

AOF1 is a histone H3K4 demethylase possessing demethylase activity-independent repression function

Ze Yang¹, Jun Jiang², David M Stewart³, Shankang Qi¹, Kenichi Yamane⁴, Jiwen Li¹, Yi Zhang⁴, Jiemin Wong¹

¹The Institute of Biomedical Sciences and School of Life Sciences, East China Normal University, Shanghai 200241, China; ²Department of Urology, Daping Hospital/Institute of Surgical Research, Third Military Medical University, Chongqing 400042, China; ³Department of Genetics, University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA; ⁴Howard Hughes Medical Institute and University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

LSD1 (KDM1 under the new nomenclature) was the first identified lysine-specific histone demethylase belonging to the flavin-dependent amine oxidase family. Here, we report that AOF1 (KDM1B under the new nomenclature), a mammalian protein related to LSD1, also possesses histone demethylase activity with specificity for H3K4me1 and H3K4me2. Like LSD1, the highly conserved SWIRM domain is required for its enzymatic activity. However, AOF1 differs from LSD1 in several aspects. First, AOF1 does not appear to form stable protein complexes containing histone deacetylases. Second, AOF1 is found to localize to chromosomes during the mitotic phase of the cell cycle, whereas LSD1 does not. Third, AOF1 represses transcription when tethered to DNA and this repression activity is independent of its demethylase activity. Structural and functional analyses identified its unique N-terminal Zf-CW domain as essential for the demethylase activity-independent repression function. Collectively, our study identifies AOF1 as the second histone demethylase in the family of flavin-dependent amine oxidases and reveals a demethylase-independent repression function of AOF1.

Keywords: AOF1, histone H3K4 demethylase, chromatin, repression, Zf-CW

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Introduction

Histone methylation is a key determinant of chromatin function, and has been shown to play crucial roles in heterochromatin formation, X-inactivation, transcription and DNA repair [1]. Methylation of specific lysine (K) residues on histones has been linked to specific functions such as transcriptional repression (H3K9 methylation) or activation (H3K4 methylation) [2, 3]. Histone methylation was considered to be irreversible until the identification of the first lysine-specific histone demethylase LSD1 [4].

LSD1 is a member of the amine oxidase family of proteins and has been shown to demethylate mono- and di-methyl K4 of histone H3 (H3K4me1 and H3K4me2) as well as that of non-histone proteins through a FAD-

dependent amine oxidation reaction [4, 5]. Biochemical studies have identified LSD1 within a number of multiprotein complexes containing, typically, HDAC1/2 and CoREST [6–8]. Both LSD1 enzymatic activity and substrate specificity are modulated by proteins that it interacts with. For example, CoREST has been shown to not only potentiate LSD1 enzymatic activity but is also required for LSD1 to demethylate nucleosomal substrates [6]. Interaction with liganded androgen receptor has been shown to switch LSD1 enzymatic activity toward repressive H3K9 methylation [9]. Functional studies have linked LSD1 to diverse biological processes, including transcription, DNA repair and cell cycle, possibly via its demethylase activity toward both histone and non-histone proteins such as p53 and DNMT1 [10–12]. More recently, a new family of proteins containing the JmjC domain was found to possess histone demethylase activity [13]. This family of demethylases demethylates mono-, di- and tri-methylated lysines through a hydroxylation reaction that requires Fe(II) and α -ketoglutarate as cofactors. Due to the intrinsic difference in the underlying

Correspondence: Jiemin Wong

Tel: +021-54345013; Fax: +021-54344922

E-mail: jmweng@bio.ecnu.edu.cn

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ing mechanism of demethylation (oxidation vs hydroxylation), LSD1 can theoretically demethylate only mono- and di-methylated lysine residues [14], whereas the JmjC family of demethylases can also act on tri-methylated lysines [15, 16]. So far a large number of histone demethylases have been identified from the JmjC domain-containing family of proteins [17-20].

Among the amine oxidase proteins, AOF1 is highly related to LSD1 in both sequence and structure [4]. Like LSD1, AOF1 also contains a SWIRM domain and a C-terminal oxidase domain (see Figure 1). AOF1 differs from LSD1 in containing a unique N-terminal Zf-CW domain. The Zf-CW domain is a type of four-cysteine zinc-finger motif named for its conserved cysteine and

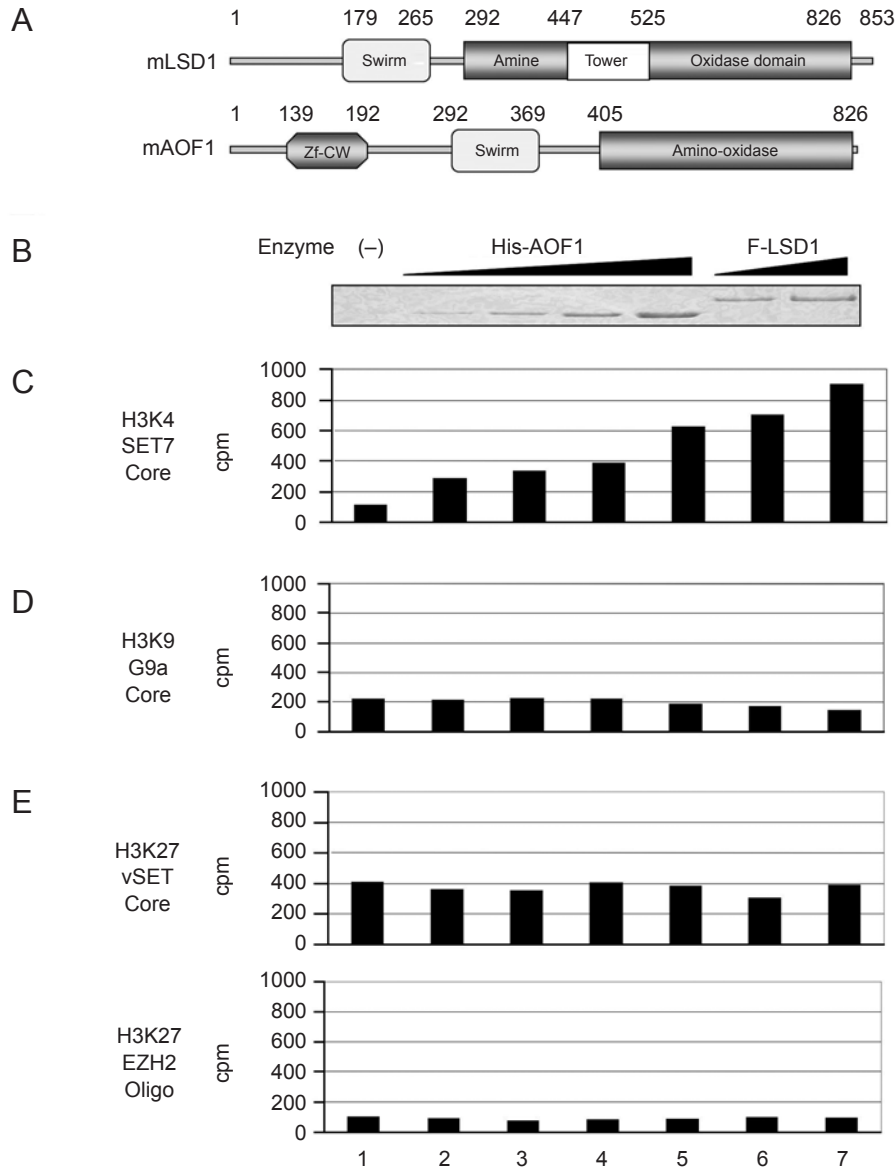


Figure 1 Recombinant AOF1 acts as an H3K4 demethylase *in vitro*. **(A)** Diagram illustrating the similarity and difference in domain organization of AOF1 and LSD1. Note that AOF1 contains a unique Zf-CW (CW-type Zinc-Finger) domain and lacks a Tower domain that has been shown to mediate the interaction of LSD1 with the corepressor CoREST. **(B)** Recombinant AOF1 and LSD1 were expressed and purified from SF9 insect cells via His- and Flag-tag, respectively. The increasing amounts of AOF1 and LSD1 used in demethylase activity assays were revealed by Coomassie blue staining. Histone demethylase assays were performed using core histones substrates containing ^3H -methyl-H3K4 **(C)**, H3K9 **(D)** or H3K27 **(E)**, respectively, and an increasing amount of AOF1 or LSD1 proteins. The demethylase activity was measured based on the release of radioactivities from labeled core histone or oligo-nucleosome substrates, as indicated.

tryptophan residues [21]. Here, we report that mouse AOF1 also possesses a histone demethylase activity specific for H3K4me1 and H3K4me2. We show that AOF1 differs from LSD1 in association with HDAC and mitotic chromosomes, and may regulate transcription in a demethylase activity-independent manner through its unique Zf-CW domain.

Results

AOF1 exhibits an H3K4-specific demethylase activity in vitro

Given the sequence and structural similarity between AOF1 and LSD1 (Figure 1A), we were interested in determining if AOF1 is also a histone demethylase. Toward this end, we expressed both human LSD1 and mouse AOF1 from baculovirus-infected Sf9 insect cells. His-tagged AOF1 was purified to near homogeneity via nickel-based affinity purification, whereas LSD1 was purified via Flag-tag using anti-Flag-M2 agarose beads (Figure 1B). For demethylation assays, we made use of the site-specificity of histone methyltransferases SET7, G9a, vSET and EZH2 and ³H-SAM (S-adenosyl-[methyl-³H]-L-methionine) to generate radiolabeled core histone substrates, methylated specifically at the K4, K9 or K27 site of histone H3, as described previously [13]. When these substrates were subjected to demethylation assay using increasing amounts of recombinant AOF1 or LSD1, we found that, like LSD1, AOF1 also exhibited an H3K4-specific demethylase activity and displayed no activity toward K9- or K27-methylated H3 substrates. In multiple experiments, we found that after normalizing the protein concentrations, the demethylase activity of AOF1 is somewhat lower than that of LSD1. Given that SET7 generated H3K4me1 and H3K4me2, this result indicates that AOF1 has a histone demethylase activity toward H3K4me1 and H3K4me2.

AOF1 exhibits an H3K4-specific demethylase activity in cells

To further substantiate the H3K4 demethylase activity of AOF1, we expressed mouse AOF1 as Flag-tagged proteins in HeLa cells and examined the demethylase activity by measuring the levels of H3K4me1 and H3K4me2 by immunofluorescence. In agreement with the *in vitro* demethylation assay results, the representative results in Figure 2B show that the cells expressing Flag-AOF1 had reduced levels of H3K4me1 and H3K4me2 in comparison to neighboring cells that did not express Flag-AOF1. Also consistent with the *in vitro* demethylation data, no demethylase activity toward H3K9 and H3K27 was observed for AOF1 (Supplementary information, Figure S1). Consistent with the chemistry of a FAD-dependent amine oxidation reaction, no H3K4me3 demethylase

activity was detected for AOF1 (Supplementary information, Figure S2). It is noteworthy that in this assay, AOF1 in general exhibited a better H3K4me2 demethylase activity than LSD1. While a clear reduction of H3K4me2 could be observed for transfected cells expressing Flag-LSD1 (Figure 2B), we often did not observe a significant reduction for H3K4me1 (data not shown). The strong demethylase activity observed for ectopically expressed AOF1 in HeLa cells is in contrary to the *in vitro* demethylase assay data in Figure 1, in which LSD1 exhibited a higher activity than AOF1. Although this discrepancy is currently not understood, one possibility is that the demethylase activity of AOF1 was enhanced by one or more proteins in HeLa cells. The purified recombinant AOF1 might lack this protein(s) and therefore its demethylase activity was compromised.

Previous studies on LSD1 indicate that K661 in the amine oxidase domain is required for its demethylase activity, presumably because it is required for binding of cofactor FAD [22, 23]. Sequence comparison with LSD1 revealed a conserved K667 in AOF1. We thus converted this residue to alanine (referred as AOF1m) and tested the effect on the enzymatic activity. We found that this mutation impaired the AOF1 demethylase activity, implying that binding of FAD is indeed essential for AOF1 demethylase activity. Taken together, we conclude that like LSD1, AOF1 also has a FAD-dependent demethylase activity toward H3K4me1 and H3K4me2.

Both Zf-CW and SWIRM domains are required for AOF1 demethylase activity

As shown in Figure 1A, AOF1 contains a unique Zf-CW domain and a SWIRM domain that is also present in LSD1. The SWIRM domain is present in several proteins involved in chromatin remodeling [24, 25]. The SWIRM domain has been shown to be required for LSD1 demethylase activity and forms a compact structure with the amine oxidase domain [22, 26]. We thus attempted to test if the SWIRM domain in AOF1 is also required for AOF1 demethylase activity. We constructed the AOF1Δ381 mutant with deletion of both SWIRM and Zf-CW domains and the AOF1Δ271 mutant with the deletion of Zf-CW domain only. These mutants were transfected into HeLa cells and tested for demethylase activity toward H3K4me1 or H3K4me2. The results in Figure 2B show that the AOF1Δ381 mutant exhibits no demethylase activity toward H3K4me1 and H3K4me2. Interestingly, we found that the AOF1Δ271 mutant with an intact SWIRM and amine oxidase domain also has no demethylase activity. One potential explanation for this result is the altered subcellular localization of AOF1Δ271 mutant. We observed that in ~80% Flag-AOF1Δ271-

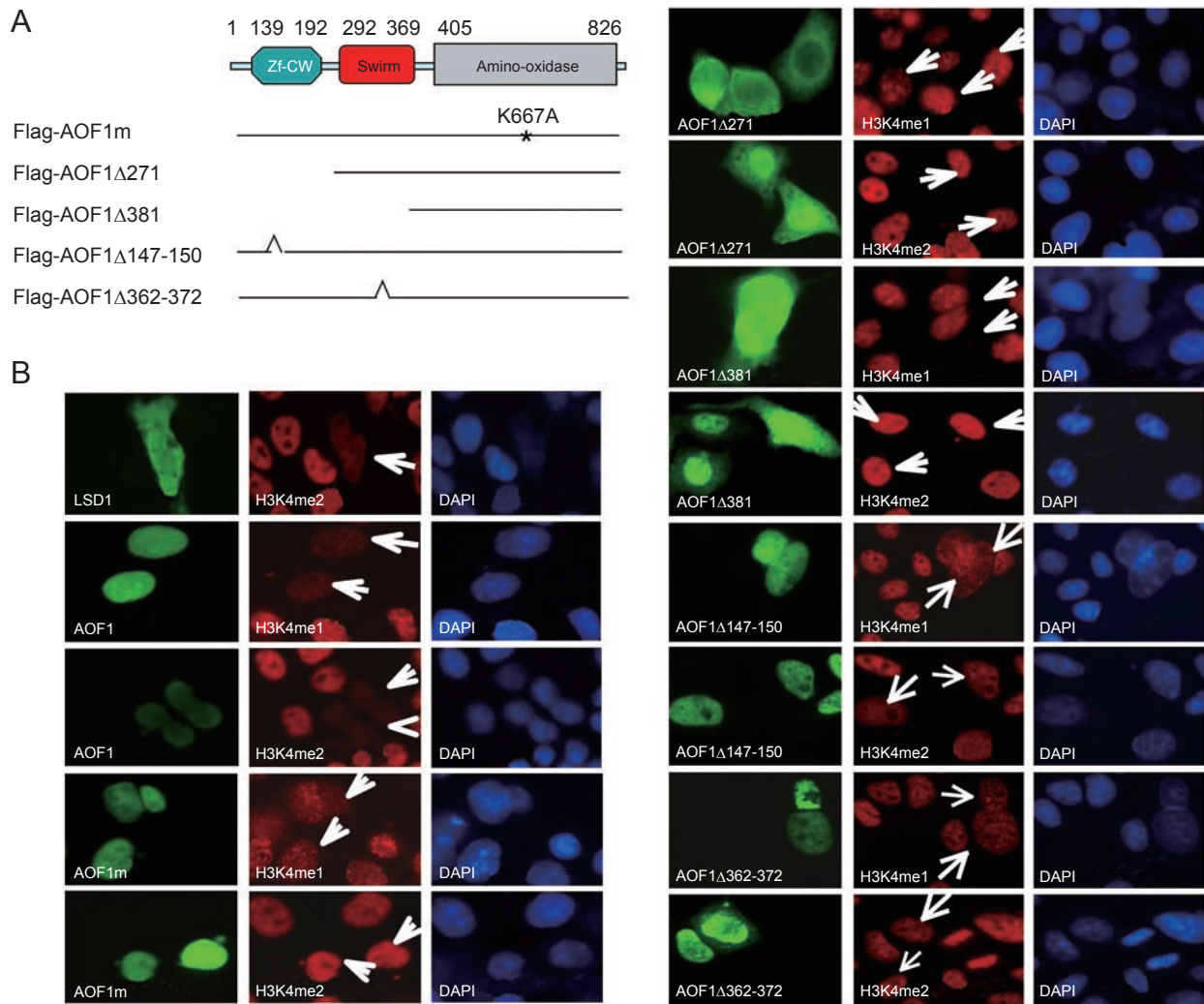


Figure 2 AOF1 acts as a mono- and di-H3K4 demethylase in cells. **(A)** Diagram showing point and deletion mutants of AOF1. **(B)** Flag-tagged AOF1 and its mutants were transfected into HeLa cells and the demethylase activity was detected by immunofluorescence using various methylated H3-specific antibodies. LSD1 served as a positive control for H3K4me2 demethylase activity. Arrows mark the cells in which the proteins of interest were expressed. Note that reduced levels of H3K4me1 and H3K4me2 were observed in cells expressing the wild-type Flag-AOF1 but not in cells expressing AOF1 K667A mutant and deletion mutants.

expressing cells Flag-AOF1Δ271 was mainly present in the cytoplasm. However, there were ~10-20% cells in which a nuclear presence of Flag-AOF1Δ271 was observed (Figure 2B), yet demethylation of H3K4me1 and H3K4me2 was not observed within these cells.

To further test the requirement of Zf-CW and SWIRM domain for AOF1 demethylase activity, we constructed two additional mutants, AOF1Δ147-150 and AOF1Δ372-382. The AOF1Δ147-150 mutant contains a deletion of four conserved amino acids within the Zf-CW domain, whereas the AOF1Δ372-382 mutant has a deletion of 11 amino acids within the conserved SWIRM domain. When tested for demethylase activity by immunofluo-

rescence (Figure 2B), we found that both mutants were inactive for demethylation of H3K4me1 and H3K4me2. These results indicate that both the Zf-CW and SWIRM domains are required for optimal H3K4 demethylase activity of AOF1.

AOF1 associates with mitotic chromosomes

From the above immunofluorescent experiments, we observed that within mitotic cells, the majority of AOF1 proteins localized to mitotic chromosomes (Figure 3A). This chromosome localization in mitotic cells is independent of the AOF1 demethylase activity, since the AOF1m mutant defective of the enzymatic activity was

also chromosome associated (Figure 3B). This result prompted us to test if LSD1 also localizes to the mitotic chromosomes. As shown in Figure 3C, the chromosome association in mitotic cells was not observed for LSD1. Interestingly, we found that the AOF1 chromosome association required its unique N-terminal Zf-CW domain, as the chromosome association was not observed for the AOF1 Δ 271 mutant (Figure 3D). This result indicates that LSD1 and AOF1 differ in the association with mitotic chromosomes and suggests that the unique Zf-CW domain is likely responsible for targeting AOF1 to mitotic chromosomes.

AOF1 does not appear to form stable protein complex with histone deacetylases

LSD1 has been identified in several multiprotein complexes containing HDAC1/2 and HDAC3 [6-8]. To investigate if AOF1 also similarly forms protein complexes with histone deacetylases, we next carried out co-immunoprecipitation assays using HeLa nuclear extracts (Figure 4A). Consistent with previous reports, a substantial amount of HDAC1/2 was found to co-immunoprecipitate with LSD1 by western blot analysis using an antibody that can immunoprecipitate HDAC1, but detect both HDAC1 and HDAC2 (the band marked by an arrow) in western blot. Similarly, LSD1 was found to co-immunoprecipitate with HDAC1 and HDAC3. In contrast, HDAC1/2 was not detected in the AOF1-immunoprecipitated fraction and AOF1 was not detected in the fraction immunoprecipitated with either HDAC1 or HDAC3 antibody, indicating that in HeLa nuclear extracts, AOF1 was not associated with HDAC1/2 and HDAC3.

To further examine if AOF1 associates with HDAC1/2, we generated a HeLa cell line stably expressing Flag-AOF1. The Flag-AOF1 was isolated from the whole cellular extracts using anti-Flag-M2 agarose beads. As a control, LSD1 was also immunoprecipitated using LSD1 antibody. The subsequent western analysis again revealed co-immunoprecipitation of HDAC1/2 with LSD1, but not with Flag-AOF1, thus confirming that AOF1 is not physically associated with HDAC1/2 (Figure 4B).

While the above results exclude the association of AOF1 with HDAC1/2, it remains a possibility that AOF1 associates with other HDACs. To test this, we subjected the immunopurified Flag-AOF1 and LSD1, as described above, to a fluorometric *in vitro* HDAC activity assay [27]. TSA, an HDAC inhibitor, was included to determine if the associated HDAC activity was derived from TSA-sensitive class I and class II HDACs. A representative result shown in Figure 4C demonstrates that, while a substantial TSA-sensitive HDAC activity is associated

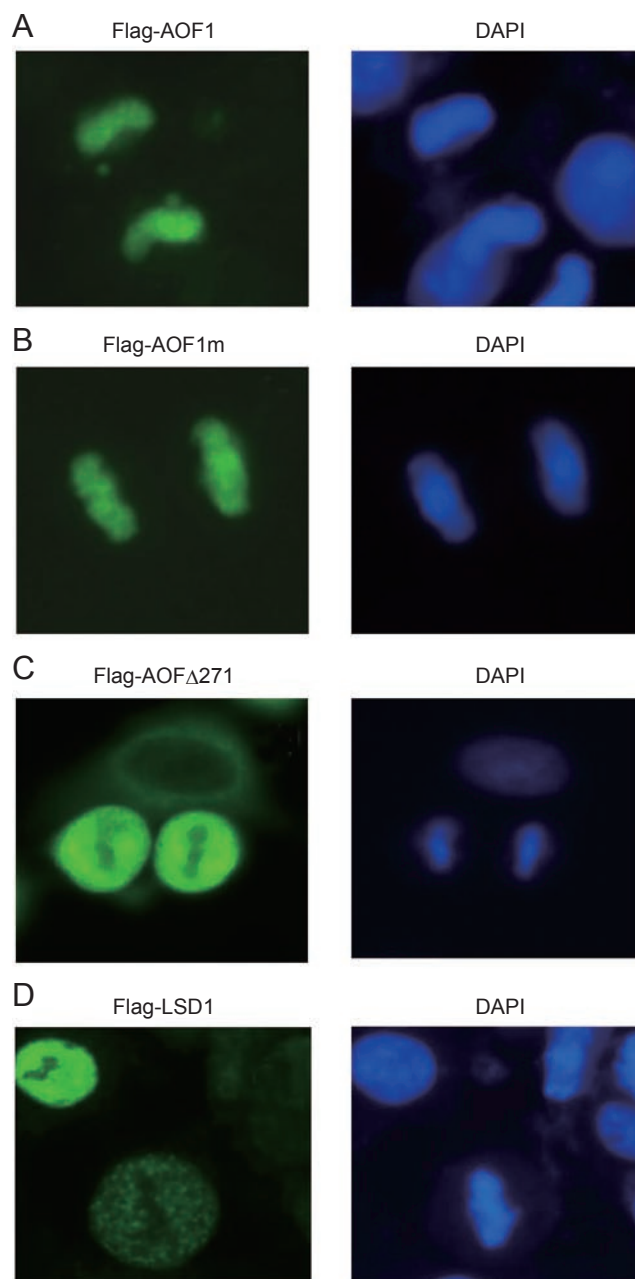


Figure 3 AOF1 associates with mitotic chromosomes during the M phase of cell cycle. Flag-AOF1 (A), Flag-AOF1m (B), Flag-AOF1 Δ 271 (C) and Flag-LSD1 (D) were transfected into HeLa cells and a representative pattern of subcellular localization in the M phase cells was shown. Note that both Flag-AOF1 and Flag-AOF1m colocalized with DAPI-stained mitotic chromosomes, whereas Flag-AOF1 Δ 271 and Flag-LSD1 were absent in the mitotic chromosomes.

with LSD1, the HDAC activity associated with AOF1 was at the background level. Taken together, our results indicate that AOF1 is unlikely to form stable protein

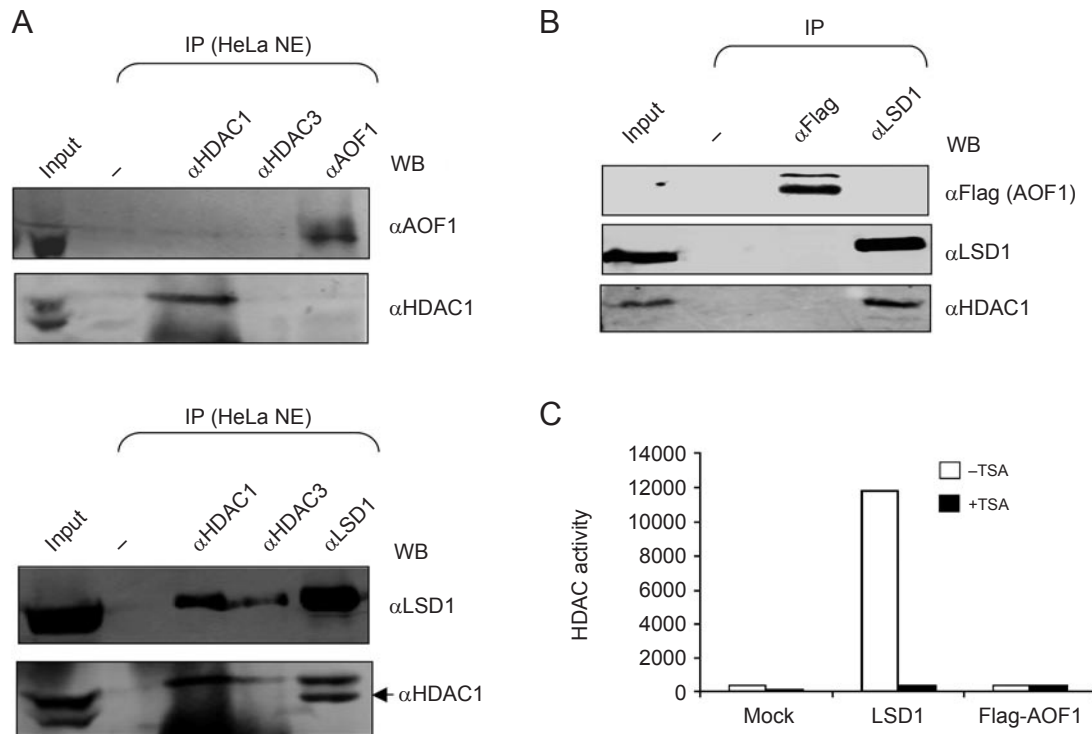


Figure 4 AOF1 differs from LSD1 in association with histone deacetylases. **(A)** HeLa nuclear extracts were immunoprecipitated with antibodies against HDAC1, HDAC3, AOF1 or LSD1, and blotted with antibodies indicated. Input 10% of extracts for IP. Note that HDAC1 antibody detected both HDAC1 and HDAC2 (marked with an arrow). **(B)** A stable Flag-AOF1 HeLa cell line was established and IP-western blot analysis was performed using whole cell extracts derived from this stable cell line. Input, 10% of whole cell extracts for IP. **(C)** Relative HDAC activity associated with LSD1 and AOF1. The whole cell extracts derived from Flag-AOF1-stable cells were first immunoprecipitated with protein G beads (mock), LSD1 antibody + protein G beads or anti-Flag M2 beads, and then subjected to a fluorometric-based HDAC activity assay. The final concentration of TSA is 1.25 μ M.

complex(es) containing histone deacetylases.

AOF1 has a histone demethylase-independent repression activity

Having established AOF1 as an H3K4 demethylase, we next wished to examine the potential role of AOF1 in transcription. As an H3K4 demethylase, AOF1 is expected to remove the active transcriptional marker and therefore confers transcriptional repression. Consistently, previous study has shown that when targeted to a transcriptional reporter as a Gal4(DBD) (DNA-binding domain) fusion protein, LSD1 functions as a repressor and this repression activity is to a certain extent dependent on its demethylase activity [4]. Using the same approach, we found that Gal-AOF1 repressed transcription from a 4 \times UAS-TK-luc reporter (Figure 5B). Surprisingly, we found that the enzymatically defective AOF1 K667A mutant (Gal-AOF1m) repressed transcription as effectively as the wild-type AOF1. The expression of both Gal-AOF1 and Gal-AOF1m was confirmed by western

blotting (Figure 5A). Thus, unlike LSD1, AOF1 appears to have a histone demethylase activity-independent repression function.

To test if the observed demethylase-independent repression may derive from recruitment of histone deacetylases, we repeated the above experiments with addition of the histone deacetylase inhibitor TSA. The results in Figure 5B lower panel show that addition of TSA resulted in an overall increase of transcriptional activity from the luciferase reporter, but did not significantly affect the repression by both Gal-AOF1 and Gal-AOF1m. This result excludes recruitment of class I and class II HDACs as a mechanism for AOF1 demethylase-independent repression and is consistent with the biochemical data that AOF1 is not associated with histone deacetylases.

The unique Zf-CW domain is required for AOF1 transcriptional repression activity

Previous studies indicate that reporter DNA introduced into the nuclei of *Xenopus* oocytes through microinjec-

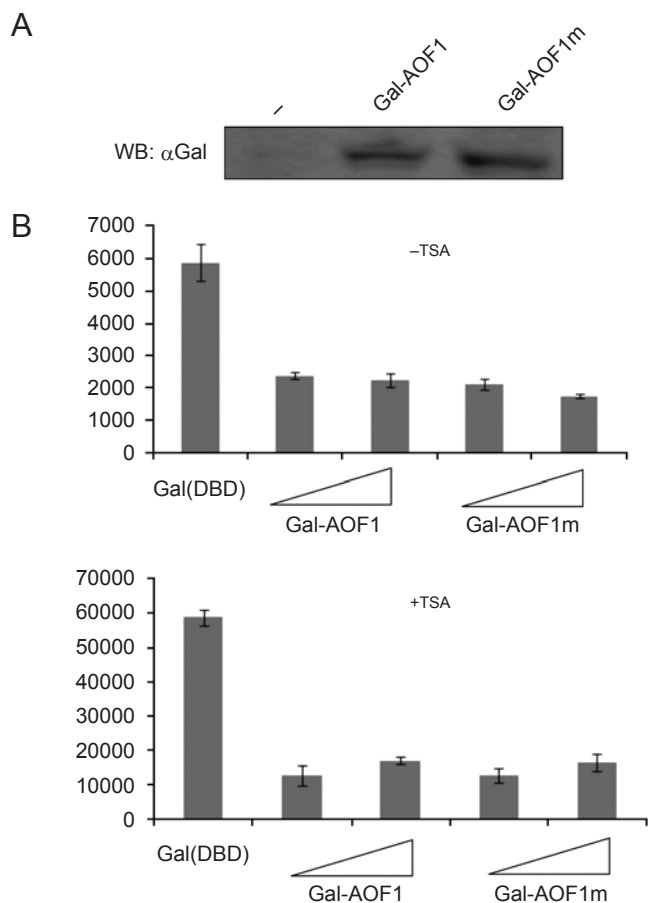


Figure 5 AOF1 possesses a demethylase- and HDAC-independent repression activity. **(A)** Plasmids encoding a Gal4(DBD) fusion of AOF1 or AOF1 H667A mutant were transfected into HeLa cells and the expression was detected by western blotting using a Gal4(DBD)-specific antibody. **(B)** HeLa cells were transfected with Gal-AOF1 or Gal-AOF1m (K667A) mutant together with a luciferase reporter (4×UAS-TK-luc) and treated with or without addition of 330 nM TSA. Amounts of plasmids used: 100 ng for 4×UAS-TK-luc, 75 ng and 150 ng for Gal-AOF1 and Gal-AOF1m. Note that addition of TSA resulted in an overall increase in luciferase activity, but had no significant effect on repression by both wild type and mutant AOF1. The relative luciferase activities are the mean ± SEM of three independent transfections.

tion will be assembled into chromatin [28], presumably as a result of abundant chromatin assembly activities and core histone proteins in *Xenopus* oocytes. *Xenopus* oocytes are also ideal for expression of exogenous proteins by microinjection of corresponding *in vitro* synthesized mRNAs [29]. *Xenopus* oocytes therefore have been extensively used as a convenient model for transcriptional analysis in the context of chromatin [30, 31]. Using an experimental scheme illustrated in Figure 6A, we ex-

pressed AOF1 as a Gal4 DBD fusion protein in *Xenopus* oocytes and tested its ability to repress transcription from three different transcription reporters. We found that expression of Gal-AOF1 led to repression of all three reporters, as revealed by primer extension analysis (Figure 6B-6D). The primer extension reactions also included a primer specific for *Xenopus* storage histone H4 mRNA, and the resulting product (cx) served as an internal loading control. As a control, expression of Gal-LSD1 also resulted in repression of all three reporters.

Having demonstrated that AOF1 broadly repressed transcription in *Xenopus* oocytes, we next wished to determine if the demethylase activity is required for repression and if not, the region responsible for the observed transcriptional repression activity. For this purpose, a new set of AOF1 deletion mutants suitable for *Xenopus* oocyte expression was generated (Figure 7A). A representative result in Figure 7C shows that the demethylase-defective Gal-AOF1m (K667A) repressed transcription as effectively as Gal-AOF1 did, indicating that the repression is again demethylase activity independent. However, deletion of the N-terminal Zf-CW alone or both ZF-CW and SWIRM domains resulted in loss of repression. On the other hand, deletion of the C-terminal amine oxidase domain has no effect on repression. Western blot analysis showed that all these AOF1 proteins were expressed to a similar level as revealed by using an anti-Gal4 (DBD) antibody (Figure 7B). Together, these results further substantiate an H3K4 demethylase activity-independent repression function for AOF1 and identify the unique Zf-CW domain as a key determinant for repression.

Discussion

AOF1 exhibits H3K4 histone demethylase activity

Among the mammalian amine oxidase family proteins, AOF1 is highly related to LSD1 and was predicted to be a nuclear protein. In this study, we tested if like LSD1, AOF1 also possesses a histone demethylase activity. Using recombinant mouse AOF1 expressed and purified from SF9 insect cells and radiolabeled core histone substrates, we detected a demethylase activity toward H3K4me1 and H3K4me2 (Figure 1). Under our experimental conditions, no demethylase activity toward H3K9 and H3K27 was detected for AOF1. This result is in agreement with the work from Andrea Mattevi's laboratory that was published during the preparation of our manuscript [32]. However, given that both the enzymatic specificity and activity of LSD1 demethylase have been reported to be modulated by the proteins that it interacts [6, 9], we also expressed AOF1 in HeLa or NIH3T3 cells

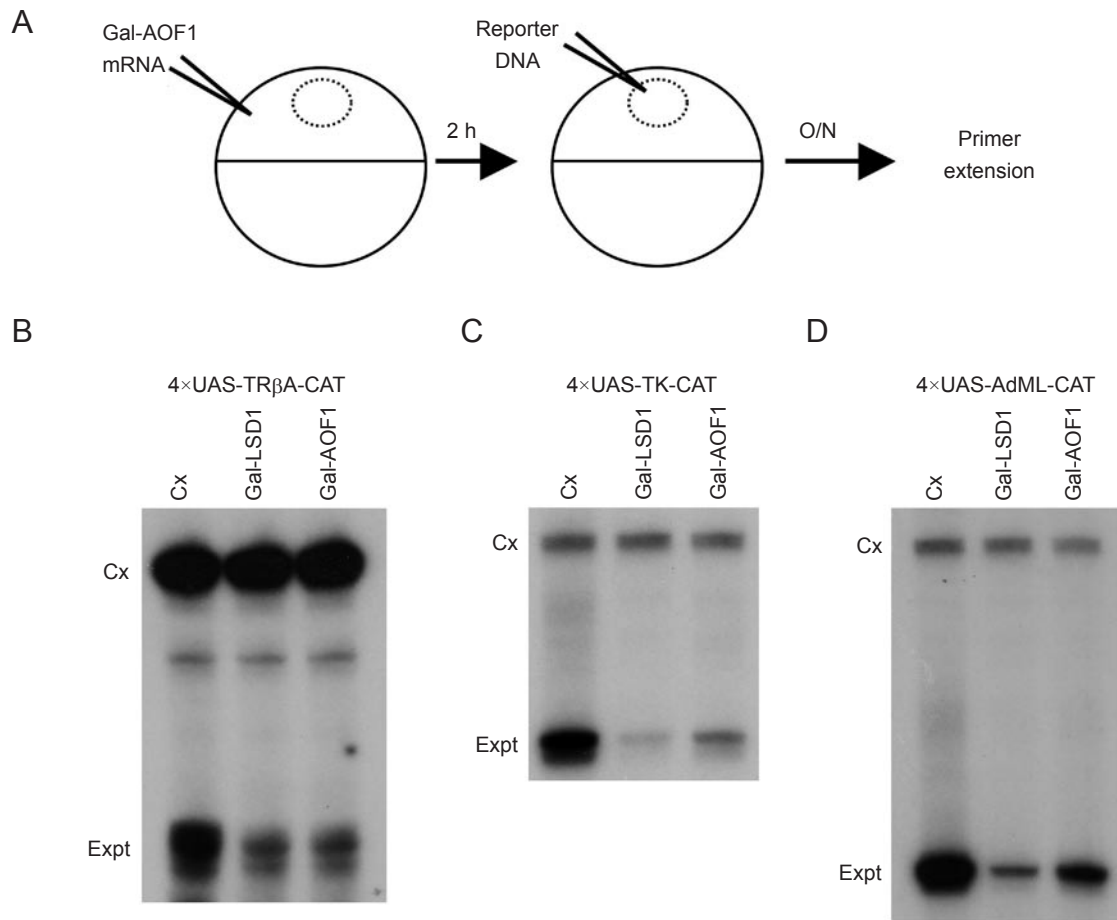


Figure 6 In *Xenopus* oocytes, both LSD1 and AOF1 exhibit a broad repression activity. **(A)** Diagram illustrating the experimental procedure, including microinjection of mRNA encoding Gal-AOF1 or Gal-LSD1 into the cytoplasm and reporter DNA into nuclei. After overnight incubation, total RNAs were prepared from injected oocytes and primer extension was performed to measure transcripts derived from the reporters. **(B)** Primer extension analysis revealed that expression of Gal-LSD1 and Gal-AOF1 repressed transcription from *Xenopus* TRβA promoter. Expt, the primer extension product from 4×UAS-TRβA-CAT; Cx, primer extension product from endogenous storage histone H4 mRNA that served as an internal control. **(C)** Expression of Gal-LSD1 and Gal-AOF1 repressed transcription from 4×UAS-TK-CAT reporter. **(D)** Expression of Gal-LSD1 and Gal-AOF1 repressed transcription from 4×UAS-AdML-CAT reporter.

and analyzed its histone demethylase activity by immunofluorescence using various antibodies against methylated histone H3. Using this approach, we again found that AOF1 has a demethylase activity toward H3K4me1 and H3K4me2, and has no demethylase activity toward H3K9, H3K27 and H3K36 methylation. Consistent with the notion that amine oxidation reaction can only lead to demethylation of mono- and di-methylated lysine residues, no demethylase activity toward H3K4me3 was detected for AOF1. Thus, in agreement with a recent work from Taiping Chen and coworkers [33], both *in vitro* and *in vivo* data indicate that AOF1 is an H3K4-specific histone demethylase.

Given the sequence and structural similarity between

AOF1 and LSD1, it is not surprising that mutation of a conserved histidine residue (K667A) in the oxidase domain impairs AOF1 demethylase activity. Previous studies indicated that the SWIRM domain is required for LSD1 demethylase activity [22, 26]. Similarly, we found that deletion of a stretch of 11 conserved amino acids within the AOF1 SWIRM domain impairs its enzymatic activity. Interestingly, we found that deletion of either the entire N-terminal unique Zf-CW domain or just four amino acids within the Zf-CW domain (AOF1Δ147-150) also impairs AOF1 demethylase activity. The Zf-CW domain is predicted to be a highly specialized four-cysteine zinc-finger that plays a role in DNA binding and/or promoting protein-protein interactions. Although

it is not clear why deletion of Zf-CW domain impairs AOF1 demethylase activity, Zf-CW domain is present in a dozen of proteins that have been implicated in the regulation of chromatin methylation status and early embryonic development.

In this study, we also find that AOF1 is associated with mitotic chromosomes, whereas LSD1 is not. The Zf-CW domain is required for chromosome association, as the AOF1 Δ 271 mutant was not associated with mitotic chromosomes. The mitotic chromosome association suggests that AOF1 may play a role in epigenetic inheritance of H3K4 methylation during cell divisions. In this regard, AOF1 was very recently reported to be required for establishing maternal genomic imprints [33]. It is thus tempting to suggest that its chromosome association may be related to its crucial role in regulating maternal genomic imprinting. Future study is thus needed to deter-

mine the functional significance of AOF1 mitotic chromosome association.

AOF1 does not appear to form an HDAC-containing protein complex

Our preliminary gel filtration analysis suggests that AOF1 in HeLa cells is likely to exist in a large protein complex (Yang and Wong, unpublished data). However, unlike LSD1, our extensive immunoprecipitation and HDAC activity assays suggest that AOF1 does not appear to associate with HDAC1/2 or other class I and class II HDACs. This result is in agreement with and is an expansion of the work from Andrea Mattevi's laboratory [32]. This difference in HDAC association may be explained by the lack of Tower domain in AOF1. The Tower domain in LSD1 is required for its interaction with CoREST and MTA1 and, therefore, the association

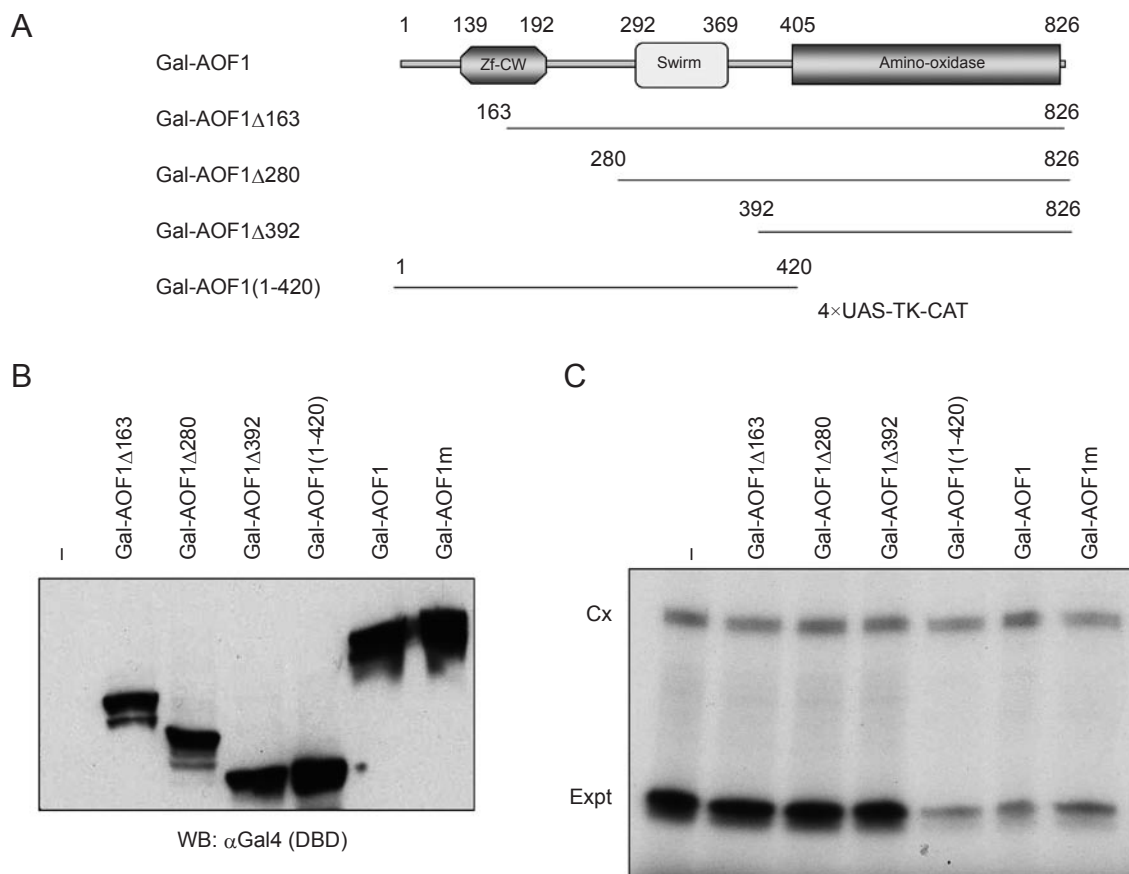


Figure 7 The unique Zf-CW domain is required for AOF1 demethylase-independent repression activity. **(A)** Diagram illustrating the structure of new set of AOF1 mutants for *Xenopus* oocyte expression. **(B)** The *in vitro* synthesized mRNAs encoding Gal-AOF1 and various mutants were injected into groups of *Xenopus* oocytes that were also injected with 4xUAS-TK-CAT reporter and the expression was detected by western blot analysis. **(C)** The total RNAs derived from the same groups of *Xenopus* oocytes as in **(B)** were analyzed by primer extension. Note that disruption of the N-terminal unique Zf-CW domain (Gal-AOF1 Δ 163) abolishes the AOF1 repression activity, indicating that the Zf-CW domain is required for repression.

with HDAC1/2 [23, 26]. The lack of HDAC association is also consistent with the observed TSA-insensitive repression by AOF1 in tethering experiments (Figure 5B).

The unique Zf-CW domain of AOF1 confers histone demethylase-independent repression function

A novel finding in this study is that AOF1 has a demethylase activity-independent repression activity, and this activity is dependent on its unique Zf-CW domain. LSD1 has been shown to repress transcription when tethered to a reporter as a Gal4 fusion protein [4]. This repression function is compromised for the LSD1 mutant defective in demethylase activity. To our surprise, we found that the enzymatically defective AOF1 H667A mutant represses transcription as effectively as the wild-type AOF1, both in mammalian cells and in *Xenopus* oocytes, suggesting that AOF1 differs from LSD1 and possesses a demethylase activity-independent repression function. Given that the AOF1 repression activity is insensitive to TSA and that AOF1 does not appear to associate with substantial HDAC activity, we conclude that this demethylase-independent repression activity is likely also HDAC independent. Further structural and functional analyses identified the N-terminal Zf-CW domain as essential for repression function. Although the Zf-CW domain is unique for AOF1, sequence comparison reveals it is conserved among AOF1 proteins from different organisms. Given that the Zf-CW domain is required for both mitotic chromosome association and repression function, it raises the question as to whether the role of AOF1 in maternal imprinting is entirely H3K4 demethylase activity dependent. Future work is required to determine the underlying mechanism of AOF1 Zf-CW domain-mediated repression and its biological function.

Materials and Methods

Plasmids, antibody, cell culture and stable cell line

To express AOF1 in SF9 cells, the full-length open reading frame of mouse AOF1 cDNA (MGC:38211) was PCR amplified, digested with *SalI* and *XbaI* and cloned into pFastBachTC vector also digested with *SalI* and *XbaI*. In addition, the full-length AOF1 was PCR amplified, digested with *HindIII* and *NheI* and cloned into a modified pcDNA5/FRT/TO vector also digested with *HindIII* and *NheI*. The sequence of AOF1 was verified by sequencing. The AOF1m (K667A) was generated using site-directed mutagenesis. The AOF1 mutants with deletion of N-terminal 271 amino acids (AOF1Δ271) or 381 amino acids (AOF1Δ381) were generated by PCR-based cloning, as described above, and verified by sequencing. The mutants with a small deletion in either the Zf-CW domain (AOF1Δ147-150) or SWIRM domain (AOF1Δ372-382) were generated using PCR-based site-directed mutagenesis and verified by DNA sequencing. To express AOF1 and AOF1m as a Gal4-DBD fusion protein, the corresponding DNA fragments were

cleaved from pcDNA5/FRT/TO vector and cloned into pCMX-Gal vector. To express AOF1 and various mutants in *Xenopus* oocytes, the corresponding DNA fragments were cloned into a modified pSP64-poly(A) vector. Three reporters (4×UAS-TRβA-CAT, 4×UAS-TK-CAT and 4×UAS-AdML-CAT) used for transcriptional analysis in *Xenopus* oocytes were obtained as described [34].

Antibodies against H3K4me1, H3K4me2, H3K9me1, H3K9me2 and H3K27me2 were purchased from Abcam (Cambridge, MA, USA). The H3K36me2 antibody was a gift from professor Degui Chen (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China). Gal4 (DBD) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Flag-tag (M2) antibodies were purchased from Sigma (St Louis, MO, USA). Rabbit polyclonal anti-HDAC1 antibody was a kind gift from Dr Jiangou Song (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China). The mouse polyclonal AOF1 antibody was from AbMart (Shanghai, China) and monoclonal LSD1 antibody was purchased from Nexus Biological (Houston, TX, USA).

HeLa cells were maintained in DMEM medium (Hyclone) supplemented with 10% fetal bovine serum and 100 U penicillin-streptomycin. The stable Flag-AOF1 expressing HeLa cell line was established by transfecting HeLa cells with pcDNA5-FRT/TO-AOF1 and followed by selection with 150 μg/ml hygromycin B for 2 weeks.

Preparation of recombinant AOF1 and LSD1

Recombinant AOF1 was expressed as His-tagged proteins in SF9 cells using the Bac-to-Bac baculoviral system (Invitrogen). The recombinant Flag-LSD1 was expressed and purified from SF9 cells as described [17].

In vitro demethylation assay

The histone demethylase assay was performed essentially as previously described [17]. Briefly, various ³H-SAM-labeled histone substrates were incubated with recombinant Flag-AOF1 or FLAG-LSD1 in histone demethylation buffer (100 mM glycine (pH 9) or 1 mM PMSF or 1 mM DTT or 50 mM KCl) at 37 °C for 1 h. For detection of the released ³H-formaldehyde, a modified NASH method was used. After trichloroacetic acid precipitation, an equal volume of NASH reagent (3.89 M ammonium acetate, 0.1 M acetic acid and 0.2% 2,4-pentanedione) was added into the supernatant, and the mixtures were incubated at 37 °C for 50 min before extraction with equal volume of 1-pentanol. The extracted radioactivity was measured by scintillation counting.

Immunoprecipitation and western blotting

To prepare whole cell extracts from stable Flag-AOF1 HeLa cells, the cells from ten 10-cm plates were washed with phosphate-buffered saline (PBS) two times and lysed with ice-cold EBC buffer (20 mM Tris-HCl (pH 8.0), 125 mM NaCl, 2 mM EDTA and 0.5% NP-40) with protease inhibitors for 15 min. The lysate was centrifuged at 13 000× g for 15 min at 4 °C to remove cell debris. Immunoprecipitation was performed with 100 μl whole cell extract and anti-Flag-M2 beads or LSD1 antibody. Western blot analysis was performed using antibodies as described. Primary antibodies were diluted in the ratio of 1:1 000, except for anti-Gal4 (DBD) and anti-Flag, which were diluted in the ratio of 1:5 000.

For western blot analysis using *Xenopus* oocyte, whole cell extracts and 5 to 10 oocytes were homogenized in 100 mM Tris/10

mM EDTA (pH 8.0) (10 µl per oocyte) and centrifuged to remove insoluble material. Western blot analyses were performed as previously described [34].

Immunofluorescence staining

For detecting AOF1 demethylase activity by immunofluorescent staining, HeLa cells were transfected with the wild-type or mutant AOF1 in pcDNA5-FRT/TO vector using lipofectamine 2000 (Invitrogen) according to manufacturers instruction. At 48 h after transfection, the cells were fixed with 4% paraformaldehyde, permeabilized in 1% Triton X-100/PBS and then processed for immunofluorescent staining as described [17]. DNA was stained with Hoechst 33342 stain. Primary antibodies were used at the following dilution: 1:250 for H3K4me1, H3K9me2, H3K27me2, H3K36me2; 1:500 for H3K4me3; 1:1 000 for H3K4me2 and 1:3 000 for anti-Flag (Sigma). Secondary goat-anti-rabbit (Alexa fluor 555) and goat-anti-mouse (Alexa fluor 488) were purchased from Molecular Probes and used at 1:500 dilution.

Dual-reporter luciferase activity assay

Transfection for luciferase activity assay was performed in 24-well plates using lipofectamine 2000 (Invitrogen). The Gal4DBD-AOF1 and Gal4DBD-AOF1 mutant plasmids were co-transfected with 4×UAS-TK-firefly luciferase plasmid and a control *Renilla* luciferase plasmid, which is under control of the thymidine kinase (TK) promoter. Luciferase activity in the cell lysates was determined by using a dual luciferase reporter assay system (Promega, WI, USA) 48 h post-transfection and the luciferase activity was normalized to the TK *Renilla* activity. Treatments with 50 nM trichostatin A was performed for 12 h before the cells were harvested for luciferase assay.

In vitro HDAC assay

To measure HDAC activity associated with AOF1, whole cell extracts were prepared from the stable Flag-AOF1 HeLa cells. Flag-AOF1 was immunoprecipitated with Flag-M2 beads (Sigma). As controls, the whole cell extracts were also immunoprecipitated with protein G alone (mock) or with protein G plus LSD1 antibody. After extensive washes, the resulting beads were subjected to HDAC activity assays using a histone deacetylase assay kit from Sigma (catalog number cs1010) according to manufacturers instruction. The excited fluorophore resulting from HDAC reactions was detected on a multi-detection microplate reader (Tecan M200).

Microinjection of *Xenopus* oocytes

Preparation and microinjection of mRNA and reporter DNA into stage VI *Xenopus* oocytes were performed as previously described [34]. All capped poly-A mRNAs used for injection were synthesized using a SP6 mESSAGE mACHINE kit (Ambion, Austin, TX, USA). mRNA was injected at a concentration of 100 ng/µl (18.4 nl per oocyte) and reporter DNA was injected at a concentration of 50 ng/µl (18.4 nl per oocyte) according to the experimental scheme described in each figure.

Primer extension analysis

Primer extension was used to analyze the RNA transcripts produced from reporter genes injected into *Xenopus* oocytes. The primer for all three reporters (4×UAS-TRβA-CAT, 4×UAS-TK-

CAT and 4×UAS-AdML-CAT) is 5'-GTG AAG GAT AAG TGA CGA GCG GAG ACG-3'. The procedure used for primer extension has been previously described [35]. The *Xenopus* oocyte storage histone H3 mRNA was used as an internal control in all primer extension assays [35]. Each experiment was repeated at least twice.

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