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LETTER TO THE EDITOR

# Silkworm (*Bombyx mori*) *Bm*Lid is a histone lysine demethylase with a broader specificity than its homolog in *Drosophila* and mammals

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**Dear Editor,**

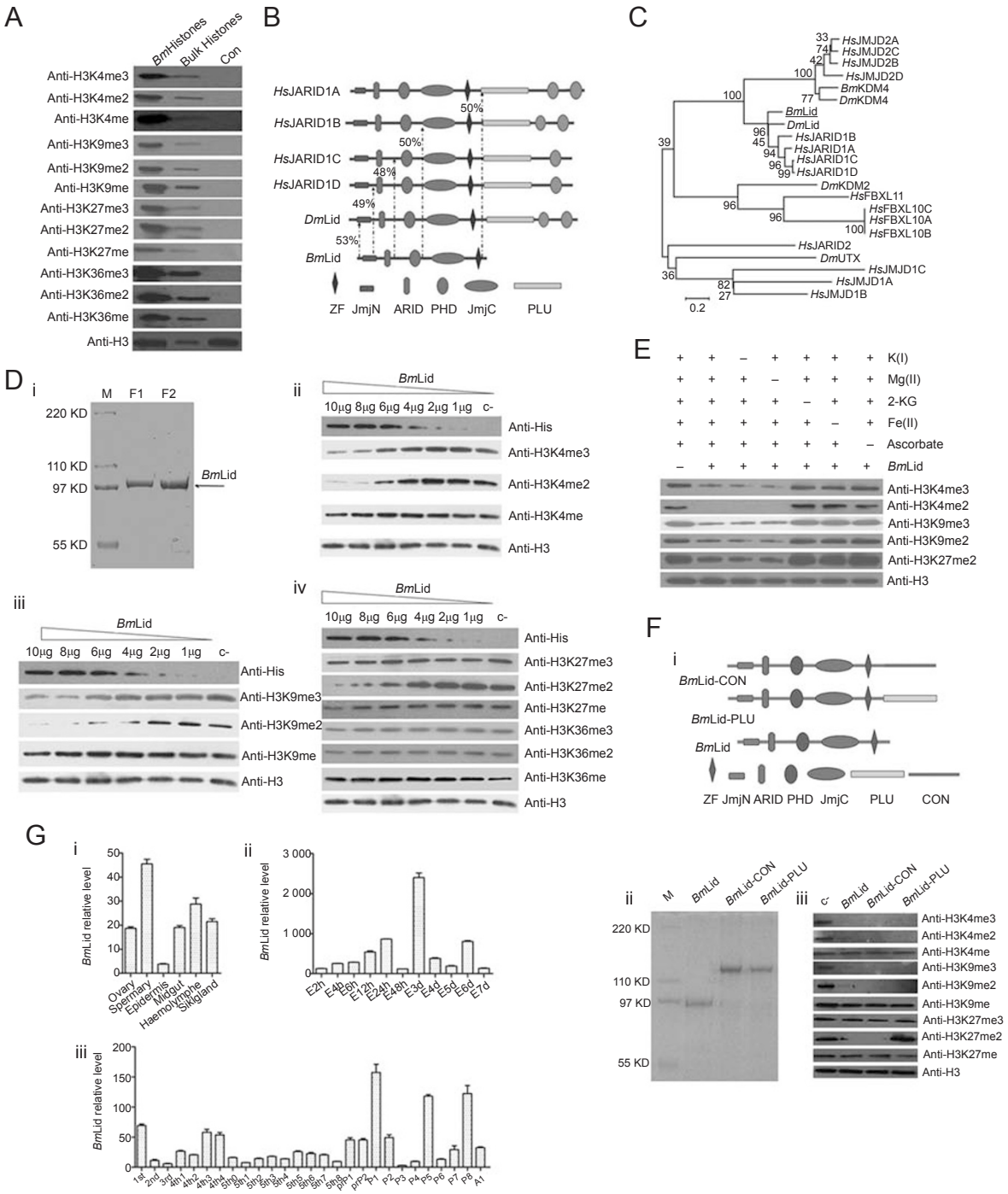
Histone methylation is a dynamic process that plays important roles in gene transcription regulation, and a number of enzymes have been shown to catalyze the removal of methyl marks [1]. Shi *et al.* (2004) identified one of the amino oxidases, lysine-specific demethylase 1 (LSD1), as the first specific demethylase for both mono- (me) and dimethylation (me2) of H3K4 and H3K9 in humans [2]. Subsequently, a total of 27 JmjC-domain-containing proteins have been discovered within the human genome, and 15 of them exhibit demethylation activities for specific lysines in the H3 tail [1]. *Dm*UTX, *Dm*KDM2, *Dm*Lid, and *Dm*KDM4 are JmjC-domain-containing histone demethylases found in *Drosophila* [3-6]. JmjC-domain-containing histone demethylases have also been reported in *Arabidopsis* [7]. Here, we examined the lysine methylation patterns of histone H3 in *Bombyx mori*, a biological model for lepidopteran insects, and showed that a putative histone H3 lysine demethylase, *Bm*Lid, has a much broader *in vitro* substrate specificity than its homolog in other species, exhibiting demethylase activity toward both tri- (me3) and dimethylated (me2) K4 and K9, as well as dimethylated K27. Evidence is also presented suggesting that the absence of a PLU domain in *Bm*Lid may be partially responsible for this broader *in vitro* H3 lysine substrate specificity.

We compared the amino acid sequence of the silkworm H3 with that of *Drosophila*, mouse, and humans (Supplementary information, Table S3). As shown in Supplementary information, Figure S1, the amino acid sequences were 100% identical. This result illustrates that histone H3 is highly conserved from insects to mammals, and suggests that the methylation pattern of lysines in silkworm H3 might be the same as that in *Drosophila* and humans. Indeed, four different lysines (K) within the N-terminal tail of silkworm histone H3 (K4, K9, K27, K36) can be methylated (Figure 1A). Furthermore, according to our MS/MS results, there are two MS/MS-detectable methylated lysine sites in histone H3 of *Bombyx mori* – K9 and K79 (Supplementary information, Figures S4-S5, Table S5-S6). These results demonstrate that the sequence as well as the lysine methylation pattern of silkworm histone H3 is evolutionary conserved.

We then performed a sequence homology search for putative demethylases in the silkworm genome (http://silkworm.genomics.org.cn/), and found only two putative JmjC-domain-containing proteins, *Bm*Lid and *Bm*KDM4 (GU365867). We cloned a JmjC-domain-containing protein with 53% (Figure 1B) similarity to *Dm*Lid and named it *Bm*Lid (GU365866). *Bm*Lid was revealed as a 3 589-bp full-length cDNA. The open reading frame (ORF) of *Bm*Lid is 2 439 bp, encoding 813 amino acid residues with a predicted molecular weight (MW) of 93.5 kDa and a calculated pI of 5.65 (Supplementary information, Figure S2). Confirmation has been made by PCR using primers derived from both 5′ and 3′ UTR of *Bm*Lid (Supplementary information, Table S1). A BLAST search based on the latest version of silkworm genome also confirmed the ORF integrity of *Bm*Lid (data not shown). Phylogenetic analysis indicated that *Bm*Lid was most closely related to *Dm*Lid (Figure 1C, Supplementary information, Table S2). Sequence similarity between *Bm*Lid and other JmjC-domain-containing proteins was as follows: *Dm*Lid (53%), *Hs*JARID1A (50%), *Hs*JARID1B (50%), *Hs*JARID1C (48%), and *Hs*JARID1D (49%) (Figure 1B). Therefore, the JmjC domain is highly conserved from insects to mammals. The deduced *Bm*Lid protein also contains a JmjN domain, an ARID/BRIGHT domain, a zf-C5HC2 zinc-finger (ZF) domain and a PHD zinc-finger domain. We also found that *Bm*Lid is shorter (813 amino acids) than members of the JARID1 subfamily and *Dm*Lid (> 1 500 amino acids); and it is missing the sequence after the ZF domain, including a PLU domain (Figure 1B) [8]. There are fewer JmjC domain proteins in silkworm than in *Drosophila* and humans [2]. The missing sequence in *Bm*Lid suggests a different demethylation mechanism in silkworms

than that in *Drosophila* and humans.

To investigate whether *Bm*Lid has histone demethylase activity, we conducted an *in vitro* H3 lysine demethylase activity assay. The recombinant *Bm*Lid (6× His-tagged at N-terminus) was purified from Sf9 cells with an approximate MW of 97 kDa, which is close to the predicted value (Figure 1Di). We then incubated the bulk core histones together with an increasing amount of the purified *Bm*Lid in the demethylation buffer and examined its demethylase activities by western blotting using 12 different antibodies that may specifically detect mono- (me), di- (me2), and trimethylation (me3) on H3 histone lysines (K4, K9, K27, and K36) (antibodies used in this paper were shown in Supplementary information, Table S4, Data S1). Interestingly, we found a gradual but clear reduction in the detection of specific bands representing



H3K4me3/me2, H3K9me3/me2, and H3K27me2. Therefore, the recombinant *Bm*Lid can individually remove the methyl mark from H3K4me3/me2, H3K9me3/me2, and H3K27me2 (Figure 1Dii-iv). The broad enzyme activities of *Bm*Lid differ from the other members of the JARID1 subfamily including *Dm*Lid, which can only remove the methyl group from H3K4me3/me2 [2]. According to homology-based searching, *Bombyx mori* only has two JmjC-domain-containing proteins (*Bm*Lid and *Bm*KDM4, GU365867), whereas *Drosophila* has at least four homolog. That might be part of the reason why *Bm*Lid needs to have a broader enzyme activity.

We incubated the purified *Bm*Lid with histones in the presence or absence of putative cofactors. Our results showed that enzymatic activity was significantly lost in the absence of 2-ketoglutarate, Fe (II), or ascorbate (Figure 1E). However, Mg (II) and K (I) were not required (Figure 1E). Both iron-binding sites are located in the JmjC domain that shares a high percentage of similarity (79%) with the other JmjC-containing demethylases. Previously, several labs have performed mutagenesis studies on the putative Fe (II) binding sites (H502, E504, and H590 shown in Supplementary information, Figure S6) in the JmjC-containing demethylases [9], and the results showed that the mutants lost the demethylase activity. Our homology alignment results suggest that H502, E504, and H590 in *Bm*Lid are key residues involved in iron binding.

Previous studies showed that domains identified in the JARID1 subfamily (JmjC, JmjN, ARID, and ZF domain) are indispensable for demethylase activity [10]. No evidence or study has been conducted on the PLU domain that is absent in *Bm*Lid and whose absence is probably or at least partially responsible for its broader enzyme activities than its homolog in *Drosophila* and humans. We thus designed two fusion mutants to address this question. As shown in Figure 1Fi, we added the *Dm*Lid PLU domain behind the *Bm*Lid-coding region (*Bm*Lid-PLU). Additionally, as a control, the sequence from behind the PLU domain in *Dm*Lid was added to construct another fusion mutant (*Bm*Lid-CON). Each mutant had 1 158 amino acid residues. When purified from Sf9 cells, both mutants showed an approximate MW of 135 kDa (Figure 1Fii). Interestingly, we found that *Bm*Lid-PLU was no longer active toward H3K27me2, but maintained its activity toward H3K4me3/me2 and H3K9me3/me2. As expected, *Bm*Lid-CON showed the same demethylase activity as *Bm*Lid (Figure 1Fiii). This result suggests that the absence of the PLU domain in *Bm*Lid is at least partially responsible for the lack of specificity of its demethylase activities.

Mutations of *Dm*Lid resulted in a small imaginal disc phenotype [5]. We then investigated the biological function of *Bm*Lid *in vivo* by checking its expression

**Figure 1** *Bm*Lid is a histone lysine demethylase with a broader specificity than its homolog in *Drosophila* and mammals. **(A)** Methylation pattern of silkworm H3 lysines. *Bm*Histones: histones isolated from silkworm; Bulk histones: core histones of bulk; con: control, the histone H3 expressed in *Escherichia coli*. The panels show the reaction probed with the indicated antibodies and assayed by western blotting. **(B)** Schematic representation of *Bm*Lid and its homolog *Hs*JARAID1 and *Dm*Lid. ARID, AT-rich interactive domain; JmjN, jumonji N domain; JmjC, jumonji C domain; PHD, plant homeotic domain; ZF, C5HC2 zinc-finger domain; PLU, PLU-1-like protein domain. **(C)** Phylogenetic analysis of the JmjC-containing protein family. Alignment of the JmjC proteins and construction of the phylogenetic tree were conducted using the neighboring-joining method in clustalx 1.8 and MEGA 4. The underline indicates the specificity reported in the study. **(D)** *Bm*Lid demethylase activity assay *in vitro*. (i) Coomassie-stained SDS-PAGE gel of His-tagged *Bm*Lid protein purified from baculovirus-infected Sf9 cells. M, molecular weight marker: F1-F2, fraction1-2 eluted from a 6xhis-affinity column. (i-iv) *Bm*Lid demethylase activity assay *in vitro*. A measure of 13 μg of histones were incubated with 1, 2, 4, 6, 8, and 10 μg of recombinant *Bm*Lid, and 10% of incubated histone was used for western blotting; c-, control; the panels show the reaction probed with the indicated antibodies and assayed by western blotting. **(E)** Effect of cofactors on *Bm*Lid activities. A measure of 13 μg of histones were incubated with or without 8 μg of recombinant *Bm*Lid in the presence or absence of certain cofactors. The panels show the reaction probed with the indicated antibodies and assayed by western blotting. 2-KG: 2-ketoglutarate. **(F)** Reduced demethylase activity of *Bm*Lid-PLU toward H3K27me2. (i) Schematic representation of the mutants with links to predicted functional domains. (ii) Fusion proteins purified by His-tag affinity chromatography. (iii) Comparison of demethylation activities of *Bm*Lid and the constructed fusion proteins. A measure of 13 μg of histones were incubated with 8 μg of recombinant *Bm*Lid, 10 μg of recombinant *Bm*Lid-con or 10 μg of recombinant *Bm*Lid-PLU. A total of 10% of incubated histone was used for western blotting. The panels show the reaction probed with the indicated antibodies and assayed by western blotting. **(G)** Expression pattern of *Bm*Lid in silkworms. (a) Relative mRNA levels of *Bm*Lid in different tissues of 3-day-old fifth-instar silkworm larvae. The data points reflect the means of three independent experiments. (b) Relative mRNA levels of *Bm*Lid in different stages of silkworm eggs. E2h-E48h, 2- to 48-h-old eggs; E3d-E7d, 3- to 7-day-old eggs. (c) relative mRNA levels of *Bm*Lid in different stages of silkworms. Shown are 1st, 1-day-old first-instar silkworm larvae; 2nd, 1-day-old second-instar silkworm; 3rd, 1-day-old third-instar silkworm; 4th, 1- to 4-day-old fourth-instar silkworm; 5th, 1 to 8-day-old fifth-instar silkworm; prP1-prP2, the 1 to 3-day-old prepupae; P1-P8, 1 to 8-day-old pupae; A1, 1-day-old adult silkworm.

pattern in silkworm. The quantitative RT-PCR results showed that the *Bm*Lid gene is abundantly expressed in spermary and its expression is distinctively lower in the cuticle (Figure 1Gi). During development, much higher expression levels of the *Bm*Lid gene were seen in eggs (embryo) than in other stages, as well as during periods approaching metamorphosis and ecdysis (Figure 1Gii-iii). These results are consistent with previous studies in *Drosophila*; the level and distribution of histone methylation are highly involved in the development process by regulating both transcriptional activation and repression [1]. We also injected *Bm*Lid dsRNA into pupae to examine its potential biological functions *in vivo*. However, silkworm pupae injected with *Bm*Lid or EGFP dsRNA did not exhibit any obvious phenotypic difference despite a clear decrease in *Bm*Lid mRNA expression induced by the specific interfering RNA (Supplementary information, Figure S3).

In conclusion, this work represents the first study on the lysine methylation patterns of histone H3 in *Bombyx mori*. We describe the characterization of a putative histone H3 lysine demethylase, *Bm*Lid, and demonstrate that recombinant *Bm*Lid has much broader *in vitro* substrate specificity than its homolog in other species, exhibiting demethylase activity toward both tri- and dimethylated K4 and K9, as well as dimethylated K27. Evidence is also presented to illustrate that the absence of the PLU domain in *Bm*Lid may be partially responsible for its broader *in vitro* H3 lysine substrate specificity. Although further studies of the *Bm*Lid gene are required, our *in vitro* study of the enzyme activities of *Bm*Lid sheds light on the mechanism of JmjC-domain-containing demethylases in eukaryotes.

Experimental materials and methods, antibodies and primers, and sequences used for alignment are depicted in the Supplementary information, Data S1.

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