assays. The cells were crosslinked as in the ChIP assay and lysed in lysis buffer for 20 min followed by centrifugation. The pellet was washed with lysis buffer for another 5 min and the two parts of supernatant were combined to obtain the whole-cell lysate fraction. The pellet was then sonicated in ChIP sonication buffer as the chromatin fraction. The hMOF recruitment to the chromatin was detected by western blot. **(B)** The SIRT1 knockdown HeLa cells and control cells were harvested as in **A**, and hMOF recruitment to the chromatin was detected by western blot. **(C)** HeLa cells were infected with retroviruses expressing wild-type hMOF, K274A, K274R or K274Q mutants, and 72 h after infection, the cells were harvested as in **A** and **B** for chromatin retention assay. **(D-E)** HeLa cells were infected with either shLuc or shMOF viruses, and knockdown efficiency of hMOF was detected by western blot using MOF antibody and H4K16Ac antibody. The mRNA level of *hMOF* was measured by qRT-PCR. **(F)** Transcription of *HoxA9* in hMOF knockdown cells or control cells was detected by qRT-PCR. **(G)** HeLa cells were infected with shLuc or shSIRT1 viruses, and the knockdown efficiency was confirmed by western blot. **(H)** Transcription of *HoxA9* was detected in SIRT1 knockdown cells and control cells by qRT-PCR. **(I, J)** MOF ChIP and H4K16Ac ChIP were performed in SIRT1 knockdown and control cells. Primers occupying the promoter region **(A)** and gene body region **(B)** of *HoxA9* locus were used for qPCR.

Compared with the wild-type hMOF, chromatin recruitment and modifying activities of K274R, which mimics the deacetylated status, did not change apparently. However, the K274Q form, which mimics the acetylated status, cannot be efficiently recruited to chromatin, and the H4K16Ac levels greatly decreased (Figure 8C). K274A mutation also caused reduction of H4K16Ac level, as the K to A mutation dramatically changed the polarity and structure of this residue, which appears critical for the enzyme activities of hMOF (Figure 8C). It should be noted that the recombinant GST-hMOF-K274R protein showed no activity on histone H4 in the *in vitro* assay (Figure 3E). This suggests that for the K274R mutant, the formation of MOF complex with other subunits is required for its activity. Therefore, we purified the mammalian hMOF-K274R complexes from the stable cell line by FLAG-IP and performed *in vitro* HAT assay. The results showed that the hMOF-K274R complexes can acetylate histone H4 *in vitro* (Supplementary information, Figure S3). To further characterize the effects of these hMOF mutants, the H4K16Ac levels of the stable cell lines were compared with that of the blank HeLa cells. The results showed that ectopic expression of hMOF leads to elevated H4K16Ac levels, and the different mutants have no dominant-negative effects (Supplementary information, Figure S4).

Next, we examined whether the modulations of hMOF autoacetylation by SIRT1 affect transcription of specific genes. To this end, we selected a previously characterized hMOF target gene *HoxA9* as the model. To confirm *HoxA9* as a target gene of hMOF, we generated hMOF knockdown HeLa cell lines, of which the RNAi efficiency was determined by western blot (Figure 8D) and Quantitative RT-PCR (qRT-PCR) (Figure 8E). Meanwhile, depletion of hMOF resulted in decreased global H4K16Ac level (Figure 8D). *HoxA9* transcription was detected by qRT-PCR and it was reduced as previously reported (Figure 8F). Then we detected *HoxA9* transcription in SIRT1 knockdown cells (Figure 8G). The qRT-PCR data showed that *HoxA9* mRNA level decreased upon depletion of SIRT1 (Figure 8H). ChIP experiment showed that the level of H4K16Ac at the promoter region of *HoxA9* locus decreased slightly, and it decreased more apparently at the gene body region (Figure 8I). Meanwhile, the ChIP experiments showed that recruitment of hMOF at the promoter region of *HoxA9* remained unchanged in SIRT1 knockdown cells, but apparently decreased at the gene body region (Figure 8J). This is consistent with the fact that the MOF-containing MSL complex binds to the gene body regions and potentially facilitates transcription elongation [22, 23].

## Discussion

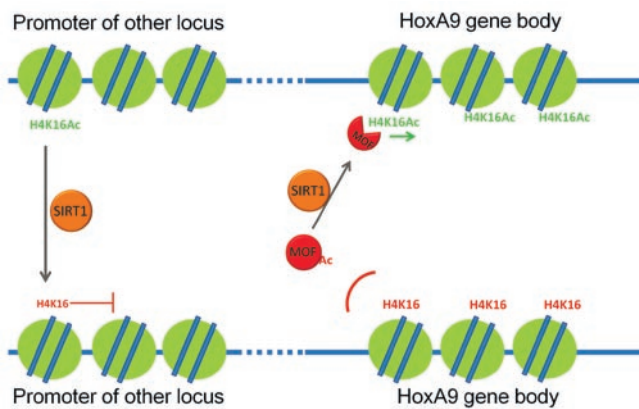
Recent studies demonstrated that hMOF participates in many biological processes, including chromatin decondensation, formation of chromatin boundaries and DNA repair [13, 24-26]. Effects of PTMs on hMOF, so far, have remained poorly studied. In this study, we have revealed that hMOF undergoes reversible autoacetylation, and the acetylation status regulates its binding activity toward nucleosomal substrates. It was also shown that SIRT1 is responsible for deacetylating acetylated hMOF, and the modulations of hMOF autoacetylation by SIRT1 contribute to regulation of hMOF recruitment and modifying activities on the chromatin. Thus, our study provides novel insight into regulatory roles of hMOF acetylation, which is a new substrate of SIRT1.

Accumulating evidence shows that posttranslational automodification of chromatin-modifying factors represents a general regulatory mechanism. PCAF, p300 and TIP60 are autoacetylated, G9a can be automethylated [27] and PARP-1 can be auto-ADP-ribosylated [28]. Here, we found that hMOF is autoacetylated both *in vitro* and *in vivo*. Although a previous study using the high-throughput mass spectrometry method identified two acetylated lysines on hMOF, our data indicate that they are not required for autoacetylation. As autoacetylation of *Drosophila* MOF was noticed by Akhtar and coworkers [12, 13], autoacetylation of MOF may be conserved among species. This is further supported by our data that the conserved MYST domain is heavily acetylated, while the N terminal region showed no acetylation. Furthermore, the multiple sequence alignment of MYST domain from five species clearly showed that K238 is not shared by hMOF and *Drosophila* MOF. Therefore, K113 and K238 receive acetyl groups from other KATs and their functions may be mammal specific. Sequence alignment also showed that there are multiple lysines within the MYST domain that are conserved in no less than four species, thus autoacetylation may occur on these lysines. By using a serial point-mutation strategy, we determined that the autoacetylation of hMOF mainly occurs on K274. Mutation of K274 to Q mimics the acetylated form, which impairs the efficient recruitment of hMOF to chromatin and its modifying activities on chromosomal H4K16. Interestingly, in addition to MOF proteins of different species, the K274 site is also conserved in Tip60, of which the K327 site (equivalent to K274 of hMOF) is among several lysines undergoing acetylation [10]. These data indicate that autoacetylation of MOF may play conserved regulatory roles for this acetyltransferase.

SIRT1 is a class III histone deacetylase that is able to deacetylate H3K9Ac and H4K16Ac and plays a role in

chromatin regulation [29]. Besides the histone substrates and many other nuclear factors, SIRT1 also deacetylates chromatin-modifying enzymes, including PCAF, p300, TIP60 and SUV39H1 [10, 30-32]. In this study, we identified hMOF as another substrate of SIRT1. It is of note that hMOF and SIRT1 share common substrates like H4K16Ac and p53 [19], suggesting their joint participations in different cellular processes. A previous study showed that depletion of SIRT1 in cancer cells reactivated some silenced tumor suppressor genes accompanied by increased H4K16 and H3K9 acetylation at their promoter regions [33]. Recruitment of SIRT1 to promoters of target genes and its deacetylation activities toward H4K16Ac and H3K9Ac contribute one means of transcriptional regulation (Figure 9). In this study, we show that modulations of hMOF autoacetylation by SIRT1 regulate hMOF recruitment and modifying activities on chromatin. This adds another means of regulation at the global level and illustrates the cooperative functions between the two nuclear enzymes (Figure 9).

So far, hMOF is known to be contained in two different complexes, MOF-MSL and MOF-MSL1v1 [15, 34]. Both the complexes have acetyltransferase activity toward nucleosomal H4, but free hMOF barely showed any activity toward HeLa nucleosomes [35, 36], even though MOF has been shown to be able to bind to the chroma-



**Figure 9** Recruitment of hMOF to the chromatin is regulated by the switch of hMOF status. SIRT1 has been shown to be recruited to specific gene promoters and directly deacetylate H4K16Ac, which then suppresses gene transcription. Here, we found that autoacetylation prevents hMOF from efficient binding to nucleosomes, probably by neutralizing positive charges on hMOF surface. The class III histone deacetylase SIRT1 can decrease hMOF acetylation and enhance its recruitment to the chromatin and further affect gene transcription by modifying H4K16 at the gene body regions. Modulations of hMOF autoacetylation by SIRT1 provide another means for concerted regulation of gene expression.

tin [37]. Our results showed that free hMOF acetylates itself in the MYST domain, and autoacetylation greatly decreased its binding to reconstituted nucleosomes or native HeLa nucleosomes. This provides at least one explanation for little activity of free hMOF toward nucleosomal H4, as the nucleosomal substrate cannot efficiently bind to the catalytic site of autoacetylated hMOF. When hMOF is complexed with other factors, however, the HAT activity can be regulated in different ways. First, certain deacetylases (for example SIRT1) may remove acetylation of hMOF and increase its interaction with the nucleosomal substrate (Figure 9). Second, the well-studied proteins in different hMOF complexes, like MSL1/2/3 and MSL1v1 [35, 36], may interfere with the autoacetylation of hMOF so as to facilitate targeting of hMOF to specific substrates, or meanwhile, stabilize its interaction with the substrates.

The mutation analyses showed that K274Q and K274A mutations greatly reduced hMOF recruitment and global H4K16Ac level, while the K274R mutant behaves similarly to the wild type. These data suggest that the structure and charge of aa 274 is important for assembly of the MOF complex, and autoacetylation may impede the process. A recent study focusing on the two MOF complexes showed that they have different substrate preferences, and proposed a model that the MSL1v1 complex is recruited to the promoters of target genes through interactions with MLL and p53, while the recruitment of the MSL complex by H3K36me3 is important for acetylating H4K16 downstream of the genes [36]. Another study in *Drosophila* also showed that the NSL complex (equivalent to MSL1v1 complex) binds to the promoters of target genes to facilitate transcription initiation, while the MSL complex is important for elongation [38]. Our ChIP analyses showed that upon SIRT1 knockdown, decreased hMOF recruitment and H4K16Ac level took place at the gene body region, indicating that the autoacetylation mainly affects the MSL complex.

During the submission of our manuscript, another study also identified K274 residue as being acetylated [39]. Their data showed that the K274A MOF was less stable in a thermal denaturation assay and not active in acetylating histone H4 N terminal peptides. Their data on the K274A mutant support our study, although they did not explore further the functional relevance of K274 acetylation because of their main focus on the effects of MSL1 mutations on the complex structure.

In addition to acetylation, other modifications of hMOF, for example, phosphorylation, also have potential regulatory roles, and may even crosstalk with acetylation. Therefore, it is important to explore further the post-translational modifications of MOF, which will lead to a



better understanding of this important acetyltransferase.

## Materials and Methods

### DNA constructs

Full-length hMOF cloned from HeLa cDNA by RT-PCR was ligated into pGEX-KG in fusion with GST tag for prokaryotic expression and into MSCV-puro in fusion with FLAG-HA tag for eukaryotic expression. The hMOF-derived fragments, including hMOF aa 1-130, 157-458 and 231-458 [16], were PCR amplified and inserted into the same vectors. Single or multiple-point mutations of GST-hMOF, as well as  $\Delta$ 314-333-aa K274A, K274R and K274Q mutants of FLAG-HA-hMOF were generated using site-directed mutagenesis facilitated by *DpnI* (NEB) selection. SIRT1 expression vector pcDNA-SIRT1 was a gift from Prof Ishikawa [40], pcDNA-myc-SIRT1 was from Professor Shengqi Wang [41] and myc-tagged SIRT1 fragments have been described earlier [42]. MBP-SIRT1 was expressed by cloning SIRT1 cDNA into pMAL-p4X vector. Oligos of shRNAs against hMOF, SIRT1 and luciferase were cloned into retroviral vector pSIREN-RetroQ at the *Bam*HI / *Eco*RI site, and their targeting sequences are 5'-GCAA-GATCACTCGCAACCAAA-3', 5'-GATGAAGTTGACCTC-CTCA-3' and 5'-CGCCTGAAGTCTCTGATTA-3', respectively.

### Generation of stable cell line

The 293T cells or HeLa cells were infected with retroviruses expressing FLAG-HA-tagged hMOF or mutant forms, and selected with puromycin (2  $\mu$ g/ml) for 2 weeks.

### Immunoprecipitation and western blot

The 293T cells were transfected with combinations of different myc-tagged SIRT1 and FLAG-HA-tagged hMOF constructs or control vectors, and were lysed in IP buffer (20 mM Tris-Cl (pH 8.0), 150 mM NaCl and 1% Triton X-100), supplemented with protease inhibitor cocktail (Sigma) for co-IP and additional 2 mM NAM and 10  $\mu$ M sodium butyrate for acetyl lysine detection. After continuous gentle agitation at 4 °C for 30 min, the lysate was cleared by centrifugation, and anti-FLAG-M2 agarose was added to capture FLAG-tagged proteins. Immunoprecipitated proteins were eluted by 0.1 M glycine (pH 3.0) and detected by western blot using anti-myc antibody (Santa Cruz, sc-40) for co-IP and anti-acetyl lysine antibody (Cell Signaling Technology, 9441) for detection of acetylation.

### In vitro acetylation and deacetylation assays

GST-tagged full-length hMOF and three hMOF fragments were purified from *Escherichia coli* according to the GE purification protocol. For each reaction, 5  $\mu$ g of substrate protein was incubated with 3  $\mu$ g of full-length hMOF in reaction buffer with or without acetyl-CoA (20  $\mu$ M) at 30 °C for 1 h, as described previously [43]. GST-hMOF was coupled to glutathione-sepharose 4B beads after *in vitro* autoacetylation, and the beads were washed four times with PBS and once with deacetylation buffer to remove traces of acetyl-CoA before subsequent deacetylation assays. The immobilized GST-hMOF was then incubated with 1  $\mu$ g purified human His-SIRT1 (Sigma, S8446) with or without NAD<sup>+</sup> (Sigma, N1663) in the deacetylation buffer (Sigma, A6480) at 30 °C for 1 h with occasional inversions. The products of the reactions were analyzed by western blot using anti-acetyl lysine antibody (Cell

Signaling Technology, 9441).

### GST pull-down assay

GST-hMOF proteins and MBP-SIRT1 were purified from *E. coli*. Different GST-hMOF proteins (full-length or fragments) and GST were coupled to GST beads, and incubated with MBP-SIRT1 in IP buffer with agitation at 30 °C for 3 h. GST beads were then washed four times with high-salt buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1% Triton X-100), and boiled with 2 $\times$  sample buffer for western blot using anti-SIRT1 antibody (Santa Cruz, sc-15404).

### Mononucleosome-binding assay

HeLa mononucleosomes were prepared as described [44]. For reconstituted mononucleosomes, recombinant HeLa histones and 194-bp satellite DNA (5' biotin labeled) were employed, and reconstitution of nucleosome core particles (NCPs) followed the salt jump-down protocol [45]. The reconstituted NCPs were further captured by streptavidin agarose. Equal amounts of Ac-GST-hMOF or GST-hMOF coupled to GST-beads were used for binding of HeLa mononucleosomes or reconstituted NCPs. Conversely, reconstituted NCPs that were immobilized on streptavidin agarose were used to bind products from parallel GST-hMOF acetylation assays in the presence of acetyl-CoA or not. All nucleosome-binding experiments were performed as described [37].

### Immunofluorescence

The 293T cells expressing FLAG-HA-tagged hMOF were fixed in 4% paraformaldehyde at room temperature for 10 min. Post-fixative permeabilization was carried out in PBS containing 0.1% Triton X-100 for 10 min. Expression of hMOF was stained with mouse anti-FLAG-M2 (Sigma, F3165) in a dilution ratio of 1:2 000, and SIRT1 was stained with rabbit anti-SIRT1 (Santa Cruz, sc-15404) in a dilution ratio of 1:200, followed by anti-rabbit IgG/TRITC (Zhongshan Goldenbridge Biotechnology) and anti-mouse IgG/FITC (Zhongshan Goldenbridge Biotechnology) staining. The nuclei were stained with DAPI.

### Quantitative RT-PCR

Total RNA from HeLa cells was isolated by using Trizol (Invitrogen) reagent and reverse transcription was carried out per the manufacturer's manual (New England Biolabs). Real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa) on RotorGene Q cycler (Qiagen). The primers used are listed in Supplementary information, Table S1.

### Chromatin immunoprecipitation assay

ChIP assays were performed as described by the Upstate protocol with some modifications. HeLa cells were crosslinked by adding formaldehyde to a final concentration of 1% at room temperature for 10 min. After washing four times with 20 ml PBS in 50 ml conical tubes, cells were scraped and swelled in hypotonic swelling buffer (25 mM HEPES (pH 7.8), 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.1% NP-40, protease inhibitor cocktail from Sigma) and incubated on ice for 10 min. Following centrifugation at 2 000 rpm for 5 min, the nuclei were lysed in SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris (pH 8.1)) and sonicated with Branson 150 sonicator. Antibodies against H4K16Ac (Millipore, 07-329) and MOF (Bethyl, A300-992A) were used for IP. PCR data



were analyzed by using the  $2^{-\Delta\Delta Ct}$  method and normalized to input samples. The results were expressed as fold changes over control groups. The primers used are listed in Supplementary information, Table S1.

#### Chromatin retention assay

HeLa cells were crosslinked with formaldehyde as in the ChIP assay, and glycine was added to the final concentration of 0.125 M to quench the crosslinking. After three washes with cold PBS, cells were extracted in the lysis buffer (20 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.2 mM EDTA, 10% Glycerol, 1% Triton X-100, protease inhibitor cocktail from Sigma) by end-to-end rotation for 30 min. After centrifugation at 3 000 rpm. and transfer of the supernatant, the pellet was again resuspended in the same lysis buffer and kept on ice for 5 min. After centrifugation, the two parts of supernatant were combined as the whole-cell lysate. The nuclei were then resuspended in ChIP-SDS buffer and sonicated as in the ChIP assay. The soluble chromatin fraction was used to detect retention of hMOF by western blot.

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(Supplementary information is linked to the online version of the paper on the *Cell Research* website.)