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The H3K4 Demethylase Lid Associates with and Inhibits Histone Deacetylase Rpd3▽

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JmjC domain-containing proteins have been shown to possess histone demethylase activity. One of these proteins is the *Drosophila* histone H3 lysine 4 demethylase Little imaginal discs (Lid), which has been genetically classified as a Trithorax group protein. However, contrary to the supposed function of Lid in gene activation, the biochemical activity of this protein entails the removal of a histone mark that is correlated with active transcription. To understand the molecular mechanism behind the function of Lid, we have purified a Lid-containing protein complex from *Drosophila* embryo nuclear extracts. In addition to Lid, the complex contains Rpd3, CG3815/*Drosophila* Pf1, CG13367, and Mrg15. Rpd3 is a histone deacetylase, and along with Polycomb group proteins, which antagonize the function of Trithorax group proteins, it negatively regulates transcription. By reconstituting the Lid complex, we demonstrated that the demethylase activity of Lid is not affected by its association with other proteins. However, the deacetylase activity of Rpd3 is greatly diminished upon incorporation into the Lid complex. Thus, our finding that Lid antagonizes Rpd3 function provides an explanation for the genetic classification of Lid as a positive transcription regulator.

Gene transcription is regulated in part by modulating the conformation of chromatin, which consists of DNA wrapped around histone proteins. Chromatin conformation can be altered by posttranslational modifications of histone tails, such as acetylation and methylation (12). The acetylation of lysine residues on histone tails likely regulates transcription in a positive manner through one of two mechanisms. First, the acetylation of histone tails may inhibit chromatin compaction, as exemplified by acetylated lysine 16 of histone H4 (31). Second, acetylated histone tails may serve as docking sites for effector proteins that promote gene transcription (19). The steady state of histone acetylation is regulated by a balance between histone acetyltransferases that add acetyl groups and histone deacetylases (HDACs) that remove these moieties from histone tails (43). One of the most prominent deacetylases is Rpd3 (reduced potassium dependency 3), which was initially identified in yeast as a transcriptional repressor with homologues in higher eukaryotes that belong to the class I HDACs (26, 42). The repressive function of Rpd3 is further underscored by the fact that it can act in concert with some Polycomb group (PcG) protein complexes to efficiently silence target genes by virtue of histone deacetylation (22, 35, 40).

The methylation of histone lysines is associated with either active or repressed gene transcription, depending on the modified lysine residues. In general, the methylation of lysines 4, 36, and 79 of histone H3 is associated with active transcription, whereas methylation on lysines 9 and 27 of histone H3, as well as lysine 20 of histone H4, correlates with gene repression (4). Although histone methylation was believed previously to be a static modification, recent studies indicated that this histone mark can also be actively removed by histone demethylases (30, 37). The majority of histone demethylases identified so far contain the evolutionarily conserved JmjC domain as a signature motif (10). Based on sequence homology in the JmjC domain and the overall architecture of associated motifs, JmjC domain-containing proteins have been classified into seven groups (10), and members of five groups have been demonstrated to possess histone lysine demethylase activity (1). The *Drosophila* protein Little imaginal discs (Lid) has been shown to possess demethylase activity toward trimethylated histone H3 lysine 4 (H3K4me3) (7, 15, 29), a mark that is enriched in promoters and that correlates with active transcription (28). Genetically, the gene *lid* has been classified as a member of the Trithorax group (TrxG) of genes (9), which act as transcriptional activators and antagonize PcG protein function (25). TrxG proteins, in particular Trithorax and Ash1 (absent, small, or homeotic discs 1), methylate H3K4 and thus contribute to active transcription (3, 5, 33). Unexpectedly, the enzymatic activity of Lid is to remove H3K4 methylation, a mark set by other members of its genetic class. This raises the question of what may be the molecular basis for the genetic classification of *lid* as a TrxG gene.

In order to study the molecular basis of transcription regulation by Lid, we have purified a Lid-containing protein complex from *Drosophila* embryonic nuclear extracts (NE). Interestingly, one component of this complex is Rpd3, a well-known HDAC. En-

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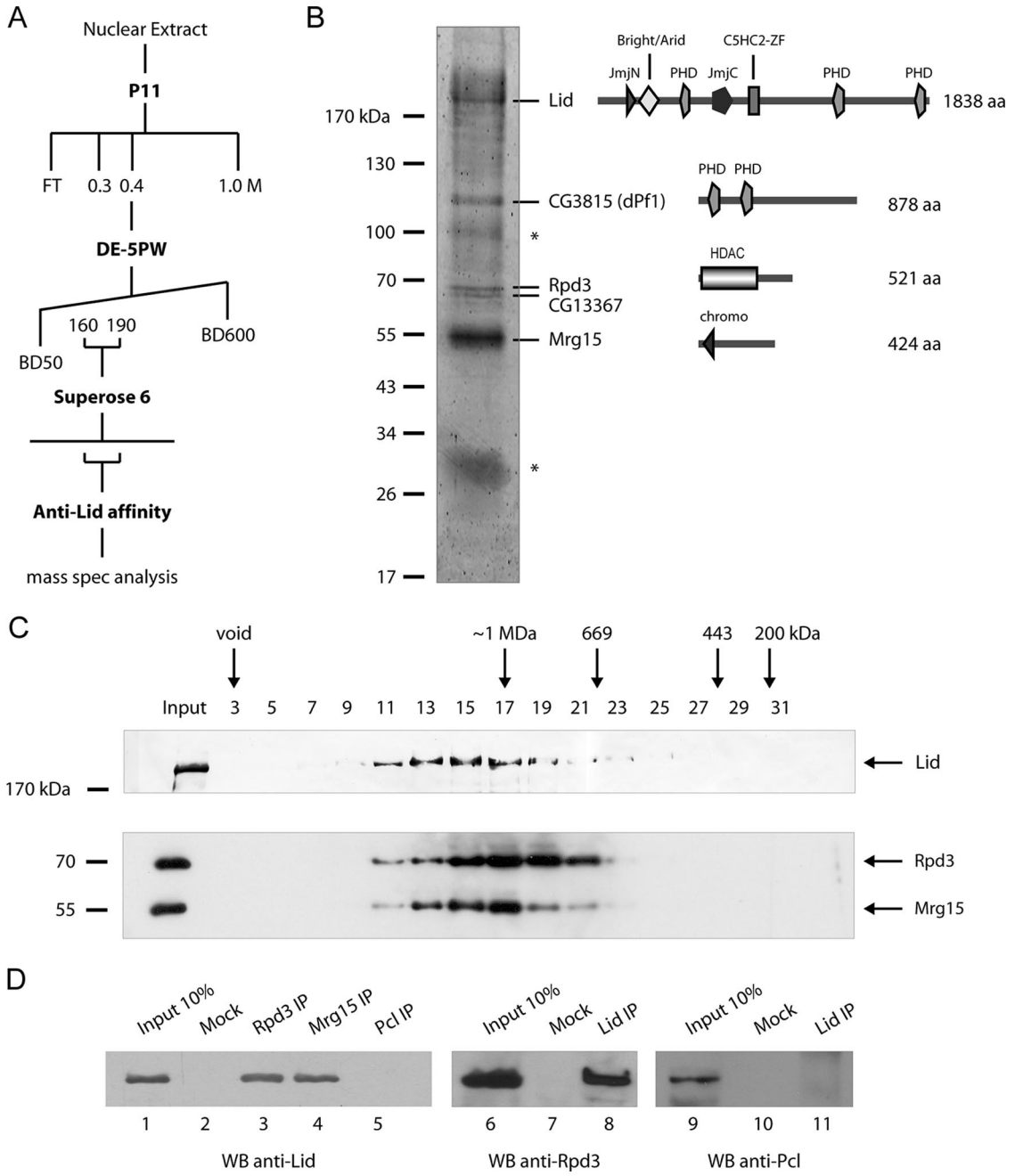

FIG. 1. Rpd3 copurifies with a Lid-containing protein complex. (A) The purification scheme for isolating a Lid-containing complex from *Drosophila* embryonic NE is shown. Molar concentrations refer to KCl in fractions. FT, flowthrough; DE-5PW, DEAE-5PW; mass spec, mass spectrometry. (B) Following anti-Lid affinity purification, proteins were resolved by SDS gel electrophoresis. Coomassie blue-stained bands were identified by mass spectrometry. Asterisks show nonspecific bands. aa, amino acids; chromo, chromodomain. (C) Rpd3 and Mrg15 coelute with Lid following gel filtration chromatography on a Superose 6 column. The elution profile of protein standards for this column and the estimated molecular mass of the Lid complex are indicated at the top. (D) Coimmunoprecipitation using embryo NE demonstrates the association of Lid with Rpd3 and Mrg15. As a control for antibody specificity, anti-Pcl antibody was included in the coimmunoprecipitation experiments. IP, immunoprecipitation; WB, Western blotting.

zymatic studies with a reconstituted complex show that the HDAC activity of Rpd3 is greatly diminished in the context of the Lid complex. This finding can be recapitulated in *Drosophila* S2 cells, suggesting that Lid may function as a transcription activator by inhibiting histone deacetylation.

MATERIALS AND METHODS

Purification of a Lid-containing complex. *Drosophila* embryonic NE was prepared from 0- to 24-h-old embryos as described previously (8). The scheme for conventional chromatography purification is outlined in Fig. 1A. Peak fractions of Lid were identified using a previously described rabbit polyclonal antibody

against the N-terminal region of Lid (15). Approximately 900 mg of NE was fractionated on a phosphocellulose P11 (Sigma) column by stepwise elution using elution buffer (40 mM HEPES [pH 7.9], 0.2 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) containing the KCl concentrations indicated in Fig. 1A. The 0.4 M fraction was loaded onto a DEAE-5PW column (TosoHaas), and the bound proteins were eluted with a linear gradient from 50 mM (BD50) to 600 mM (BD600) ammonium sulfate in elution buffer. Fractions containing the Lid complex eluting at BD160 to BD190 were pooled and further separated by gel filtration chromatography on a Superose 6 column (Amersham) using elution buffer containing 300 mM KCl. For affinity purification of Lid-associated proteins, anti-Lid antibody was coupled to protein A-agarose beads (RepliGen). The resin was incubated overnight at 4°C with fractions 11 to 16 from the Superose 6 fractionation (see Fig. 1C) and washed extensively. The immunoadsorbed complex was separated by sodium dodecyl sulfate (SDS) gel electrophoresis, and the resulting protein bands were subjected to mass spectrometry analysis. For complex verification, rabbit anti-Rpd3 and anti-Mrg15 antibodies (kind gifts from James Kadonaga, University of California, San Diego, and Thomas Kusch, Rutgers, respectively) were used at a 1:5,000 dilution for Western blot analysis.

Reconstitution of the Lid complex. Clones of the Lid complex component cDNAs (Lid clone no. LD40310, *Drosophila* Pf1 [dPf1] clone no. GH06635, CG13367 clone no. RH61522, Rpd3 clone no. GM14158, and Mrg15 clone no. LD22902) were obtained from the Drosophila Genomics Resource Center. The cDNAs were cloned into the pFastBac-HTB vector (Invitrogen) expressing an N-terminal His tag or into a modified version of this vector expressing an N-terminal Flag tag to generate baculoviruses for protein expression in Sf9 cells. The optimal viral titer for each component was empirically determined and used for purifying the reconstituted complex by Flag immunoprecipitation (Flag-IP) using anti-Flag M2 affinity beads (Sigma) and gel filtration chromatography. To purify Rpd3-containing subcomplexes, Sf9 cells were infected with baculovirus expressing Flag-Rpd3, along with baculovirus expressing His-Mrg15, His-CG13367, or His-Lid. Cell lysates were subjected to nickel-nitrilotriacetic acid (Ni-NTA) affinity purification according to the instructions of the Ni-NTA manufacturer (Qiagen), followed by Flag-IP. For Western blot analysis, anti-Flag (Sigma) and anti-His (Santa Cruz) antibodies were used at a 1:1,000 dilution.

Histone demethylase reaction. The histone demethylase reaction was carried out in demethylase buffer [50 mM HEPES (pH 7.9), 25 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM α-ketoglutarate, 2 mM ascorbate) using synthetically methylated histone substrates generated as described by Simon et al. (32). The reaction mixtures were incubated for 3 h at 30°C, and the reactions were stopped by the addition of SDS loading buffer and subsequently analyzed by Western blotting using rabbit anti-dimethylated H3K4 (anti-H3K4me2) antibody (product no. ab7766 [Abcam]; 1:500 dilution) and mouse anti-H3 antibody (product no. ab10799 [Abcam]; 1:2,000 dilution).

HDAC reaction. Histone deacetylation was carried out in deacetylation buffer (75 mM Tris [pH 7.5], 150 mM NaCl, 2 mM dithiothreitol, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) using HeLa cell core histones, which were prepared as described by Zhang et al. (47), as substrates. For the deacetylation reactions involving the mixing of Rpd3 with other proteins (see Fig. 3E), Flag-Rpd3 enzyme was preincubated for 30 min at 30°C with the other proteins prior to the addition of core histones. The deacetylation reaction mixtures were incubated at 30°C for 1 h and analyzed by Western blotting using rabbit anti-acetylated histone H3 (anti-AcH3) antibody (product no. 06-599 [Upstate]; 1:1,000 dilution). Alternatively, differently acetylated histone forms were resolved by Triton-acetic acid-urea (TAU) gel electrophoresis followed by Coomassie blue staining as described previously (16). To determine the level of histone H4 acetylation by Western blotting, anti-acetylated histone H4 (anti-AcH4) antibody (product no. 06-866; Upstate) was used at a 1:2,000 dilution.

Cell culture and immunostaining. S2 cells (obtained from Greg Rogers, University of North Carolina) were grown in Sf-900 II serum-free medium (Gibco) at room temperature. Mrg15, CG13367, wild-type Lid, and H637A mutant Lid (Lid^{H637A}) cDNAs including sequences encoding N-terminal Flag tags were cloned into the pAc5.1 vector (Invitrogen), and cells were transfected using Effectene according to the instructions of the manufacturer (Qiagen). For immunostaining, cells were fixed in 2% paraformaldehyde in Brower buffer (50 mM PIPES [pH 6.9], 1 mM $MgSO_4$, 0.5 mM EGTA, 0.5% NP-40) for 2 h at 4°C. Afterward, cells were washed in PBNT (0.5 M NaCl, 10 mM $NaPO_4$ [pH 7.0], 1% bovine serum albumin [BSA], 0.1% Triton X-100) and blocked in PBNT containing 10% goat serum for 30 min. Mouse anti-Flag antibody was used at a 1:2,000 dilution, and rabbit anti-Odd antibody (a kind gift from James Skeath, Washington University in St. Louis) was used at a 1:100 dilution.

RNA isolation and RT-PCR. RNA from S2 cells was isolated using Trizol (Invitrogen), and cDNA for reverse transcriptase PCR (RT-PCR) was generated using SuperScript III according to the instructions of the manufacturer (Invitrogen). Primers used for RT-PCR were as follows: for *ftz*, 5′-CGAGGAGACTT TGGCATCAG-3′ and 5′-ACGCCGGGTGATGTATCTATT-3′; for *eve*, 5′-CC CTGGTTGTGGACCTCTT-3′ and 5′-ACTGGATAGGCATTCATTTGG-3′; for *odd*, 5′-AGCAACATAACCGTGGATGAC-3′ and 5′-AGCATGGGGGAG AGACTTG-3′; for *lid*, 5′-ACGGCCTTTATCGGGTATTT-3′ and 5′-TTGAA CAGCAACACCACCAG-3′; and for *rp49*, 5′-TGCACCAGGAACTTCTTGA AT-3′ and 5′-ATACAGGCCCAAGATCGTGAA-3′.

ChIP. Chromatin immunoprecipitation (ChIP) was carried out using a dual cross-linking approach with DSG [di(*N*-succinimidyl) glutarate] followed by formaldehyde as described online at www.epigenome-noe.net (PROT29). The following primers were used to quantify the immunoprecipitated material by real-time PCR: for *ftz*, 5′-TGCACATCGCAGAGTTAGAGA-3′ and 5′-ATGT TGTCGGCGTAGCTGTAG-3′; for *eve*, 5′-AGAGCGCAGCGGTATAAAA G-3′ and 5′-GGCAGTTAGTTGTTGACTGTGC-3′; and for *odd*, 5′-AAAGC AAAAGCAAAAGCAACA-3′ and 5′-ACGCTTGAGAATCGAAGTGAA-3′.

Immunostaining of polytene chromosomes. The *yw* strain of *Drosophila melanogaster* was used as the wild type. The salivary gland-specific AB1-Gal4 line was obtained from the Bloomington stock collection (stock no. 1824). To generate transgenic flies that overexpress Lid, Lid cDNA including a sequence encoding an N-terminal Flag tag was cloned into the pUAST vector. For Gal4-driven overexpression, flies were reared at 27°C. Polytene chromosomes were immunostained as described previously (23). In brief, salivary glands of mid-third-instar larvae were dissected and fixed in a solution of 45% acetic acid and 4% paraformaldehyde for 8 min on poly-L-lysine-coated slides before being squashed. The slides were washed twice in phosphate-buffered saline (PBS), incubated in PBS–1% Triton X-100 for 10 min, and blocked in PBS–5% nonfat dried milk for 1 h. Primary antibodies (rabbit anti-Rpd3, 1:200; mouse anti-Flag, 1:1,000; and rabbit anti-AcH3, 1:250) were diluted in PBS–1% BSA and incubated for 2 h at room temperature. Secondary antibodies (anti-rabbit immunoglobulin G [IgG]-Cy3, 1:400, and anti-mouse IgG-AF488, 1:400) were diluted in PBS containing 2% normal donkey serum and incubated for 1 h at room temperature. The slides were washed in washing buffer (PBS containing 0.2% Tween 20 and 0.2% NP-40) with NaCl concentrations of 300, 400, and 500 mM. Chromosomes were stained in DAPI (4′,6-diamidino-2-phenylindole; 100 ng/ml) and mounted with fluorescent mounting medium (DakoCytomation).

RESULTS

Purification of a Lid-containing complex. Lid has been genetically identified as a member of the TrxG of proteins (9), which act as transcriptional activators. Contrary to the genetic classification of Lid, the biochemical activity of the protein is to demethylate H3K4, thereby removing a mark of active transcription. To resolve this contradiction, we sought to identify functional partners of Lid. Using conventional chromatography, we purified a Lid-containing protein complex from *Drosophila* embryonic NE (Fig. 1A). Mass spectrometry analysis identified Lid-associated proteins as CG3815, Rpd3, CG13367, and Mrg15 (Fig. 1B). CG3815 (hereinafter referred to as dPf1) is homologous to mammalian Pf1 (PHD zinc finger protein 1), which has been reported to associate with HDACs such as Rpd3 (44). CG13367 is an uncharacterized protein distantly related to mammalian ocular development-associated gene/GATA zinc finger domain-containing protein 1 (ODAG/Gatad1) (38). Mrg15 contains an N-terminal chromodomain whose mammalian homologue has been shown to bind to H3K36me3 (46). Additionally, Mrg15 is part of the *Drosophila* Tip60 complex that functions in histone exchange (13), and Mrg15 is also found in a mammalian HDAC-containing complex together with Pf1 (45).

To verify that these proteins form a complex, we compared the elution profile of Lid with those of Rpd3 and Mrg15 by using a gel filtration column. Western blot analysis confirmed that Rpd3 and Mrg15 coelute with Lid (Fig. 1C). We could not examine the elution patterns of dPf1 and CG13367 because

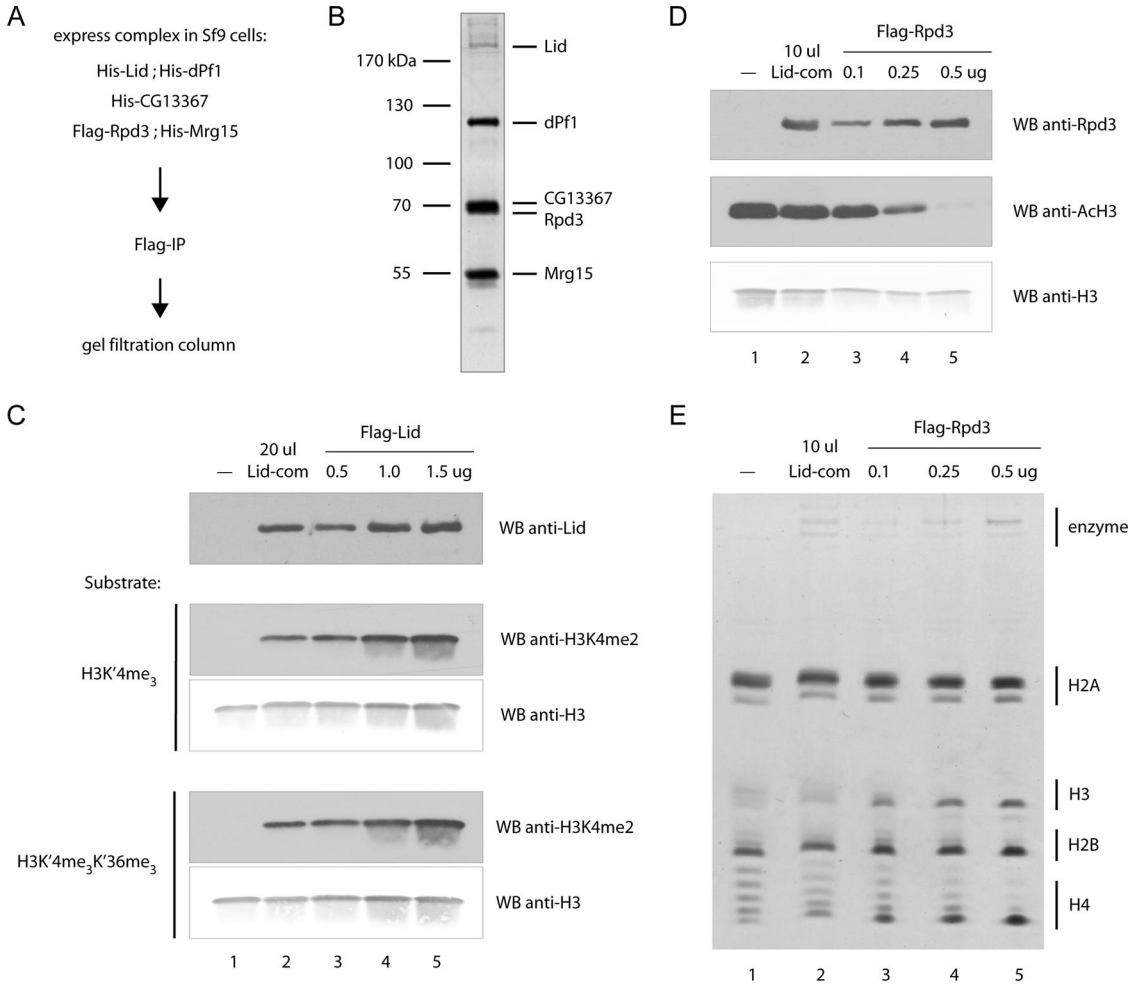

FIG. 2. The incorporation of Rpd3 into the Lid complex reduces the deacetylase activity of Rpd3. (A) Reconstitution scheme for the Lid complex. Sf9 cells were infected with baculoviruses expressing His-tagged Lid, His-dPf1, His-CG13367, Flag-Rpd3, and His-Mrg15. The complex was immunoprecipitated from cell lysate by using Flag antibodies and further purified with a Superose 6 column. (B) Silver-stained gel with the reconstituted Lid complex obtained as depicted in panel A. (C) Histone demethylase assay with the Lid complex (Lid-com) and recombinant Lid. The amount of Lid in the complex was determined (top), and the complex was subsequently subjected to histone demethylation reactions using synthetic H3K'4me3 (middle) or H3K'4me3K'36me3 (bottom) as a substrate. Western blotting (WB) using anti-H3K4me2 antibody was carried out to detect the reaction product. Western blotting with anti-H3 antibody served as a loading control. −, no Lid. (D) The HDAC activity of Rpd3 is inhibited by its incorporation into the complex. The amount of Rpd3 in the complex was determined (top), and HDAC reactions were carried out by incubating the Lid complex and recombinant Rpd3 with core histones and then performing Western blot analysis using anti-AcH3 antibody (middle). Western blotting with anti-H3 antibody served as a loading control (bottom). (E) Core histones were incubated with the Lid complex or with increasing amounts of recombinant Rpd3. The reaction products were analyzed by electrophoresis on a TAU gel to visualize differentially acetylated histone forms. While as little as 0.1 μg of recombinant Rpd3 is sufficient to show deacetylase activity, a larger amount of Rpd3 incorporated into the Lid complex is unable to deacetylate core histones.

antibodies against these proteins were not available. To further verify the association of these proteins, we carried out coimmunoprecipitation studies of Lid with Rpd3 and Mrg15. Antibodies against both Rpd3 and Mrg15 coimmunoprecipitated Lid from embryonic NE (Fig. 1D, lanes 3 and 4). Reciprocal immunoprecipitation using a Lid antibody confirmed the association of Lid with Rpd3 (Fig. 1D, lane 8). The coimmunoprecipitation of Mrg15 was not evaluated due to the similarity of its molecular weight to those of antibody heavy chains. These associations are specific as the Lid antibody did not immunoprecipitate Polycomb-like (Pcl) protein, which is a component of a PcG complex, and vice versa (Fig. 1D, lanes 5 and 11) (21, 27).

Reconstitution and characterization of the demethylase activity. To characterize the enzymatic activities of the Lid complex, we attempted to reconstitute the complex in vitro. To this end, we generated baculoviruses that expressed Flag-Rpd3 along with the remaining components, each harboring a His tag. After the coinfection of Sf9 cells, the complex was first affinity purified by Flag-IP and then subjected to gel filtration chromatography (Fig. 2A). The overexpressed proteins copurified as a protein complex (Fig. 2B), demonstrating that complex formation with the identified subunits can be recapitulated.

Previous studies have shown that the activities of histone-modifying enzymes can be modulated through association with

other proteins (6, 14). To determine whether the enzymatic activity of Lid is modulated by its association with other components, we first quantified the amount of Lid in the complex by comparing the complex to given amounts of recombinant Lid by Western blot analysis (Fig. 2C, top panel). We then compared the demethylase activity of the complex to that of recombinant Lid alone. For the demethylase assays, we employed chemically methylated histones as a substrate (32) and monitored the demethylation reaction by Western blot analysis using methylation state-specific histone antibodies. We adopted this approach because the use of homogeneously methylated histone substrates proved to offer the most sensitive readout for the demethylase activity. We generated chemically trimethylated lysine 4 analogues of histone H3 (H3K'4me3, where the apostrophe denotes the lysine analogue) and monitored the generation of H3K4me2 by Western blot analysis using an H3K4me2-specific antibody. Results shown in Fig. 2C (middle panels) indicate that the Lid complex and Lid alone have similar enzymatic activities when equal amounts of Lid are compared. Thus, we conclude that the incorporation of Lid into the complex does not significantly alter its demethylase activity under our assay conditions. Previous studies have demonstrated that Mrg15 binds to H3K36me3 through the Mrg15 chromodomain (46). This finding prompted us to test whether methylation on H3K36 might help the recruitment of the Lid complex to its substrate and consequently lead to enhanced H3K4 demethylase activity. To this end, synthetic histones that were methylated on both lysine 4 and lysine 36 were generated and used as substrates. Results shown in Fig. 2C (bottom panels) demonstrate that doubly methylated histones are no better substrates for the Lid complex. However, we cannot rule out the possibility that under physiological conditions, methylation at H3K36 might stimulate the demethylase activity through the interaction of Mrg15 with methylated histones. Furthermore, the lack of enhanced enzymatic activity of the Lid complex in vitro may be due to the lack of other as yet unidentified factors in our assay system.

The HDAC activity of Rpd3 is inhibited by the association of Rpd3 with the Lid complex. Next, we analyzed whether the deacetylase activity of Rpd3 is affected by the incorporation of Rpd3 into the Lid complex. To this end, the reconstituted Lid complex and increasing amounts of recombinant Flag-Rpd3 were incubated with core histones and the reaction products were analyzed by Western blotting using an antibody against AcH3. Results shown in Fig. 2D indicate that the incorporation of Rpd3 into the Lid complex greatly inhibited the deacetylase activity of Rpd3 (cf. lanes 2 and 5). We independently confirmed this finding by TAU gel electrophoresis, as this technique allows the visualization of differentially acetylated histone forms. While as little as 0.1 μg of recombinant Rpd3 showed detectable enzymatic activity, the Lid complex containing fivefold the amount of Rpd3 was unable to deacetylate core histones (Fig. 2E, cf. lanes 2 and 3).

Next, we attempted to resolve which subunit of the Lid complex is responsible for the inhibition of Rpd3. To this end, we first determined which subunit interacts with Rpd3 by coinfecting Sf9 cells with baculoviruses expressing Flag-tagged Rpd3 and a His-tagged version of each component of the Lid complex. Proteins that associate with Rpd3 were coimmunoprecipitated using anti-Flag antibody and detected by Western blotting using anti-His antibody. Rpd3 was found to interact with Mrg15, CG13367, and Lid, while it did not interact with dPf1 under the same conditions (Fig. 3A). Next, we reconstituted Rpd3-containing subcomplexes that contained Rpd3 in association with each of its interacting partners by using Ni-NTA affinity followed by Flag-IP (Fig. 3B) and compared the deacetylase activities of these two-component subcomplexes with that of Rpd3 alone. Unexpectedly, we found that the association of Rpd3 with any of the three components of the Lid complex inhibited its HDAC activity (Fig. 3C).

To rule out that the observed inhibition of Rpd3 was caused by the purification procedure involving Ni-NTA affinity and Flag-IP, we purified each component bearing a Flag tag from Sf9 cells (Fig. 3D, left panel) and added the components individually to the deacetylation reaction mixtures. Results shown in Fig. 3E (left panel) demonstrate that the addition of each of the Rpd3 binding partners to the reaction mixtures inhibited the deacetylase activity of Rpd3. To show that the inhibitory nature of these proteins toward Rpd3 is specific, we added unrelated proteins, such as BSA and other JmjC domain-containing proteins (Flag-JHDM3A and His-JHDM1A) (Fig. 3D, right panel) (11, 37) to the deacetylase assay mixtures. Results shown in Fig. 3E (middle panel) demonstrate that the addition of the above-mentioned proteins did not affect the deacetylase activity. Moreover, to show that the inhibition of Rpd3 is caused by direct interaction with its binding partner and is not due to aggregation, we carried out deacetylation reactions with increasing amounts of Lid (Fig. 3E, right panel). Taken together, these results indicate that the inhibitory effects of Mrg15, CG13367, and Lid on Rpd3 are specific.

Lid inhibits the activity of Rpd3 at an Rpd3 target gene. As Mrg15, CG13367, and Lid inhibit the activity of Rpd3 in vitro, we examined whether this inhibition is also observed in vivo. To this end, we transfected *Drosophila* S2 cells with constructs expressing Flag-tagged Mrg15, CG13367, and Lid and analyzed whether the overexpression of these proteins results in the inhibition of Rpd3 activity by monitoring the global AcH3 level. We found that none of these proteins when overexpressed are able to elicit an obvious increase in the global H3 acetylation level (data not shown). One possible explanation for the lack of global HDAC inhibition is the inability of Rpd3 proteins, which are preassembled into existing Rpd3-containing complexes, to associate with the overexpressed proteins.

Nevertheless, we asked whether the inhibition of Rpd3 could occur in a gene-specific manner. Previous studies have shown that Rpd3 regulates segmentation genes, such as *fushi tarazu* (*ftz*), *even-skipped* (*eve*), and *odd-skipped* (*odd*), during embryogenesis (18, 34). To determine whether any of these genes are regulated by Rpd3 in S2 cells in an HDAC activity-dependent manner, we treated S2 cells with the HDAC inhibitor trichostatin A (TSA) and checked whether these segmentation genes become upregulated upon TSA treatment. RT-PCR analysis showed that only *odd* was derepressed after TSA treatment (Fig. 4A), indicating that *odd* may be an Rpd3-responsive target gene in S2 cells. We then examined whether the overexpression of Mrg15, CG13367, or Lid is able to upregulate *odd* expression and consequently result in an enhanced protein level. Immunostaining with antibodies against Odd (41) demonstrated that the levels of Odd protein were increased in cells overexpressing Lid (34 of 55 cells; 62%) but not in cells over-

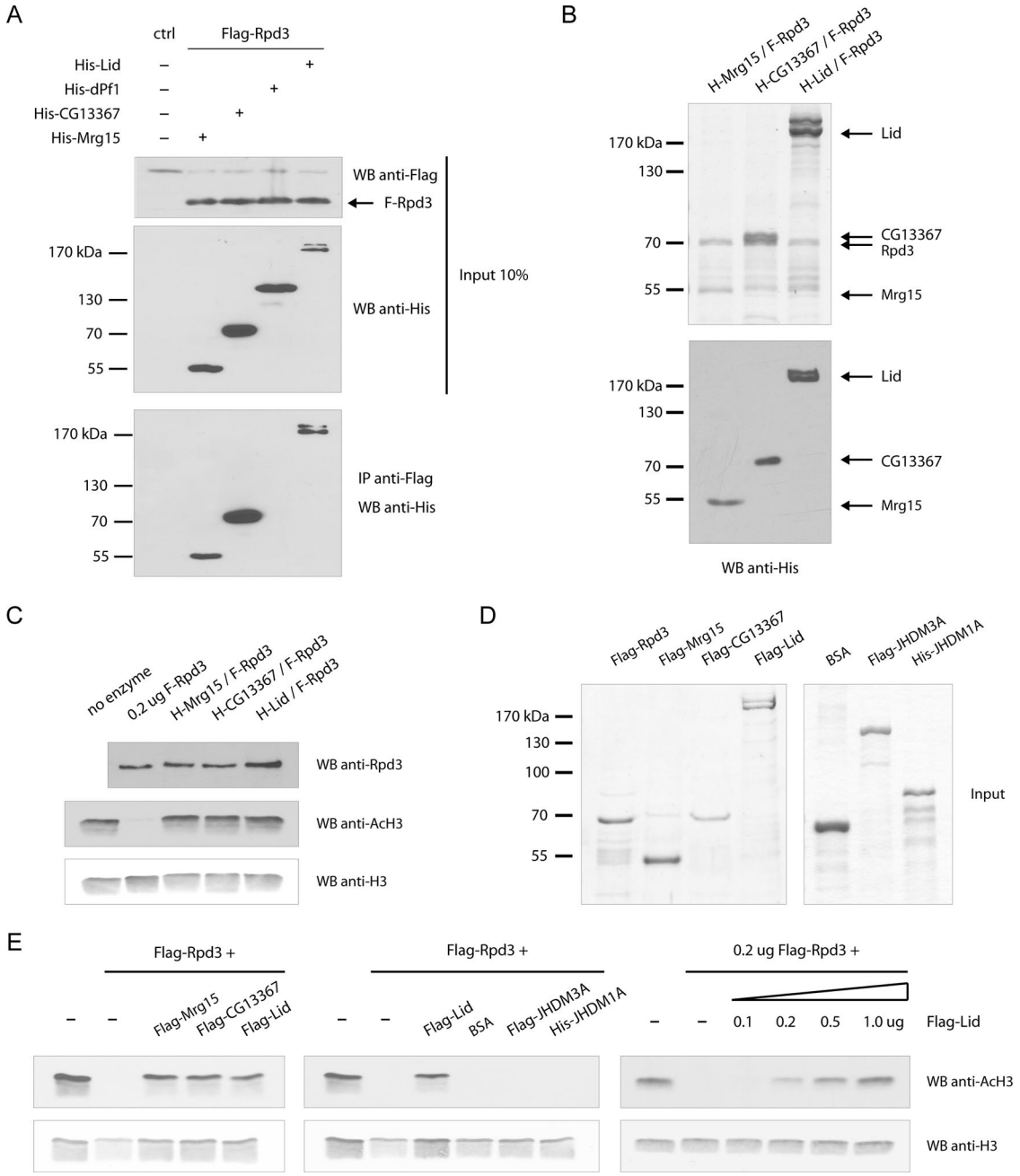

FIG. 3. Association with Mrg15, CG13367, and Lid inhibits Rpd3 activity in vitro. (A) Rpd3 interacts with Mrg15, CG13367, and Lid. Sf9 cells were coinfected with baculoviruses expressing Flag-Rpd3 (F-Rpd3) in combination with His-tagged proteins of the Lid complex. Cell lysates were subjected to immunoprecipitation (IP) with Flag antibody, and interaction partners were detected by Western blot (WB) analysis using anti-His antibody. ctrl, control (no Flag-Rpd3); −, absent; +, present. (B) Reconstitution of Rpd3-containing subcomplexes. Sf9 cells were coinfected with baculoviruses expressing the indicated proteins, and Rpd3-containing subcomplexes were purified from cell lysates using Ni-NTA affinity followed by Flag-IP. A Coomassie blue-stained gel (top) and a Western blot with Rpd3 subcomplexes detected by anti-His antibody (bottom) are shown. H, His; F, Flag. (C) Core histones were incubated with Rpd3 subcomplexes, and HDAC activity was analyzed by Western blotting using anti-AcH3 antibody. Comparable amounts of Rpd3 were used in all reactions, as shown by Western blotting with anti-Rpd3 (top). Association with Mrg15, CG13367, and Lid diminishes the deacetylase activity of Rpd3 in vitro (middle). Anti-H3 Western blotting served as a loading control (bottom). (D) Coomassie blue-stained SDS gels with the indicated Flag-tagged proteins purified from baculovirus-infected Sf9 cells, BSA, and His-JHDM1A purified from *E. coli* are shown. (E) The inhibition of Rpd3 activity can be achieved by mixing individual interaction partners of Rpd3 in vitro. Flag-Rpd3 (0.2 μg) and equimolar amounts of Mrg15, CG13367, and Lid were added to the deacetylation reaction mixtures prior to the addition of core histones. Western blot analysis using anti-AcH3 antibody showed that the addition of each of the interacting partners could inhibit the deacetylase activity of Rpd3 (left panel) but that unrelated proteins did not show any inhibition (middle panel). Increasing amounts of Lid as indicated were added to 0.2 μg of Rpd3 to show dose-dependent inhibition of HDAC activity (right panel). Western blotting with anti-H3 antibody served as a loading control.

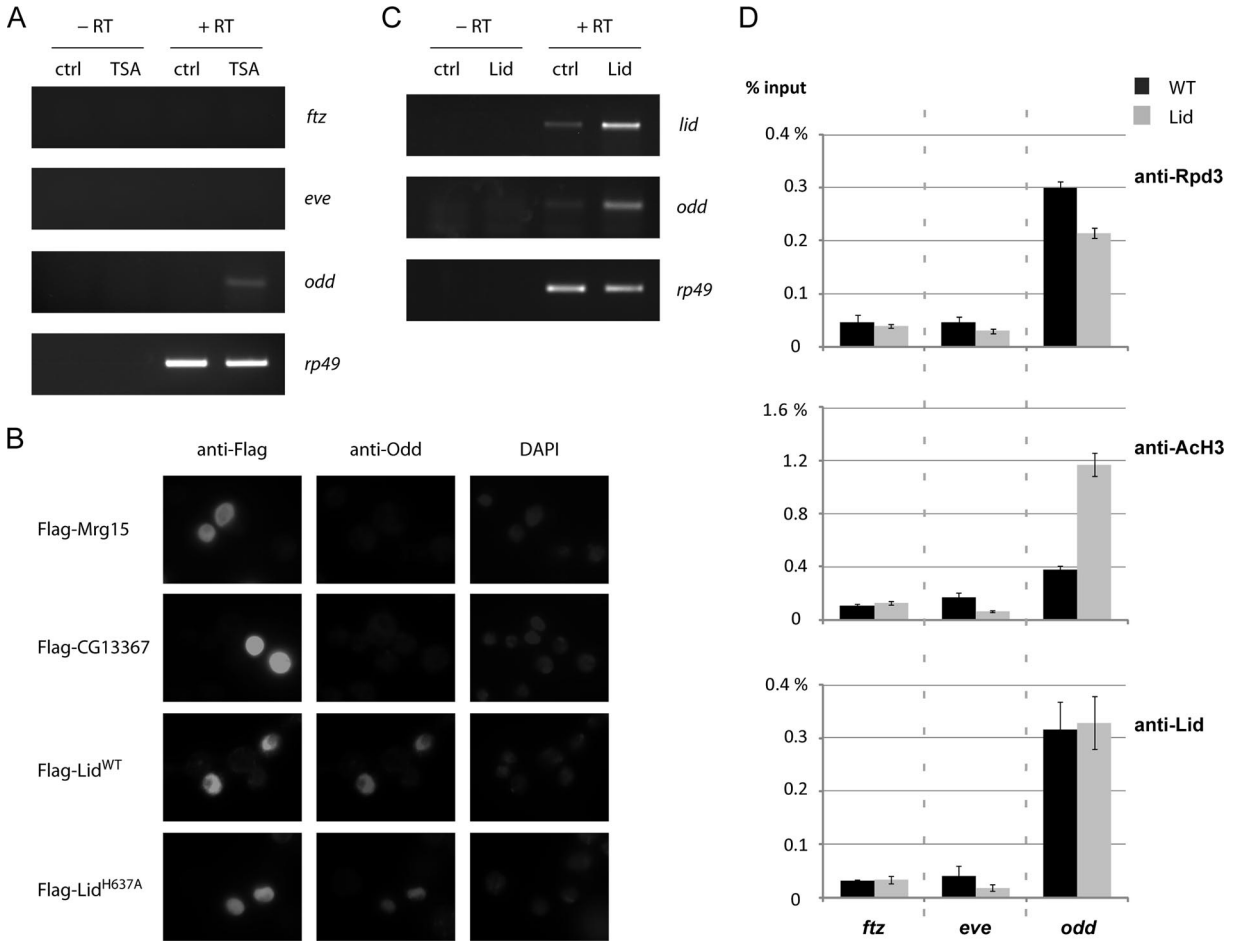

FIG. 4. Lid inhibits the HDAC activity of Rpd3 in vivo. (A) Identification of Rpd3 targets in S2 cells. S2 cells were treated with 100 ng/ml TSA for 48 h to repress deacetylation by Rpd3. RNA was isolated from dimethyl sulfoxide-treated control cells (ctrl) or TSA-treated cells (TSA) and examined for the reactivation of Rpd3 target genes by RT-PCR. Of the three tested segmentation genes, only *odd* was derepressed by TSA treatment. *rp49* served as a loading control. +, with; −, without. (B) S2 cells were transfected with constructs expressing Flag-tagged Mrg15, CG13367, wild-type Lid (Flag-Lid^{WT}), or catalytically inactive Lid (Flag-Lid^{H637A}). Cells were stained with anti-Flag antibody, anti-Odd antibody, and DAPI. Only cells overexpressing wild-type Lid (34 of 55 cells; 62%) and Lid^{H637A} (37 of 67 cells; 58%) showed ectopic staining of Odd at comparable frequencies. (C) The levels of *odd* mRNA in Lid-overexpressing cells are increased. S2 cells were transfected with constructs expressing Lid and GFP at a ratio of 3:1. Lid-overexpressing cells were isolated by FACS analysis using GFP fluorescence. RNA was isolated from control cells (GFP negative) and Lid-overexpressing cells (GFP positive) and subjected to RT-PCR to detect transcripts of *lid* and *odd*. *rp49* served as a loading control. (D) ChIP analysis for the promoter regions of the *ftz*, *eve*, and *odd* gene locus. ChIP was carried out with anti-Rpd3, anti-AcH3, and anti-Lid antibodies and anti-IgG antibody (data not shown) by using chromatin from wild-type (WT) and Lid-overexpressing (Lid) cells. Real-time PCR was employed to quantify the immunoprecipitated material relative to the input.

expressing Mrg15 or CG13367 (Fig. 4B). To rule out the possibility that the derepression of *odd* was due to the histone demethylase activity of Lid and thus independent of the HDAC activity of Rpd3, we overexpressed a catalytically inactive form of Lid (Lid^{H637A}) (15). Results shown in Fig. 4B (bottom panels) demonstrate that the overexpression of the mutant Lid also led to increased levels of Odd at a comparable frequency (37 of 67 cells; 58%), indicating that the derepression of *odd* was independent of the demethylase activity of Lid. To evaluate whether the upregulation of *odd* was due to enhanced transcription and not caused solely by protein stabilization, we cotransfected S2 cells with constructs expressing Lid and green fluorescent protein (GFP) at a ratio of 3:1 and isolated Lid-overexpressing cells by fluorescence-activated cell sorter (FACS) analysis based on GFP fluorescence. We found that the FACS step was necessary, as the transfection efficiency was less than 15%. RT-PCR analysis showed that *odd* transcripts were upregulated in cells overexpressing Lid (Fig. 4C). Considering the facts that *odd* is a known Rpd3 target (18, 34) and that TSA treatment can upregulate the expression of *odd* in S2 cells (Fig. 4A), in combination with the fact that Lid can inhibit the activity of Rpd3, the upregulation of *odd* mediated by the overexpression of Lid is likely to be due to the inhibition of the HDAC activity of Rpd3.

To further examine whether the upregulation of *odd* is caused by Lid-mediated inhibition of Rpd3, we performed ChIP assays analyzing the localization of Rpd3, AcH3, and Lid at the *odd* locus. As a control, we included the promoter regions of *ftz* and *eve*. Expectedly, we observed Rpd3 localization at the *odd* promoter region and its absence at the *ftz* and

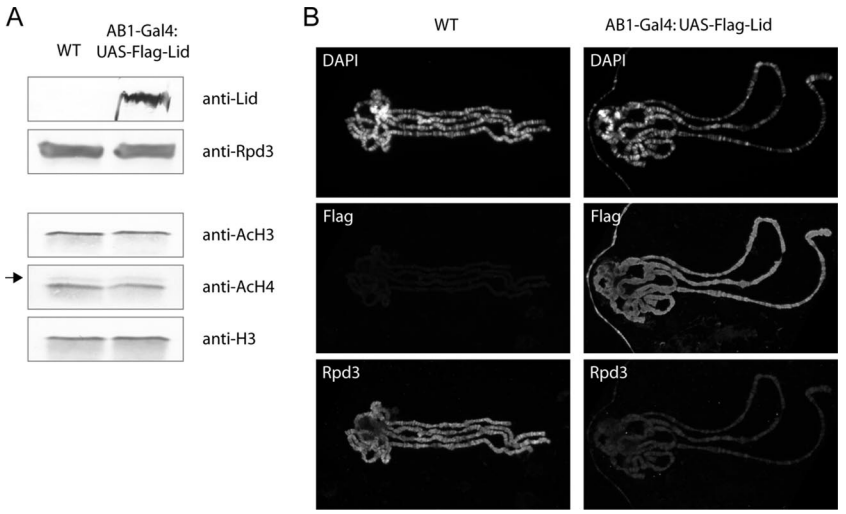

FIG. 5. The overexpression of Lid reduces the binding of Rpd3 to polytene chromosomes. (A) Western blot analysis of protein extracts derived from wild-type (WT) and Lid-overexpressing salivary glands. Ten pairs of salivary glands from mid-third-instar larvae were used in the analysis to detect the levels of Lid, Rpd3, AcH3, and AcH4. The arrow indicates cross-reacting acetylated H2B. Western blotting using anti-H3 antibody served as a loading control. UAS, upstream activation sequence. (B) Immunostaining of polytene chromosomes from wild-type and Lid-overexpressing larvae. Polytene chromosomes were immunostained using anti-Rpd3 and anti-Flag antibodies to detect ectopically expressed Lid.

eve promoters in wild-type S2 cells (Fig. 4D, top panel), consistent with the transcriptional responsiveness of the *odd* gene to TSA treatment. Interestingly, we also found Lid to be present at the *odd* promoter but absent at the promoters of *ftz* and *eve* (Fig. 4D, bottom panel). Following Lid overexpression, the level of AcH3 at the *odd* promoter increased significantly, consistent with the transcriptional reactivation of *odd* (Fig. 4D, middle panel). The levels of AcH3 at the *ftz* and *eve* promoters were unaffected. Intriguingly, we observed that the occupancy of Lid at the *odd* promoter was unchanged upon Lid overexpression and that the amount of Rpd3 binding was decreased moderately but reproducibly. These results suggest that an excessive nuclear pool of Lid may sequester Rpd3 from its target gene. The merely moderate decrease in Rpd3 occupancy may reflect the fact that a heterogeneous population of cells was analyzed by ChIP, as only approximately 60% of cells overexpressing Lid displayed ectopic *odd* expression (Fig. 4B). The unchanged level of Lid at the *odd* locus despite its overexpression may be explained by the fact that the recruitment of Lid requires additional limiting factors. Taken together, these results indicate that the reduced binding of Rpd3 is sufficient to elicit the observed increase in the level of acetylated histones and, thus, elevated transcription.

Lid antagonizes the function of Rpd3 in a transgenic fly line. A recent study has shown that the levels of acetylated histones on larval polytene chromosomes in *lid* mutants are severely reduced (17). Based on our finding that Lid can inhibit the HDAC activity of Rpd3 in vitro, the reduced levels of acetylated histones may be caused by overactive Rpd3 in the absence of Lid in these mutants. To further substantiate a link between Lid and histone acetylation, we generated a transgenic fly line in which Flag-tagged Lid was under the control of a Gal4-inducible upstream activation sequence promoter. Using the salivary gland-specific AB1-Gal4 driver, we observed robust overexpression of Lid in salivary gland tissue, while the level of Rpd3 remained unaffected (Fig. 5A, upper panels). Immunostaining of polytene chromosomes of wild-type larvae with anti-Rpd3 antibody showed a distribution of Rpd3 on less condensed interbands throughout all chromosome arms and its absence from the chromocenter, as described previously (Fig. 5B, left panels) (24). Notably, the overexpression of Lid resulted in a marked decrease in Rpd3 binding on polytene chromosomes, suggesting that excessive Lid is able to displace Rpd3 from chromatin (Fig. 5B, right panels). To rule out that the reduced binding of Rpd3 was caused by compromised chromosome integrity upon Lid induction, we coimmunostained the polytene chromosomes with anti-Flag antibody. The distribution of the ectopic Flag-tagged Lid did not differ from the previously reported distribution of endogenous Lid (15), as it localized to interbands and was absent from the chromocenter. The decreased binding of Rpd3 to polytene chromosomes, however, was not accompanied by elevated levels of AcH3, as analyzed by Western blotting and immunostaining of polytene chromosomes (Fig. 5A, lower panels, and data not shown). In addition, we examined whether the global level of AcH4 was altered by Lid overexpression and concomitant reduction in Rpd3 localization. As the acetylation of lysine 16 of histone H4 is enriched on the male X chromosome as a consequence of dosage compensation (39), we analyzed histones of salivary glands from male and female larvae separately. However, in neither case did we observe an elevated level of AcH4 (Fig. 5A). These results suggest that the temporary removal of Rpd3 from chromatin alone in this tissue is not sufficient to increase the level of acetylated histones.

DISCUSSION

To shed light on the molecular mechanism of how the histone demethylase Lid regulates transcription, we have purified a Lid-containing protein complex, which includes dPf1, Rpd3, CG13367, and Mrg15, from *Drosophila* embryonic NE. Previous studies have shown that the activities of chromatin-modi-

fying enzymes can be modulated through association with other proteins in a complex. Although we did not observe an alteration in histone demethylase activity upon the formation of the Lid complex compared to the activity of recombinant Lid alone, we cannot rule out the possibility that additional factors are required to mediate this stimulatory effect. As we could not use nucleosomes as a substrate for reasons of sensitivity, it is possible that the Lid complex is irresponsive to enhanced demethylase activity on methylated histones. On the other hand, a different Lid-containing complex that is primarily responsible for histone demethylation may exist. Previously, Lid has been reported to interact with dMyc and another TrxG protein, Ash2, in larval eye imaginal discs (29), implying that other, tissue- and developmental stage-specific Lid-containing complexes may exist.

Intriguingly, we observed inhibition of the HDAC activity of Rpd3 in the Lid complex. In this respect, the major function of Lid in this particular complex may be to counteract the transcriptional repression mediated by the deacetylase activity of Rpd3. Notably, Rpd3 has been shown previously to interact with the PRC2 (Polycomb repressive complex 2) complex and to enhance PcG-mediated gene silencing through histone deacetylation (36, 40). As the H3K4 demethylase activity of Lid is not required for *odd* gene activation (Fig. 4B), it is tempting to speculate that the genetic characterization of *lid* as a TrxG gene is due in part to its inhibitory effect on Rpd3. By inhibiting the HDAC activity of Rpd3, Lid may counteract the full extent of PcG-mediated suppression of gene expression, providing an explanation for the contradictory genetic classification of *lid* as a TrxG gene and the enzymatic activity of Lid to remove an active histone mark. From this point of view, it appears possible that the histone demethylase activity of Lid is developmentally dispensable. However, we have observed that *lid* homozygous mutant flies can be rescued only by a transgene encoding wild-type Lid and not by a transgene encoding a catalytically inactive mutant form of Lid (N. Lee and Y. Zhang, unpublished observations), indicating that H3K4 demethylation is developmentally important. Thus, Lid appears to fulfill two possibly distinct functions during development, and these functions may act independently of each other. One function is to demethylate H3K4, whereas the other is to antagonize HDAC activity to promote transcription.

The findings of a recent study substantiate the antagonistic behavior of Lid toward Rpd3. Lloret-Llinares et al. reported that *lid* mutant alleles act as an enhancer of position effect variegation (17), whereas some mutations in *Rpd3* have been found to confer suppressor-of-variegation phenotypes (20). Moreover, polytene chromosomes of *lid* mutants have been shown to have reduced levels of AcH3 (17), which is consistent with our finding that the overexpression of Lid is able to reduce the binding of Rpd3 to polytene chromosomes. Thus, in the absence of Lid, the balance between Lid and Rpd3 would be tilted toward Rpd3, resulting in reduced levels of AcH3.

A similar HDAC complex containing Pf1 and Mrg15 in mammals has been described previously (45). We can envisage that Lid is recruited to a core HDAC complex consisting of Rpd3, dPf1, and Mrg15 (and possibly including additional factors that are part of the HDAC complex) and thereby inhibits the HDAC activity. The recruitment of Lid to the sites of the HDAC complex may act as a switch to turn on the expression of target genes during development. We have shown on a gene-specific level for the *odd* gene in S2 cells by ChIP analysis and on a global level by the immunostaining of polytene chromosomes that the overexpression of Lid results in a marked decrease in Rpd3 binding, suggesting that excessive Lid is able to interact with and displace Rpd3 from its target sites (Fig. 4D and Fig. 5B). We have to point out, however, that our findings are based on conditions of robust overexpression of Lid and that our observations need to be confirmed for target genes of the Lid complex in the context of development.

It is surprising that we find Mrg15 to negatively regulate the HDAC activity of Rpd3 in vitro, because Mrg15 has been shown previously to contribute to transcription repression (45). In this regard, it is possible that the interaction solely between Rpd3 and Mrg15 results in enzymatic inhibition and that interaction with additional factors, such as Sin3, may be required to restore the HDAC activity. Provided that the Lid complex identified in this study does play a role in regulating dynamic histone methylation, another role for Mrg15 is conceivable. The chromodomain of Mrg15 may potentially be involved in recruiting the Lid complex to target genes. The trimethylation of H3K4 peaks in the promoter region, whereas the trimethylation of H3K36 is enriched in the 3′ region of genes (2, 28). During the process of transcription, the chromodomain of Mrg15 may target the Lid complex to the bodies of genes through its interaction with H3K36me3 and induce the removal of H3K4 trimethylation, resulting in the enrichment of the 5′ region of genes with this modification. In the absence of Lid, this distinct border of the different methyl marks would not be sustained and transcription efficiency would deteriorate, thus offering an explanation for the function of Lid in active transcription.

Future genome-wide location studies of Lid and the other components of the complex will reveal which target genes are controlled by this complex. Furthermore, it will be interesting to find out where within target genes the complex is located. Does the complex bind to the bodies of genes to demethylate H3K4, or does the binding take place at promoter regions to regulate dynamic histone deacetylation? The identification of Lid-associated proteins has set the stage for these detailed studies, which will reveal insight into the mechanism underlying transcription regulation by Lid.

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