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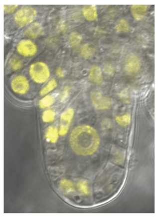

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RESEARCH PAPER

Coordinate activation of a target gene by KDM1C histone demethylase and OTLD1 histone deubiquitinase in Arabidopsis

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ABSTRACT

Potential functional coordination between histone deubiquitinases and histone lysine demethylases represents one of the least studied aspects of epigenetic control of transcriptional outcomes. Here, this question was addressed using Arabidopsis histone modification erasers deubiquitinase OTLD1 and demethylase KDM1C known to interact with each other in plant cells. Characterization of gain- and loss-of-function mutants of *OTLD1* and *KDM1C* showed that both enzymes associate with the promoter chromatin of their target gene *AN3* and function as coactivators of its expression. This transcriptional outcome was underlain by demethylation of the H3K9 repression mark, presumably by the KDM1C histone demethylase activity. Association of KDM1C and OTLD1 with the target chromatin was interdependent such that OTLD1 was not detected at the *AN3* in the absence of KDM1C and KDM1C displayed a different and non-functional pattern of association in the absence of OTLD1. Thus, OTLD1 and KDM1C may crosstalk with each other to assemble a functional coactivator complex at the *AN3* promoter chromatin and set the KDM1C specificity for the methylated H3K9 to determine the correct transcriptional outcome.

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Chromatin; chromatin remodeling; epigenetics; histone modifications

Introduction

Posttranslational histone modifications are central to gene regulation in all eukaryotic organisms, determining the active or inactive state of the chromatin. Furthermore, one histone modification often promotes generation of another, a phenomenon termed histone crosstalk, that shapes the specific histone modification landscape and, ultimately, transcriptional outcomes (1,2). Well-studied cases of histone crosstalk are illustrated by the mammalian MLL3/4-UTX activator complex that coordinates histone lysine 27 (H3K27) demethylation and H3K4 methylation [3], Drosophila repressor complexes PcG that coordinates H3K27 methylation and H2AK119 ubiquitylation [4] and dRAF that links H2AK119 ubiquitylation with H3K36 demethylation [5], and yeast Rad6/Bre1 ubiquitin ligases and Dot1/Set1 histone methyltransferases that coordinate H2BK123 ubiquitylation and H3K4/H3K79 methylation [6]. In plants, studies of histone crosstalk are still lacking, although mutations in the Arabidopsis UBP26 have been shown to increase H2BK143 ubiquitylation and H3K4 methylation and decrease H3K9 methylation, resulting in gene silencing [7]. One of the least studied cases, in plant and non-plant model systems, is the possible functional coordination between histone deubiquitinases and histone lysine demethylases in transcriptional regulation of their target genes. Here, we addressed this question using Arabidopsis histone modification erasers deubiquitinase OTLD1 and demethylase KDM1C.

OTLD1 is a member of the ovarian tumor (OTU) deubiquitinase family [8,9] that deubiquitylates monoubiquitylated H2B [10] and acts mainly as gene repressor, whose target genes include *GA20OX, WUS, OSR2, ARL*, and *ABI5* involved in plant growth and development [11]. However, OTLD1 also can activate gene expression; specifically, OTLD1 induces expression of a plant developmental regulator gene *AN3/GIF* [12]. Interestingly, OTLD1 can interact with the KDM1C histone demethylase in plant cells [10]. KDM1C is an Arabidopsis member of the SWIRM (Swi3p, Rsc8p, Moira) [13]/polyamine oxidase (PAO) domain histone lysine demethylase family [14], exemplified by the animal LSD1/KDM1A enzyme, the founding member of the KDM1 family [15]. Most

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Supplemental data for this article can be accessed here.

studies suggested that diverse, plant and non-plant KDM1 enzymes function as corepressors by demethylating the euchromatic marks on H3K4 in the target chromatin, both in vivo and in vitro [15–19]. Yet, the animal LSD1/KDM1A has been shown also to act as a coactivator, removing the repressive marks on H3K9 [20–25]. Using gain-of-function and loss-of-function Arabidopsis alleles of *OTLD1* and *KDM1C*, we demonstrated coordinate action of both histone modification erasers in transcriptional activation of their common target gene *AN3*.

Results

Transcriptional regulation of the *AN3* gene in *KDM1C* loss-of-function and gain-of-function plants

We examined whether KDM1C affects the expression of *AN3*, the target gene activated by OTLD1 [12]. First, we utilized a previously described loss-of-function mutant *kdm1c-1* (SALK_142477) [26]. RT-qPCR analysis of this line showed statistically significant (p-values < 0.05) reduction in the *KDM1C* gene expression as compared to the wild type plants (Figure 1(a)). The *kdm1c-1* line also exhibited substantial decrease in accumulation of the *AN3* transcripts (Figure 1(b)). We then generated several independent homozygous transgenic lines of Arabidopsis that express *KDM1C* tagged with an hexahistidine epitope from a general CaMV 35S promoter; one of these gain-of-function lines, designated *KDM1C* OE-1, was analyzed in detail. Figure 1(d) shows that the *KDM1C* OE-1 plants accumulated 7-fold higher amounts of *KDM1C* transcripts than the wild-type plants; this relatively moderate increase in expression allowed us to analyze the gain-of-function effects of *KDM1C* while avoiding unnecessarily high expression amounts. Specifically, the *KDM1C* OE-1 plants displayed, with statistical significance (p-values < 0.05) an increase in the *AN3* transcript levels (Figure 1(e)). The expression levels of the internal reference genes *ACT7* and *UBQ10* showed no statistically significant differences between any of the tested plant lines (Figure 1(c,f)).

Next, we used quantitative chromatin immunoprecipitation (qChIP) to investigate potential association of KDM1C with the *AN3* chromatin in the *KDM1C* OE-1 plants in the general promoter area

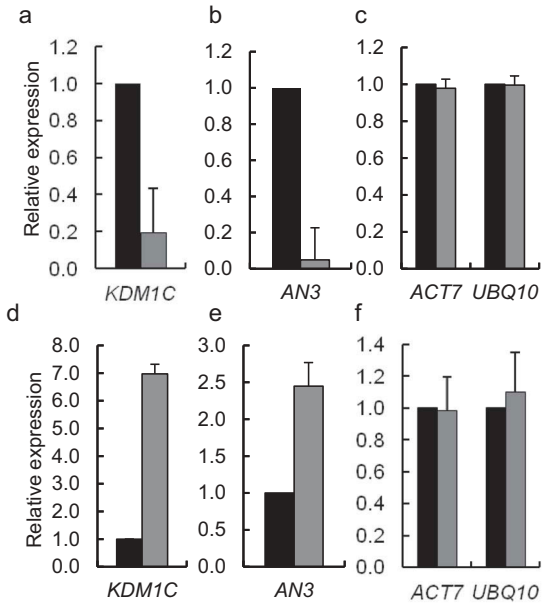

Figure 1. Effect of loss- and gain-of-function of *KDM1C* on *AN3* transcript levels. Loss-of-function *kdm1c-1* plants. (a) Expression of *KDM1C*. (b) Expression of *AN3*. (c) Expression of the reference genes *ACT7* and *UBQ10*. Gain-of-function *KDM1C* OE-1 plants. (d) Expression of *KDM1C*. (e) Expression of *AN3*. (f) Expression of the reference genes *ACT7* and *UBQ10*. Relative expression levels of the indicated genes in the wild-type plants (black bars) and *kdm1c-1* plants or *KDM1C* OE-1 plants (gray bars) were analyzed by RT-qPCR. The expression level in the wild-type plants is set to 1.0. Error bars represent s. e. m. of three independent biological replicates, N = 3; p-values <0.05 for statistical significance of differences between the wild-type plants and *kdm1c-1* plants in (a) and (b) or *KDM1C* OE-1 plants in (d) and (e) whereas differences between all tested plants in (c) and (f) were not statistically significant.

previously shown to be occupied and deubiquitylated by OTLD1 [12]. Figure 2(a) shows statistically significant (p-values < 0.05) association of KDM1C with two distinct regions, one located ca. 500 bp upstream of the translation initiation codon (*AN3-C*) and the other flanking the translation initiation codon (*AN3-A*); no association of KDM1C was observed with two other tested promoter regions. In positive control experiments using previously described transgenic lines *OTLD1* OE-1 and *OTLD1* OE-2 that express a hexahistidine-tagged OTLD1 [12], OTLD1, known to bind to the *AN3* chromatin [12], indeed associated with the promoter areas of *AN3* (Figure 2(b)). This qChIP analysis utilized the same four *AN3* promoter regions used for testing the binding of KDM1C (Figure 2(a)); interestingly, in both *OTLD1* OE-1 and *OTLD1* OE-2 plants, OTLD1 associated with those two chromatin regions (Figure 2(b)) that were not

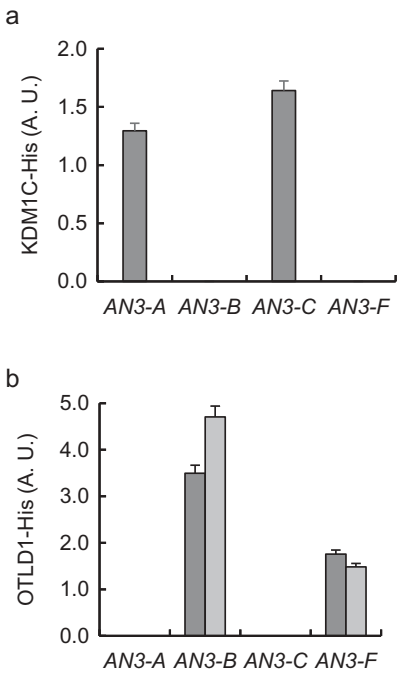

Figure 2. KDM1C and OTLD1 associate with *AN3* chromatin. (a) Association of His-tagged KDM1C with the *AN3* chromatin. Wild-type plants, black bars; *KDM1C* OE-1 plants, dark gray bars. (b) Association of His-tagged OTLD1 with the *AN3* chromatin. *OTLD1* OE-1 plants, dark gray bars; *OTLD1* OE-2 plants, light gray bars. Locations of the indicated regions within the promoter area of *AN3* used for qChIP analyses are detailed in Table S1. A. U., arbitrary units. Error bars represent s. e. m. of three independent biological replicates, N = 3; *p*-values <0.05 for statistical significance of differences between the wild-type and *KDM1C* OE-1, *OTLD1* OE-1, or *OTLD1* OE-2 lines.

occupied by KDM1C in the *KDM1C* OE-1 plants (Figure 2(a)). Taken together, the data in Figures 1 and 2 support the role of KDM1C in transcriptional activation of the *AN3* gene.

KDM1C reduces methylation of H3K9, but not of H3K4, in the *AN3* chromatin

Based on the substrate specificity of KDM1 enzymes, which includes mainly H3K4 [15–19] or, in some cases, H3K9 [20–25], we used qChIP to compare the content of the heterochromatic H3K9me2 and euchromatic H3K4me3 marks in the *AN3* chromatin in the loss-of-function *kdm1c-1* plants and in the wild-type plants. From four tested chromatin regions of the *AN3* promoter, two became hypermethylated at H3K9 in a statistically significant fashion (*p*-values < 0.05) in the *kdm1c-1* line as compared to the wild-type plants whereas two other regions showed no statistically significant changes in the H3K9 methylation (Figure 3(a)). No statistically significant differences in H3K9 methylation were detected in the chromatin of the internal reference *ACT7* and *UBQ10* genes (Figure 3(b)). Our qChIP analysis of the H3K4me3 content in the *AN3* chromatin in the *kdm1c-1* and the wild-type plants revealed statistically significant (*p*-values < 0.05) hypomethylation of H3K4 in the loss-of-function line (Figure 3(c)) with no changes in H3K4 methylation in the reference *ACT7* and *UBQ10* chromatin (Figure 3(d)). These data correlate the absence of the KDM1C demethylase with the increased levels of the H3K9me2 repressive mark, but not of the euchromatic H3K4me3 mark, in the target *AN3* chromatin, suggesting that activation of *AN3* transcription involves H3K9 demethylation by KDM1C.

Next, we performed the qChIP analysis of the H3K9me2 and H3K4me3 content of the *AN3* chromatin in the gain-of-function *KDM1C* OE-1 line. The *KDM1C* OE-1 plants exhibited statistically significant (*p*-values <0.05) hypomethylation of H3K9 relative to the wild-type plants (Figure 4(a)). In contrast, we did not detect statistically significant changes in the H3K4me3 levels between both plant lines (Figure 4(c)). Reference *ACT7* and *UBQ10* chromatin exhibited no changes in its H3K9me2 and H3K4me3 content (Figure 4(b,d)). These data are consistent with the notion that the heterochromatic H3K9 represents the main target of the KDM1C demethylase during transcriptional activation of the *AN3* gene.

Both KDM1C and OTLD1 are required for transcriptional activation of *AN3*

Our data suggest that the KDM1C demethylase is involved in transcriptional activation of the *AN3* gene, potentially through occupying its promoter chromatin and demethylating its H3K9 repressive marks (Figures 1–4). On the other hand, also the OTLD1 deubiquitinase has been shown to activate *AN3* via association with its chromatin and H2B deubiquitylation [12]. Potentially connecting these observations is the ability of KDM1C and OTLD1 to interact with each other physically in the plant cells [10]. Thus, we endeavored to examine whether KDM1C and OTLD1 cooperate functionally during activation of the *AN3* expression. To this end, we generated two types of transgenic

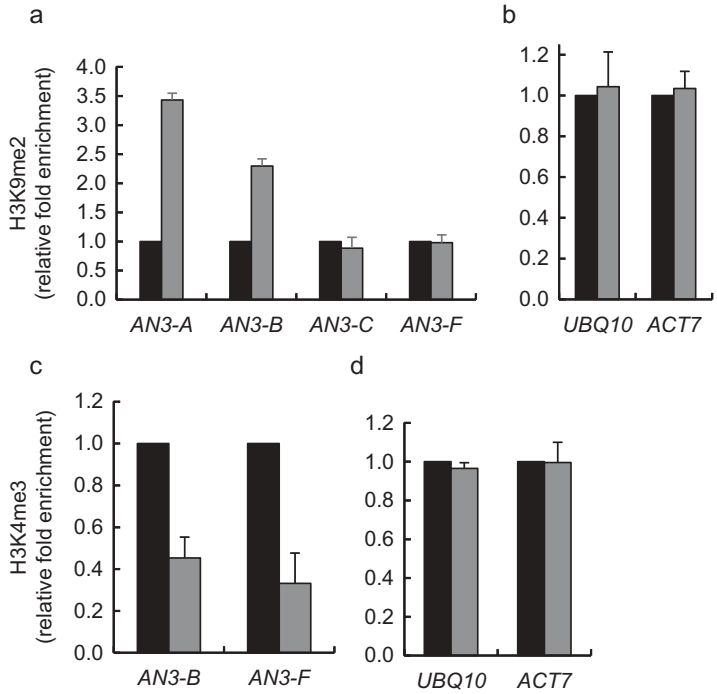

Figure 3. Effect of loss-of-function of *KDM1C* on H3K9 dimethylation and H3K4 trimethylation of *AN3* chromatin. (a) H3K9 methylation of the *AN3* chromatin. (b) H3K9 methylation of the reference genes *ACT7* and *UBQ10*. (c) H3K4 methylation of the *AN3* chromatin. (d) H3K4 methylation of the reference genes *ACT7* and *UBQ10*. Wild-type plants, black bars; *kdm1c-1* plants, gray bars. Locations of indicated regions within the promoter area of *AN3* used for qChIP analyses are detailed in Table S1. The corresponding methylation level in the wild-type plants is set to 1.0. Error bars represent s. e. m. of three independent biological replicates, N = 3; p-values <0.05 for statistical significance of differences between the wild-type and *kdm1c-1* plants in (a) and (c) whereas differences between all tested plants in (b) and (d) were not statistically significant.

plants: OTLD1 gain-of-function/KDM1C loss-of-function and KDM1C gain-of-function/OTLD1 loss-of-function. Specifically, two independent lines of each type, i.e., *OTLD1* OE-3/*kdm1c-1* and *OTLD1* OE-4/*kdm1c-1* lines and *KDM1C* OE-2/*otld1-1* and *KDM1C* OE-3/*otld1-1* lines, were produced and analyzed. Figure 5(a) shows, with statistical significance (p-values < 0.05), that both *OTLD1* OE-3/*kdm1c-1* and *OTLD1* OE-4/*kdm1c-1* lines exhibited reduced expression of the *KDM1C* gene and increased expression of the *OTLD1* gene. Both of these lines also were unable to activate expression of *AN3* (Figure 5(b)) but did not display any changes in the expression of the *ACT7* and *UBQ10* reference genes (Figure 5(c)). In reciprocal experiments, the *KDM1C* OE-2/*otld1-1* and *KDM1C* OE-3/*otld1-1* lines exhibited elevated expression of *KDM1C* and low levels of expression of *OTLD1* (Figure 5(d)). Yet, these lines also did not activate the expression of the *AN3* gene (Figure 5(e)) while not altering the expression of the control, reference genes (Figure 5(f)). This is in contrast to the observations that individual gain-of-function mutants of *KDM1C* (Figure 1(e)) or *OTLD1* [12] in the wild-type genetic background activate *AN3* expression. Thus, these data suggest that both KDM1C and OTLD1 are required to transcriptionally activate their target *AN3* gene whereas each of these individual histone modifying enzymes alone is insufficient for activation, suggesting that KDM1C and OTLD1 act in a coordinate fashion.

Association of KDM1C and OTLD1 with the target *AN3* chromatin is interdependent

Coordinate action of KDM1C and OTLD1 on the *AN3* gene expression may be the result of coordinate association of these proteins with the target chromatin. To test this idea, we performed the qChIP analysis of occupation of the *AN3* promoter chromatin by KDM1C and OTLD1 in the *OTLD1* OE-3/*kdm1c-1*, *OTLD1* OE-4/*kdm1c-1*, *KDM1C* OE-2/*otld1-1*, and *KDM1C* OE-3/*otld1-1* lines. Figure 6(a) shows that,

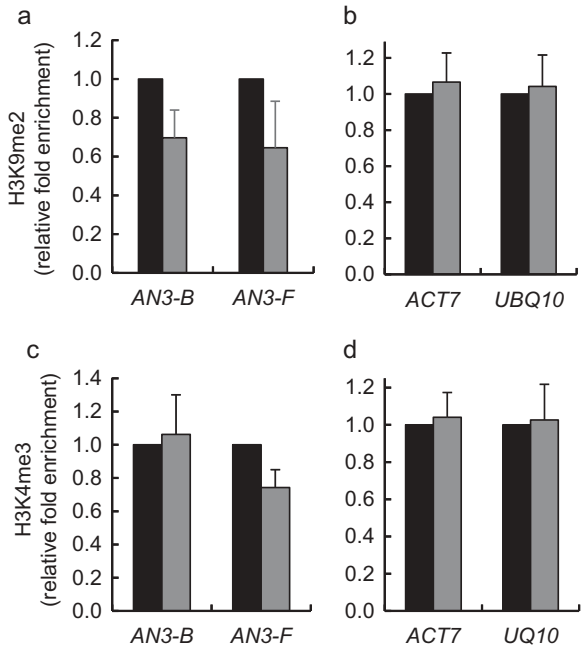

Figure 4. Effect of gain-of-function of *KDM1C* on H3K9 dimethylation and H3K4 trimethylation of *AN3* chromatin. (a) H3K9 methylation of the *AN3* chromatin. (b) H3K9 methylation of the reference genes *ACT7* and *UBQ1*. (c) H3K4 methylation of the *AN3* chromatin. (d) H3K4 methylation of the reference genes *ACT7* and *UBQ1*. Wild-type plants, black bars; *KDM1C*-OE-1 plants, gray bars. Locations of indicated regions within the promoter area of *AN3* used for qChIP analyses are detailed in Table S1. The corresponding methylation level in the wild-type plants is set to 1.0. Error bars represent s. e. m. of three independent biological replicates, N = 3; p-values <0.05 for statistical significance of differences between the wild-type and *KDM1C*-OE-1 plants in (a) and (c) whereas differences between all tested plants in (b) and (d) were not statistically significant.

in both *OTLD1* OE-3/*kdm1c-1* and *OTLD1* OE-4/*kdm1c-1* plants, OTLD1 failed to associate with the *AN3* chromatin with statistical significance, exhibiting only very low levels of the qChIP signal. These data are consistent with the inability of these plant lines to activate *AN3* expression (Figure 5(b)). However, in the *KDM1C* OE-2/*otld1-1* and *KDM1C* OE-3/*otld1-1* plants, KDM1C retained its ability to associate with the target chromatin (Figure 6(b)). Interestingly, this association was detected mainly with those chromatin regions of the *AN3* promoter that showed no association with KDM1C in the presence of wild-type levels of OTLD1 (Figure 2(a)), and most likely it was non-functional as these plant lines did not support induction of *AN3* expression (Figure 5(e)). Thus, association of OTLD1 with the *AN3* promoter chromatin appears to require the presence of KDM1C whereas KDM1C can associate with the target chromatin in the absence of OTLD1, albeit this association does not faithfully replicate the association of KDM1C in the presence of the wild-type OTLD1. This notion, that KDM1C association with the *AN3* chromatin in the absence of OTLD1 is not functional, is lent further support by the observation that elevated expression of KDM1C in the *KDM1C* OE-2/*otld1-1* and *KDM1C* OE-3/*otld1-1* lines did not result in hypomethylation of H3K9 in the *AN3* chromatin (Figure 7) as we observed in the *KDM1C* gain-of-function plants (Figure 4(a)).

Discussion

One fascinating feature of some chromatin modifying enzymes is their ability to function both as corepressors and coactivators, depending on the specific chromatin target. Both OTLD1 and KDM1C belong to this category of dual-function enzymes. Historically, OTLD1 and KDM1C were first characterized as corepressors [10,11,26,27]. However, LSD1/KDM1A, the animal homolog of KDM1C, has been found also in activator complexes [20–25], although this activity has not been demonstrated for any of the plant KDM1 family members, including KDM1C. More recently, OTLD1 was shown to act as a coactivator [12]. Based on these data and on the ability of OTLD1 to interact with KDM1C [10], we hypothesized that KDM1C and OTLD1 may act together, in a coordinate manner as coactivators of their target gene expression. Our data here support this notion, demonstrating that KDM1C activates transcription of the *AN3* gene by associating with its promoter chromatin where KDM1C most likely prevents accumulation of the repressive mark H3K9me2 through its demethylase activity, allowing accumulation of the acetylated H3 histone marks of the active chromatin [12] (Figure 8(a)). This coactivator action of KDM1C is dependent on the presence of OTLD1 which also associates with the *AN3* promoter chromatin and deubiquitylates its monoubiquitylated H2B [12] whereas the *AN3*-activating action of OTLD1 is dependent on the presence of KDM1C. This functional interdependence of KDM1C and OTLD1 is not simple. Although their ability to promote transcriptional activation of *AN3* strictly depends on each other's presence, their association with the target chromatin shows a more complex relationship. OTLD1 association with the *AN3*

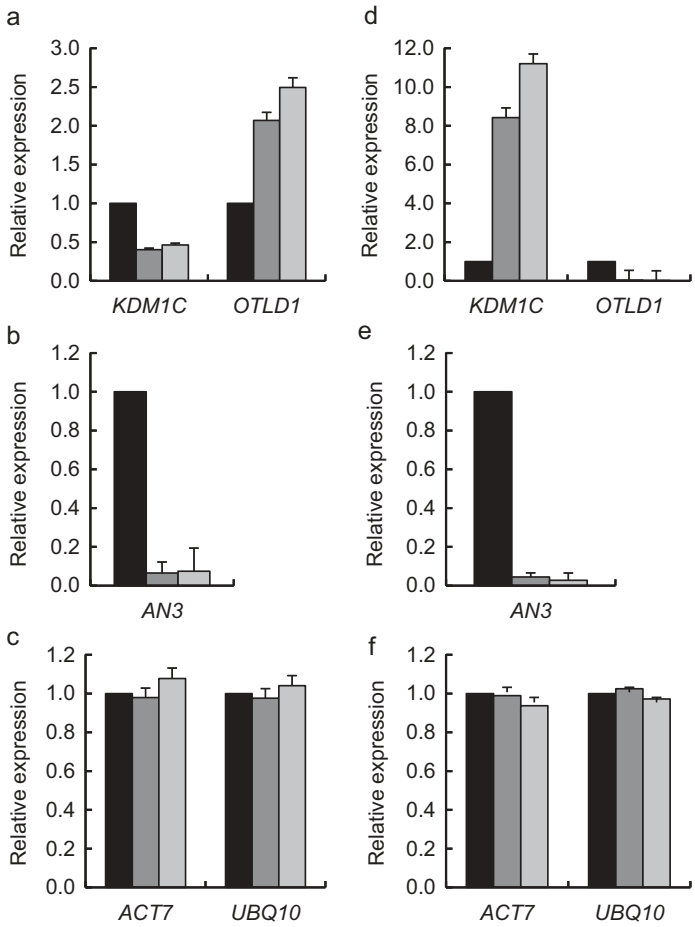

Figure 5. Effect of loss-of function of *KDM1C* or *OTLD1* on *AN3* transcript levels in *OTLD1* or *KDM1C* gain-of-function plants. *OTLD1* OE-3/*kdm1c-1* and *OTLD1* OE-4/*kdm1c-1* plants. (a) *KDM1C* and *OTLD1* transcript levels. (b) *AN3* transcript levels. (c) Reference gene *ACT7* and *UBQ10* transcript levels. *KDM1C* OE-2/*otld1-1* and *KDM1C* OE-3/*otld1-1* plants. (d) *KDM1C* and *OTLD1* transcript levels. (e) *AN3* transcript levels. (f) Reference gene *ACT7* and *UBQ10* transcript levels. Relative expression levels of the indicated genes in the wild-type plants (black bars), *OTLD1* OE-3/*kdm1c-1* plants and *KDM1C* OE-2/*otld1-1* plants (dark gray bars), and *OTLD1* OE-4/*kdm1c-1* and *KDM1C* OE-3/*otld1-1* plants (light gray bars) were analyzed by RT-qPCR. The expression level in the wild-type plants is set to 1.0. Error bars represent s. e. m. of three independent biological replicates, N = 3; *p*-values <0.05 for statistical significance of differences between the wild-type and *OTLD1* OE-3/*kdm1c-1* and *OTLD1* OE-4/*kdm1c-1* in (a) and (b) or *KDM1C* OE-2/*otld1-1* and *KDM1C* OE-3/*otld1-1* plants in (d) and (e) whereas differences between all tested plants in (c) and (f) were not statistically significant.

chromatin could not be detected in the absence of KDM1C (Figure 8(b)), but, in the absence of OTLD1, KDM1C was still detected at the target chromatin (Figure 8(c)). This observation suggests that KDM1C may recruit OTLD1 to the AN3 chromatin. However, the target chromatin association of KDM1C in the presence of OTLD1 differs from that in the absence of OTLD1; specifically, when OTLD1 is unavailable, KDM1C associates with the promoter regions with which it does not associate when OTLD1 is present (Figure 8). One potential explanation for this difference is that, when KDM1C attaches to the target chromatin in complex with OTLD1, OTLD1 may affect which chromatin regions are accessible to KDM1C. It is association of KDM1C with these OTLD1-defined chromatin regions of *AN3* that correlates with transcriptional activation of this gene.

Another interesting aspect of the *AN3* regulation by KDM1C/OTLD1 is that it most likely involves demethylation of the H3K9 repressive mark by KDM1C. To our knowledge, this is the first report of a coactivator role for a plant KDM1-type histone demethylase as well as of its potential ability to demethylate H3K9. Recently, the animal LSD1/KDM1A has been suggested to alter its substrate specificity from H3K4 euchromatic mark to H3K9 heterochromatic mark and act as a co-activator in the presence of its interactor protein [25]. Thus, OTLD1 and KDM1C may crosstalk with each other to assemble a functional coactivator complex at the *AN3*

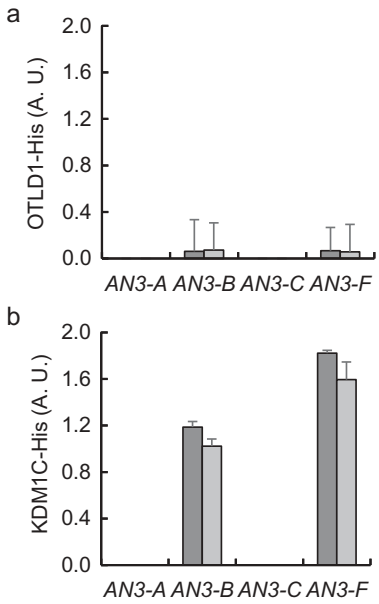

Figure 6. Effect of loss-of function of *KDM1C* or *OTLD1* on OTLD1 or KDM1C association with *AN3* chromatin. (a) Association of His-tagged OTLD1 with the *AN3* chromatin in *KDM1C* loss-of-function plants. *OTLD1* OE-3/*kdm1c-1* plants, dark gray bars; *OTLD1* OE-4/*kdm1c-1* plants, light gray bars. (b) Association of His-tagged KDM1C with the *AN3* chromatin in *OTLD1* loss-of-function plants. *KDM1C* OE-2/*otld1-1* plants, dark gray bars; *KDM1C* OE-3/*otld1-1* plants, light gray bars. Locations of the indicated regions within the promoter area of *AN3* used for qChIP analyses are detailed in Table S1. A. U., arbitrary units. Error bars represent s. e. m. of three independent biological replicates, N = 3; *p*-values <0.05 for statistical significance of differences between the wild-type and *OTLD1* OE-3/*kdm1c-1*, *OTLD1* OE-4/*kdm1c-1*, *KDM1C* OE-2/*otld1-1* or *KDM1C* OE-3/*otld1-1* lines.

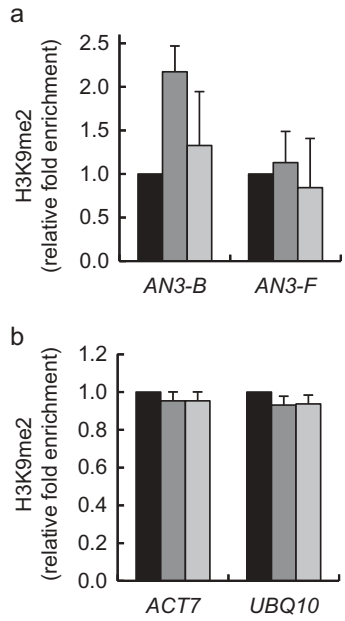

Figure 7. Effect of gain-of-function of *KDM1C* on H3K9 dimethylation of *AN3* chromatin in *OTLD1* loss-of-function plants. (a) H3K9 methylation of the *AN3* chromatin. (b) H3K9 methylation of the reference genes *ACT7* and *UBQ1*. Wild-type plants, black bars; *KDM1C* OE-2/*otld1-1* plants, dark gray bars; *KDM1C* OE-3/*otld1-1* plants, light gray bars. Locations of the indicated regions within the promoter area of *AN3* used for qChIP analyses are detailed in Table S1. The methylation level in the wild-type plants is set to 1.0. Error bars represent s. e. m. of three independent biological replicates, N = 3; *p*-values <0.05 for statistical significance of differences between the wild-type and *KDM1C* OE-2/*otld1-1* or *KDM1C* OE-3/*otld1-1* lines.

promoter chromatin and set the KDM1C specificity for the methylated H3K9 to determine the correct transcriptional outcome.

Methods

Plants

Wild-type *Arabidopsis thaliana* (ecotype Col-0) and T-DNA insertional mutant lines *kdm1c-1* (formerly s*wp1-1*) (SALK_142477) [26] and *otld1-1* (SALK_028707) [10] plants were grown at 22°C under long-day conditions (16-h light/8-h dark cycle at 140 µE $sec^{-1}m^{-2}$ light intensity) as described previously [11,12].

Production of transgenic plants

For gain-of-function expression of *KDM1C* or *OTLD1*, their coding sequences were amplified from *Arabidopsis* cDNA using primers detailed in Table S1, cloned into the XhoI-KpnI sites of pSAT4-HIS-C1 [28], excised and inserted into the I-SceI site of the binary pPZP-RCS2 vector [29]. These constructs were used to transform either wild-type Arabidopsis plants or *kdm1c-1* or *otld1-1* mutants as described [11], and the T2 progeny of the resulting *KDM1C* OE-1, *OTLD1* OE-3/*kdm1c-1*, *OTLD1* OE-4/*kdm1c-1*, *KDM1C* OE-2/*otld1-1*, and *KDM1C* OE-3/*otld1-1* lines was used for further analyses. The *OTLD1* OE-1 and *OTLD1* OE-2 lines have been described previously [11].

Quantitative RT-PCR (RT-qPCR)

Total RNA (1 µg) extracted from aerial parts of 21-days-old plants using the NucleoSpin RNA Plant Kit (Macherey-Nagel GmbH & Co.) was reverse transcribed using the RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific Inc.). The resulting cDNA sample (2 µl) was amplified

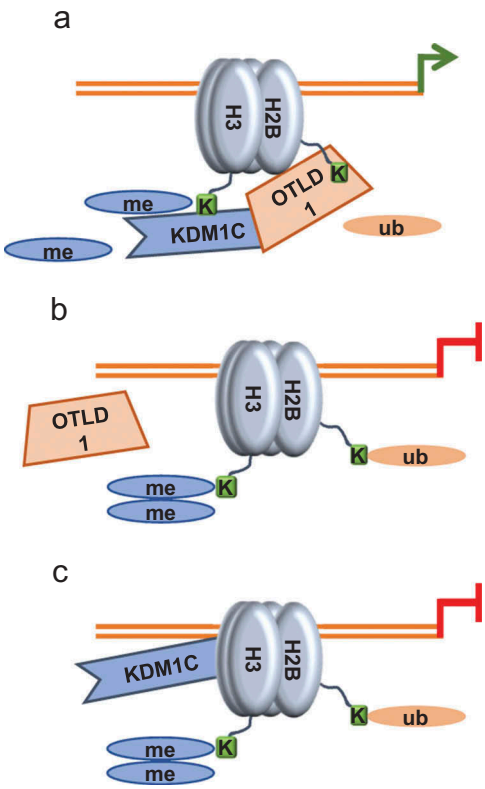

Figure 8. Schematic illustration of coordinate action of KDM1C and OTLD1 in histone modification of the *AN3* chromatin and transcriptional activation of the *AN3* gene. (a) KDM1C and OTLD1 associate with the *AN3* promoter chromatin and demethylate H3K9 and deubiquitylate H2B, respectively, promoting accumulation of the heterochromatic histone mark H3ac and transcriptional activation of *AN3*. These functional effects of KDM1C and OTLD1 on the AN3 chromatin are interdependent. (b) In the absence of KDM1C, OTLD1 does associate with and modify the target chromatin and is unable to activate *AN3* transcription. (c) In the absence of OTLD1, KDM1C still associates with the target chromatin, albeit at different locations, but is unable to modify it or to activate *AN3* transcription.

using Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific Inc.) and specific primers (Table S1) in a StepOnePlus real-time PCR system (Applied Biosystems) with previously-described program cycles [11,12]. Each sample was analyzed in three biological replicates using the reference genes *UBQ10* (At4g05320) and *ACT7* (At5g09810) [11,12] to normalize the data by the comparative C_t method [30]. All quantitative data were analyzed by the Student's *t*-test with *p*-values < 0.05, corresponding to the statistical probability of >95%, considered statistically significant. Standard error of the mean (s. e. m.) and *t*-test calculations were performed using Excel 2010 (Microsoft Corporation Inc.).

Quantitative chromatin immunoprecipitation (qChIP)

qChIP was performed as described [11]. Briefly, cell nuclei were isolated from 21-days-old plants cross-linked by 1% formaldehyde (v/v). Chromatin was sheared by sonication, pre-incubated with pre-equilibrated protein A agarose beads (16–157, MilliporeSigma), centrifuged, and the supernatant was incubated at 4°C for overnight with 1:133 dilution of the appropriate antibody [anti-acetyl-histone H3K9me2 (07–212, MilliporeSigma), anti-trimethyl H3K4 (8580, Abcam), or anti-penta-His (34,660, Qiagen)], combined with protein A agarose (60 µl) and further incubated at 4°C for 2 h. The immunocomplexes immobilized on the protein A agarose beads were treated as described [11], DNA eluted and analyzed by qPCR using the appropriate primers (Table S1) as described for RT-qPCR. For anti-His qChIP, non-specific background immunosignal control obtained with wild-type *Arabidopsis* plants that do not express the His epitope was subtracted from the qPCR data.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author Contributions

I.K. designed, performed, analyzed, and discussed all the experiments and data in this study and wrote the manuscript. V.C. and M.L. provided conceptual guidance, secured required funding, and reviewed and edited the manuscript.

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