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The trithorax-group protein Lid is a histone H3 trimethyl-Lys4 demethylase

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Recent studies have demonstrated that histone methylation can be dynamically regulated through active demethylation. However, no demethylase specific to histone H3 trimethyl-Lys4 (H3K4me3) has been identified. Here we report that the *Drosophila melanogaster* protein 'little imaginal discs' (Lid), a JmjC domain–containing trithorax group protein, can demethylate H3K4me3. Consistent with its genetic classification, Lid positively regulates Hox gene expression in S2 cells.

Histone methylation contributes to diverse biological processes[1]. Recent studies indicate that histone methylation, like other histone modifications, is dynamically regulated through active demethylation by two distinct classes of enzymes. The first class of histone demethylase, exemplified by LSD1, catalyzes demethylation of H3K4me2 and H3K4me1 in a flavin adenine dinucleotide (FAD)-dependent manner[2]. The second class of histone demethylase encompasses a large protein family[3] and uses a conserved JmjC domain to catalyze demethylation in an Fe(II)- and α-ketoglutarate–dependent hydroxylation reaction[4]. Several JmjC domain–containing proteins with specificity toward various methylation states of H3 Lys9 and Lys36 (H3K9 and H3K36) have been characterized[4–9]. However, no H3K4me3-specific demethylase has been identified thus far, leaving open the possibility that H3K4me3 might not be a reversible modification.

To facilitate identification of novel histone demethylases, we performed a phylogenetic analysis of the JmjC domain–containing proteins in six model organisms[3]. This analysis allowed us to divide the JmjC domain–containing proteins into seven subfamilies on the basis of conservation in the JmjC domain and overall protein domain architecture[3]. Members from three of the seven subfamilies have been shown to encode active histone demethylases. Of the remaining four subfamilies, the JARID1 subfamily is of particular interest because members of this subfamily contain multiple conserved functional domains (**Supplementary Fig. 1a** online), some of which are reported to participate in transcriptional regulation[10,11]. Notably, the sole

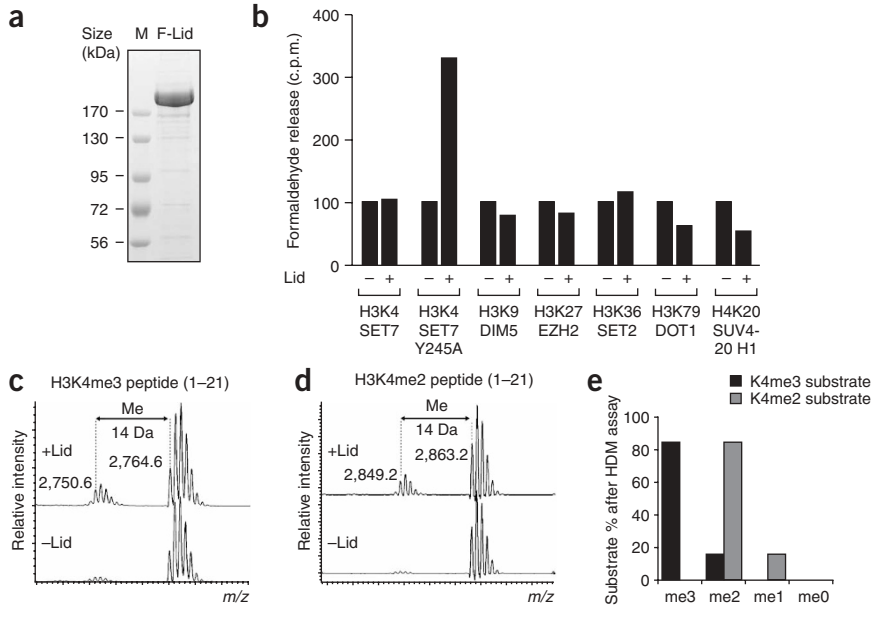

Figure 1 Lid is a histone demethylase with specificity for H3K4me3. (**a**) Coomassie-stained SDS-PAGE gel of Flag-tagged Lid protein purified from baculovirus-infected Sf9 cells. M, marker; F-Lid, Flag-tagged Lid. (**b**) Recombinant Lid can demethylate substrates generated by a SET7 mutant. Various radiolabeled methylhistone substrates were generated and used in demethylation assays with recombinant Lid. The release of radioactively labeled formaldehyde was used to measure enzymatic activity. Owing to steric constrains of the SET7 catalytic site, the wild-type SET7 can only monomethylate H3K4, whereas the Y245A mutant can di- and trimethylate H3K4. (**c**,**d**) Lid demethylates both di- and trimethylated H3K4 peptides. Histone peptides (residues 1–21) containing either tri- (**c**) or dimethylated (**d**) H3K4 were subjected to demethylation reactions in the presence or absence of Lid followed by mass spectrometric analysis. (**e**) Quantification of the mass spectrometry results in **c** and **d**. Relative percentage of peptide substrates at different methylation states after demethylation reaction is shown. Lid has similar efficiency in converting H3K4me3 to H3K4me2 and H3K4me2 to H3K4me1 *in vitro*.

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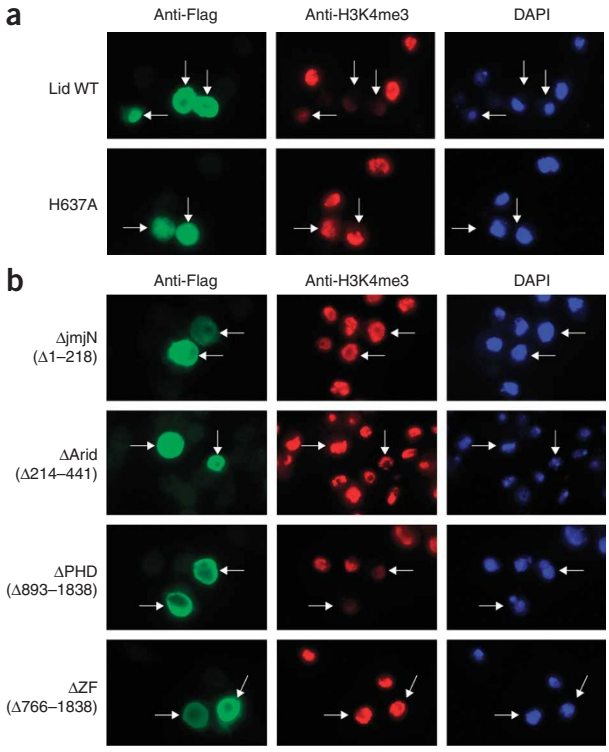

Figure 2 Multiple domains contribute to Lid enzymatic activity. (**a**) Overexpression of wild-type Lid (top images), but not the H637A mutant (bottom images) results in a global decrease of H3K4me3 levels. *Drosophila* S2 cells were transfected with Flag-tagged Lid and costained with antibodies against Flag tag and H3K4me3. (**b**) Demethylase activity of Lid mutants was monitored by H3K4me3 staining in transfected cells. Deletion of the JmjN, Arid and the zinc-finger domains abolished the enzymatic activity, whereas deletion of the C-terminal PHD fingers did not affect Lid's enzymatic activity.

Drosophila homolog of this subfamily, Lid, has been identified genetically as a trithorax group (trxG) protein[12]. The Lid protein contains all the functional domains found in the mammalian JARID1 proteins, and its JmjC domain is also highly conserved (**Supplementary Fig. 1b**). To test whether Lid is an active demethylase, we expressed an N-terminally Flag-tagged Lid protein in Sf9 cells and purified it to near homogeneity (**Fig. 1a**). Incubation of the recombinant Lid protein with various radiolabeled histone substrates in a demethylase assay resulted in release of radioactive formaldehyde only when substrates were generated with a mutant SET7 (**Fig. 1b** and **Supplementary Methods** online), which specifically di- and trimethylates (me2/3) H3K4 (ref. 13). Notably, Lid did not demethylate substrates generated by the wild-type SET7, which only monomethylates H3K4 (ref. 13), nor did it demethylate other known histone methylation sites (**Fig. 1b**). These results indicate that Lid is an H3K4me2/3-specific demethylase. To confirm the substrate specificity of Lid, synthetic peptides containing di- or trimethylated H3K4 were incubated with Lid and analyzed by mass spectrometry. In agreement with the formaldehyde-release assay, Lid was able to demethylate both H3K4me3 and H3K4me2 peptides (**Fig. 1c**,**d**). Quantification of the demethylated peptide product indicated that Lid has similar efficiency toward di- and trimethylated peptides (**Fig. 1e**). Collectively, the above results indicate that Lid is an H3K4me2/3-specific demethylase *in vitro*.

To evaluate whether Lid functions as an active H3K4 demethylase *in vivo*, we transfected a Flag-tagged Lid expression plasmid into *Drosophila* S2 cells and analyzed the effect on H3K4 methylation by indirect immunofluorescence using H3K4 methylation–specific antibodies. Overexpression of Lid greatly reduced H3K4me3 levels (**Fig. 2a**, top images). The reduction was dependent on the demethylase activity of Lid, as overexpression of a Lid mutant (H637A) predicted to disrupt Fe(II) binding had no effect on H3K4me3 levels (bottom images). Although Lid is capable of demethylating an H3K4me2 peptide *in vitro* (**Fig. 1b**), overexpression of Lid did not cause a noticeable change in the H3K4me2 or H3K4me1 levels (**Supplementary Fig. 2a** online). Similarly, trimethylation on H3K9, H3K27 and H3K36 were also unaffected (**Supplementary Fig. 2b**). Collectively, these data indicate that Lid can specifically demethylate H3K4me3 *in vivo*.

The Lid protein contains several other functional domains in addition to the JmjC domain, including JmjN, Arid, PHD and a zinc finger (**Supplementary Fig. 1a**). We have previously demonstrated that, in addition to the JmjC domain, the zinc finger of

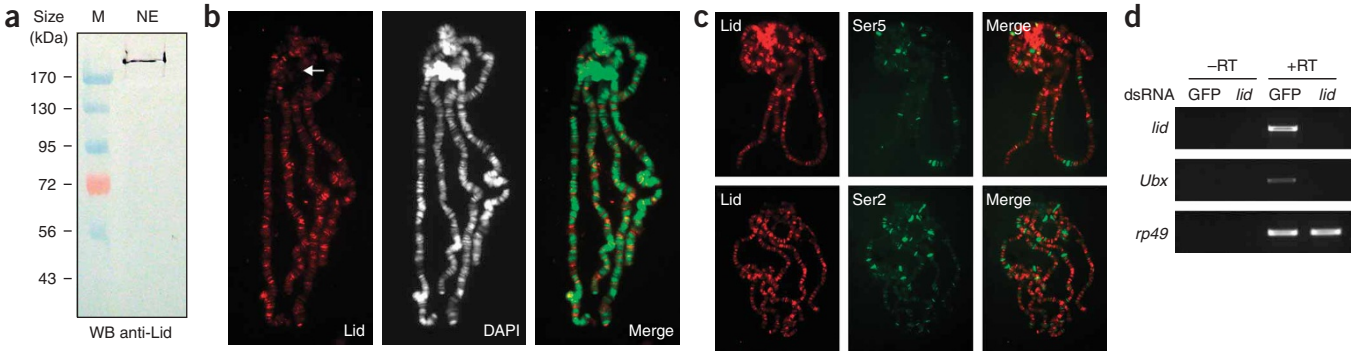

Figure 3 Lid localizes to interbands but does not colocalize with active RNA polymerase II. (**a**) Western blotting analysis of *Drosophila* embryo nuclear extracts (125 μg) indicates that the Lid antibody is specific for Lid. M, protein marker; NE, nuclear extract. (**b**) Distribution of Lid on larval polytene chromosomes. Immunostaining reveals that Lid is localized to interbands but excluded from the chromocenter (arrow) and heavy DAPI bands. (**c**) Lid does not colocalize with active RNA polymerase II. Costaining of Lid with the Ser5- or Ser3-phosphorylated form of RNA polymerase II is shown. (**d**) *lid* knockdown in S2 cells results in decreased *Ubx* expression. S2 cells were treated with double-stranded RNAs (dsRNAs) that target *lid* or GFP. Knockdown was verified by reverse-transcription PCR. GFP dsRNA serves as a control for the RNA interference treatment. *rp49* serves as a control for equal input. The numbers of PCR cycles for *lid*, *Ubx* and *rp49* were 32, 40 and 25, respectively.

JHDM2A (ref. 9) and the JmjN domain of JHDM3A (ref. 7) are essential for enzymatic activity. To define the domain requirements for Lid enzymatic activity, we generated four expression constructs with deletions of the JmjN, Arid, the zinc finger and the two C-terminal PHD fingers (**Supplementary Fig. 3** online). In contrast to expression of the wild-type Lid (**Fig. 2a**), expression of the deletion mutants had no effect on H3K4me3 levels, with the exception of the PHD-finger deletion mutant, which retained demethylase activity (**Fig. 2b**). On the basis of these results, we conclude that although the C-terminal PHD fingers are dispensable for activity, all other domains contribute to the demethylase activity of Lid. It is likely that these domains contribute either to substrate recognition or folding of the JmjC domain.

To gain insight into the potential function of Lid, we generated a polyclonal antibody against Lid protein. After demonstration of the antibody specificity by western blotting of *Drosophila* embryo nuclear extracts (**Fig. 3a**), we investigated the distribution of Lid on *Drosophila* polytene chromosomes. Lid is broadly distributed on these chromosomes (**Fig. 3b**), suggesting that Lid may regulate methylation at many genomic loci. However, Lid is excluded from the chromocenter (left image, arrow) and 4′,6-diamidino-2-phenylindole (DAPI)-heavy regions (**Fig. 3b**, right image and **Supplementary Fig. 4** online). The fact that Lid is excluded from DAPI-heavy regions prompted us to investigate whether Lid is enriched in actively transcribed regions of chromatin. To this end, we costained Lid with RNA polymerase II phosphorylated at Ser5 and Ser2 in the C-terminal domain, modifications that mark transcriptional initiation or elongation, respectively. The strongest bands for active RNAPII do not show concomitant Lid staining (**Fig. 3c**). This is in direct contrast to other TrxG proteins such as Brm and Kis, which show extensive colocalization with active RNAPII[14].

The polytene staining data presented above suggest that Lid may not directly participate in active transcription because it does not overlap with active RNA polymerase II. However, the genetic classification of the *lid* gene as a trxG gene indicates that Lid positively regulates Hox gene expression. Available *lid* mutant alleles seem to be hypomorphic and show highly variable phenotypes[12]. Some *lid* homozygotes die as embryos, whereas others survive to late larval or pupal stages. Those with more severe postembryonic phenotypes have extremely small imaginal discs, which makes molecular analysis of the mutants technically difficult. Therefore, we analyzed the effect of Lid on Hox gene expression in S2 cells using double stranded RNA–mediated knockdown. Consistent with the genetic classification of *lid* as a trxG gene, Lid seems to positively regulate the Hox gene *Ubx*, as knockdown of *lid* decreased the abundance of *Ubx* transcripts (**Fig. 3d**).

H3K4me3 is enriched in promoters of active genes[15,16]; consequently, H3K4me3 is regarded as a mark of active transcription. Consistent with this notion, several trxG proteins involved in maintaining the 'on' state of Hox genes have H3K4 methyltransferase activity[17,18]. Therefore, it was surprising that the biochemical activity of Lid antagonizes those of other trxG proteins. Classification of *lid* as a trxG gene is based on the observation that *lid* mutant alleles respectively suppress and enhance homeotic phenotypes produced by PcG and trxG mutant alleles[12]. Consistent with its genetic classification, Lid positively regulates the *Ubx* gene in S2 cells. To reconcile its biochemical activity with its genetic classification, we favor the possibility that Lid indirectly regulates Hox gene expression. However, we cannot rule out the possibility that an H3K4 methylation-demethylation cycle may be an integral part of the gene activation process. A similar situation has been reported for H2B ubiquitination, where both ubiquitination and deubiquitination are required for efficient transcription[19,20]. Identification of direct target genes of Lid will allow us to differentiate between these two possibilities in the future.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

N.L. and Y.Z. designed the experiments. N.L., J.Z., R.J.K., H.E.-B. and P.T. performed the experiments. Y.Z., N.L. and R.S.J. wrote the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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1. Martin, C. & Zhang, Y. *Nat. Rev. Mol. Cell Biol.* **6**, 838–849 (2005).
2. Shi, Y. *et al. Cell* **119**, 941–953 (2004).
3. Klose, R.J., Kallin, E.M. & Zhang, Y. *Nat. Rev. Genet.* **7**, 715–727 (2006).
4. Tsukada, Y. *et al. Nature* **439**, 811–816 (2006).
5. Cloos, P.A. *et al. Nature* **442**, 307–311 (2006).
6. Fodor, B.D. *et al. Genes Dev.* **20**, 1557–1562 (2006).
7. Klose, R.J. *et al. Nature* **442**, 312–316 (2006).
8. Whetstine, J.R. *et al. Cell* **125**, 467–481 (2006).
9. Yamane, K. *et al. Cell* **125**, 483–495 (2006).
10. Benevolenskaya, E.V., Murray, H.L., Branton, P., Young, R.A. & Kaelin, W.G., Jr. *Mol. Cell* **18**, 623–635 (2005).
11. Lu, P.J. *et al. J. Biol. Chem.* **274**, 15633–15645 (1999).
12. Gildea, J.J., Lopez, R. & Shearn, A. *Genetics* **156**, 645–663 (2000).
13. Xiao, B. *et al. Nature* **421**, 652–656 (2003).
14. Srinivasan, S. *et al. Development* **132**, 1623–1635 (2005).
15. Ng, H.H., Robert, F., Young, R.A. & Struhl, K. *Mol. Cell* **11**, 709–719 (2003).
16. Santos-Rosa, H. *et al. Nature* **419**, 407–411 (2002).
17. Beisel, C., Imhof, A., Greene, J., Kremmer, E. & Sauer, F. *Nature* **419**, 857–862 (2002).
18. Sedkov, Y. *et al. Nature* **426**, 78–83 (2003).
19. Henry, K.W. *et al. Genes Dev.* **17**, 2648–2663 (2003).
20. Kao, C.F. *et al. Genes Dev.* **18**, 184–195 (2004).