**Tetrahymena thermophila JMJD3 Homolog Regulates H3K27 Methylation and Nuclear Differentiation**

Pei-Han Chung and Meng-Chao Yao

Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan, and Institute of Molecular Biology, Academia Sinica, Nankang, Taipei, Taiwan

Histone H3K27me3 modification is an important regulator for development and gene expression. In *Tetrahymena thermophila*, the complex chromatin dynamics of H3K27me3 marks during nuclear development suggested that an H3K27me3 demethylase might exist. Here, we report an H3K27me3 demethylase homolog, JMJD3, in *Tetrahymena*. During conjugation, JMJD3 expression is upregulated and the protein is localized first in the parental macronucleus and then in the new macronucleus. In conjugating cells, knockdown of JMJD3 expression resulted in a severe reduction in the production of progeny, suggesting that JMJD3 is essential for *Tetrahymena* conjugation. Furthermore, knockdown of JMJD3 resulted in increased H3K27 trimethylation in the new macronucleus and reduced transcription of genes related to DNA elimination, while the DNA elimination process was also partially blocked. Knockdown of the H3K27 methyltransferase EZL2 but not that of EZL1 partially restored progeny production in JMJD3 knockdown cells and reduced abnormal H3K27me3 accumulation in the new macronucleus. Taken together, these results demonstrate a critical role for JMJD3 in regulating H3K27me3 during conjugation and the importance of JMJD3 in regulating gene expression in the new macronucleus but not in regulating the formation of heterochromatin associated with programmed DNA deletion.

Several different covalent modifications, such as methylation, acetylation, phosphorylation, and ubiquitination, modify the N terminus of histone tails. Depending on the modification status and specific residues, the overall accessibility of chromatin and transcriptional activities of genes are affected by these covalent modifications (13, 36). Among these covalent modifications, the methylation of histones at lysine residues is particularly interesting, since lysine methylation can serve as a mark for transcription activation or repression, depending on which residue is methylated (63). Generally speaking, histone lysine methylation at H3K4, H3K36, and H3K79 is associated with transcriptional activation, while histone lysine methylation at H3K27, H3K9, and H4K20 is associated with transcriptional repression (23). In mammals, H3K27me3 is catalyzed by the SET domain-containing histone methyltransferase EZH2, which is the mammalian homolog of the *Drosophila* polycomb group protein E(z) (enhancer of zeste) (14). H3K27 methylation is recognized by other histone modification reader proteins, which can promote heterochromatin formation (79). The methylation state of H3K27me3 is also involved in many important cellular processes, including developmental fate decisions, imprinting, embryonic development, and cell growth control (20, 41, 45, 65).

In early studies, the turnover rate of histone methylation was measured using radioactive methyl groups, and the results indicated that the turnover was slow or the methylation was irreversible (6, 9, 11). Nevertheless, the recent identification of lysine-specific demethylase 1 (LSD1) and the jumonji C (JmjC) domain-containing histone demethylases suggested that the removal of the histone methylation mark may also be under active control (62, 72). These histone demethylases have the ability to remove stable histone methylation marks and reprogram gene expression or heterochromatin formation of cells during developmental transitions (1).

To date, with the exception of LSD1 and LSD2, all the identified histone demethylases contain a JmjC domain (57). Two recently identified JmjC domain-containing H3K27me3 demethylases, JMJD3 and UTX, revealed the importance of active H3K27me3 demethylation. Both have been shown to remove the repressive H3K27me3 marks and function as transcriptional activators (2, 26, 30, 38, 39). Binding of UTX and JMJD3 to HOX gene loci promotes transcriptional activation of HOX genes and inhibits H3K27 methylation (2, 26, 38). For example, mutation of the UTX homolog in zebrafish disrupts proper activation of *hox* genes and body patterning, and mutation of the JMJD3 homolog in *Caenorhabditis elegans* disrupts proper gonadal development and organization (2, 38). In addition to HOX genes, JMJD3 and UTX are also required for the activation of other genes essential for differentiation. For instance, JMJD3 is required for the activation of an H3K27me3-suppressed gene, *brachyury*, during embryonic stem cell differentiation (22). Furthermore, JMJD3 is also required for the activation of inflammation-induced genes in mouse macrophages, the activation of genes critical for neuronal differentiation, and the activation of genes involved in wound healing in murine skin repair (10, 25, 61). Similar to JMJD3, UTX is required for the activation of myogenesis genes (60). In response to cellular oncogenic stress, JMJD3 is required for the activation of genes associated with senescence, while UTX controls cell arrest through activating the retinoblastoma pathway (3, 7, 69, 74). Taken together, these data indicate the importance of H3K27 demethylases for dynamic regulation of H3K27me3 marks.

Striking nuclear differentiation, which involves global genome rearrangements and transcriptional activation, occurs during conjugation in *Tetrahymena thermophila* and provides a unique...
opportunity to study the possible roles of H3K27me3 dynamics in gene regulation and DNA rearrangements, both of which are related to heterochromatin formation. Like most ciliates, *Tetrahymena* contains a silenced germ line micronucleus (Mic) and a transcriptionally active macronucleus (Mac) (33). During the sexual reproduction phase of the life cycle, *Tetrahymena* destroys its old macronucleus and generates a new macronucleus from the descendant of the micronucleus. During this process, the macronucleus undergoes meiosis, nuclear fusion, and mitosis and finally differentiates from a silenced nucleus into an active macronucleus (76). The genome of the new macronucleus goes through extensive remodeling during this process. For example, about 33% of the micronucleus sequences are sequenced in heterochromatin-like structures in the developing macronucleus and eventually eliminated from the mature macronucleus. This process can be viewed as the ultimate form of heterochromatin silencing (16).

A correlation among small RNA production, H3K27me3 methylation, and the formation of heterochromatin has been implicated in the *Tetrahymena* DNA elimination process (17, 51). First, double-stranded RNAs are generated from sequences to be eliminated and are then further processed into small RNAs (18, 44, 50, 52). EZL1p, which is a *Tetrahymena* homolog of Drosophila histone methyltransferase E(z), is targeted to eliminated sequences by these small RNAs and catalyzes H3K27 methylation at these sites, which leads to H3K9 methylation (42). Finally, these histone modifications recruit downstream proteins, including the chromo domain-containing proteins Pdd1p and Pdd3p (42, 68), and promote heterochromatin formation, which eventually leads to DNA elimination (43). The dynamics of H3K27me3 in EZL1p-deficient strains also suggest that the H3K27me3 mark of *Tetrahymena* is under active turnover by an unknown mechanism (42).

These reports collectively raised the question of whether an H3K27 demethylase in *Tetrahymena* plays a role in modulating H3K27me3 during conjugation. This H3K27 demethylase in *Tetrahymena* could regulate conjugation by regulating gene expression, which is a major step of differentiation from the silent micronucleus-like structure to the actively transcribing macronucleus, and/or by affecting the heterochromatin formation that is associated with DNA elimination. In this study, we investigated the biological function of JMJ1, an H3K27me3 demethylase homolog in *Tetrahymena*. Knockdown of JMJ1 expression during conjugation abrogates progeny production during *Tetrahymena* conjugation, suggesting that JMJ1 is essential for *Tetrahymena* conjugation. JMJ1 is also required for the proper activation of the DNA elimination-related genes and the completion of the DNA elimination process. Finally, the JMJ1-knockdown phenotype was partially rescued by coexpression of an H3K27 methylase, EZL2. Collectively, these findings identified an essential function for JMJ1 in the *Tetrahymena* developmental process by removing H3K27me3 to regulate essential gene expression.

**MATERIALS AND METHODS**

**Cell culture.** Inbred strains B2086 II, CU428 [Mpr/Mpr [6-methylpurine sensitive (mp-s), VI]], and CU427 [Cdx/Cdx [cycloheximide sensitive (cy-s), VI]] were obtained from Peter Bruns (Cornell University, Ithaca, NY). *Tetrahymena* strains were maintained as previously described (56). For conjugation, *Tetrahymena* cells with different mating types were first grown in SPP (0.2% dextrose, 0.1% yeast extract, 1% protease peptone, 0.005% Sequestrene) medium at 30°C, washed with 10 mM Tris-HCl (pH 7.4) buffer, and incubated overnight before mixing to initiate conjugation.

Phylogenetic analysis and protein domain alignment. The amino acid sequences of JmjC domain-containing protein were retrieved from the ChromDB database (http://www.chromdb.org/). Additional *Tetrahymena* JmjC domain-containing protein sequences were retrieved from the *Tetrahymena* Genome Database (TGD) by searching for matches with the JmjC domain. The ClustalW program was used to generate alignments of the JmjC domain-containing proteins (70). The MEGA (version 5.05) package was used to generate phylogenetic trees using the neighbor-joining method with a Poisson correction model and a bootstrap of 1,000 replicates (67). The domains in JMJ3/UTX/UTY group proteins were identified using the hidden Markov model (HMM) algorithm in the Pfam database (http://pfam.wustl.edu/). The accession numbers of genes used for phylogenetic analysis are shown in Table S3 in the supplemental material.

**Reverse transcription-PCR (RT-PCR) and quantitative PCR.** RNA samples were extracted using a High Pure RNA purification kit (Roche Indianapolis, IN) and reverse transcribed into cDNA using Transcriptor reverse transcriptase (Roche) with oligo(dT) primers. Quantitative PCR was performed using a Roche LightCycler carousel-based PCR system with a LightCycler FastStart DNA Master plus SYBR green kit (Roche). The sequences of primers for individual genes are provided in Table S4 in the supplemental material. Each quantitative PCR was performed in triplicate. To standardize the amount of cDNA, the α-tubulin gene was used as an internal control. Data are presented as mean ± standard deviation.

Creation of JMJ1-GFP-Neo4 strains. To examine the localization of Jmjlp, we first cloned a 0.9-kb fragment of the JMJ1 gene into the pNeo4 vector (49), and a green fluorescent protein (GFP) fragment was inserted in front of the stop codon. Next, a 1.6-kb 3′ flanking genomic fragment of JMJ1 gene was cloned downstream of the Neo4 cassette to create a pJM11-GFP-Neo4 construct. Finally, the pJM11-GFP-Neo4 construct was used for bioinformatic transformation of *Tetrahymena*. Transformants were selected by paromycin and examined for integration at the micronuclear JMJ1 locus.

Construction of hairpin RNA strains. To knock down JMJ1, EZL1, EZL2, and EZL3, the coding region of the individual gene (~500 bp) was amplified by PCR. The JM11/EZL2 double-knockdown vector was constructed by amplifying a PCR product that contained 236 bp of the JMJ1 coding region and 254 bp of the EZL2 coding region. To generate the hairpin cassette, these PCR products were cloned into the pCR1-L3 vector using two different sets of primers, a forward set with the Pmiel-SmaI site and a reverse set with the XhoI-ApaI site (see Table S4 in the supplemental material for oligonucleotide sequences). The hairpin cassette was cloned into the pBF ribosomal DNA (rDNA) vector at the Pmel-Apal site (see Table S4 in the supplemental material for oligonucleotide sequences). The hairpin cassette was cloned into the pBF ribosomal DNA (rDNA) vector at the Pmel-Apal site. The expression of hairpin RNA was controlled by a CdCl2-inducible metallothionein promoter. Matings of CU427 and CU428 were transformed by electroporation using 10 μg of the hairpin vector to generate hairpin RNA-expressing strains.

Northern blotting. Total RNA was extracted from vegetative, starved, or mating *Tetrahymena* cells using TRizol reagent (Invitrogen, Paisley, United Kingdom). Northern blot analysis was performed as previously described (19). Briefly, the RNA sample was transferred to an Immobilon-Nyton+ membrane (Millipore, Billerica, MA) and cross-linked with UV. DNA probes were generated from gel-purified PCR products of the coding region of JMJ1. The PCR products were used as the templates for random primed labeling. The membrane was hybridized with probes at 42°C in hybridization buffer (Roche) and washed several times with 2× to 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Quantity One software (Bio-Rad, Richmond, CA) was used to quantify bands.

Evaluation of hairpin RNA-silencing phenotype. To determine the progeny production of *Tetrahymena* strains, two different mating types of *Tetrahymena* strains transformed with the hairpin construct were starved and mated with each other. Hairpin RNA expression was induced at 2 h postmating using 0.05 μg/ml of CdCl2. After induction, 132 individual pairs were isolated in drops of SPP medium, incubated at 30°C for 48 h, and tested for drug resistance and growth phenotypes that distinguished...
progeny from parents. To examine the developmental stages, conjugating cells were fixed with 2% paraformaldehyde and stained with DAPI (4',6-diamidino-2-phenylindole; 1 mg/ml) to visualize the nuclei.

**Western blotting.** *Tetrahymena* cell cultures (~10^6 cells) were centrifuged and washed with 10 mM Tris-HCl (pH 7.4) buffer. Cell pellets were lysed by boiling for 5 min in 100 μl of 2× SDS loading buffer (4% SDS, 160 mM Tris-HCl, pH 6.8, 20% glycerol, 0.0025% bromophenol blue, 10% 2-mercaptoethanol). Protein extracts were separated in a 15% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (PerkinElmer, Waltham, MA) with a semidry electroblotter (Owl Separation Systems, Portsmouth, NH). Blots were blocked in 5% milk, 0.3% Tween 20, 1× phosphate-buffered saline (PBS) and then incubated with primary antibodies overnight at 4°C. The primary antibodies used were anti-H3K27me3 (1:2,000; ABE44; Millipore) and anti-H3K4me2 (1:5,000; 07-030; Abcam, Cambridge, MA). Blots were extensively washed in 1× PBS with 0.3% Triton X-100 (PBT), incubated with 1:10,000-diluted secondary horseradish peroxidase-conjugated anti-rabbit IgG antibodies (GE Healthcare, Chalfont St. Gills, United Kingdom), and visualized by using an ECL kit (GE Healthcare). Loading of total protein extract was monitored by staining with anti-α-tubulin antibodies (1:2,000; DM1A; Abcam).
**Immunofluorescence analysis.** Conjugating cells were harvested and fixed with 2% paraformaldehyde, immobilized on a slide, incubated with H3K27me3 (1:500; ABE44; Millipore) primary antibodies, and washed with PBT. Next, the slide was incubated with secondary Cy5-conjugated AffiniPure F(ab')2, fragment goat anti-rabbit IgG (1:500; 111-176-003; Jackson ImmunoResearch, West Grove, PA). After incubation with secondary antibodies, the slide was washed with PBT and stained with DAPI. Digital images were collected using a Zeiss Axio Imager microscope (Zeiss, Oberkochen, Germany) and processed using Adobe Photoshop CS5 software (Adobe Systems, San Jose, CA).

**IES elimination assays.** To evaluate the efficiency of internal eliminated sequence (IES) elimination and chromosome breakage in pooled mating cells using PCR, genomic DNAs were extracted from conjugating cells at 36 h postmixing. PCR primer sets specific for IES elimination elements (the M, R, Cam, and TIR elements) and chromosome breakage sites (Chs 819 and Chs 5-2) were used to amplify the processed and unprocessed form of elements (see Table S4 in the supplemental material for oligonucleotide sequences).

To determine the efficiency of IES elimination in individual progeny pairs, mating pairs were isolated and deposited into 10 mM Tris-HCl (pH 7.4) buffer at 10 h postmixing. Total DNA samples from mating pairs were extracted, and IES elimination assays were analyzed by nested PCR as previously described (4). To ensure the completion of conjugation, additional PCR primers were used to detect the presence or absence of the parental hairpin RNA plasmid. This assay relied on the fact that progeny of mating strains degraded the parental macronuclei, thus losing their hairpin RNA plasmids. The mating pairs that contained parental hairpin RNA plasmids were omitted from this study.

**ChIP.** Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (19). For IES elements, conjugating cells were collected at 10 h postmixing, cross-linked in 1% paraformaldehyde, and washed (24). For DNA elimination-related genes, conjugating cells were collected at 9, 12, and 16 h postmixing. Next, cells were sonicated for 4 min in 15-s bursts at 5 × 10^6 cells/ml in 0.1% SDS lysis buffer (15) and immunoprecipitated using anti-H3K27me3 (1:200; ABE44; Millipore), anti-H3K4me2 (1:200; 07-030; Abcam), or anti-Pdd1p (1:100; ab5338; Abcam) antibodies. The immunoprecipitated complexes were pulled down by protein A agarose (16-157; Millipore) and washed. DNA was eluted, purified by phenol-chloroform extraction, and ethanol precipitated. Purified DNA was subject to quantitative PCR (42). The oligonucleotide sets and the amplified region for the individual loci are provided in Table S4 in the supplemental material. To normalize the input material, DNA isolated from the lysate without immunoprecipitation was used as a quantification standard. Data are presented as mean ± standard deviation.

**RESULTS**

**Domain organization and sequence homology analysis of JmjC-containing proteins of *T. thermophila*.** We searched the Tetrahymena Genome Database for proteins that have potential histone demethylase activities toward trimethylated histone H3K27 or H3K9, which is important for *Tetrahymena* conjugation (42, 68). The members of junonius C (JmjC) class enzymes remove trimethyl groups on histone H3K27 or H3K9, and the JmjC domain is the catalytic domain of the JmjC class enzyme (73).

Through bioinformatics analysis, four putative JmjC domain-encoding genes, *JMJ1, JMJ2, JMJ3*, and *JMJ4* (*Tetrahymena* JmjC demethylases 1, 2, 3, and 4), were identified from the *Tetrahymena* Genome Database (TGD; [http://www.ciliate.org/]). Based on the sequence alignment of the JmjC domain, we generated a phylogenetic tree of *Tetrahymena* and human JmjC domain-containing proteins (Fig. 1A). The JmjC proteins in *Tetrahymena* could be separated into distinct groups: the *JMJ3/UTX/UTY* group, *JARID1* group, *JMJ2* group, and *JMJ6* group. Two lines of evidence suggest that the *Tetrahymena* *JMJ1* is the active H3K27 demethylase during conjugation. First, phylogenetic analysis revealed that *Tetrahymena* Jmj1p belongs to the *JMJ3/UTX/UTY* group, which is shown to actively demethylate histone H3K27me2/me3 in other organisms (57). Second, the expression pattern in the *Tetrahymena* Gene Expression Database (TGED; [http://tged.ihb.ac.cn/]) showed that *JMJ1* was the only one among the four JmjC genes that is specifically expressed during conjugation. Thus, we decided to focus our study on *Tetrahymena JMJ1*.

We next investigated the protein domain organization and conservation within the JmjC catalytic domain. The organization of *Tetrahymena* Jmj1p is similar to that of *JMJ3* homologs in other species, which lack the tetratricopeptide repeat (TPR) domain present in the UTX homologs (Fig. 1B) (57). Sequence analysis showed that Jmj1p shared sequence similarity only around the JmjC catalytic domain. However, the key cofactor binding amino acid residues of *Tetrahymena* Jmj1p is highly conserved (Fig. 1C), indicating that *JMJ1* likely possesses demethylase activities (2, 26, 30, 38).

**JMJ1 expression and localization.** To further investigate the role of *JMJ1* during *Tetrahymena* conjugation, we first used real-time RT-PCR analysis to examine the expression of *JMJ1* mRNA at different stages of the *Tetrahymena* life cycle. The result showed that *JMJ1* mRNA was not expressed during growth or starvation.

**FIG 2 Expression pattern of *Tetrahymena* JMJ1 and the subcellular localization of the encoded protein. (A) Expression of *JMJ1* by quantitative RT-PCR. Total RNA was extracted from vegetative (V), starved (S), and conjugating (at 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 h postmixing) cells and used as the template. The α-tubulin mRNA was used as an internal control. (B) Localization of Jmj1p-GFP in conjugating cells. C-terminal GFP-tagged Jmj1p was expressed from the endogenous *JMJ1* locus. The Jmj1p-GFP-tagged strain was mated with a wild-type strain. Representative GFP- and Hoechst-stained image series are shown as indicated. M, parental macronucleus; m, micronucleus; An, developing macronucleus. Bars, 10 μm.
but was significantly upregulated starting from 4 h postmixing and highly expressed at late conjugation (Fig. 2A), which was also the time of new macronucleus development. These results suggested that \textit{JMJ1} might regulate histone demethylation from early to late conjugation.

We next examined the localization of Jmj1p by using GFP-tagged Jmj1p proteins. The GFP-tagged Jmj1p proteins were generated by fusing GFP at the C terminus of the endogenous \textit{JMJ1} locus. When crossed with a wild-type partner, Jmj1p-GFP was first localized at the parental macronucleus during early conjugation stages (Fig. 2B) but became localized in the new macronucleus as it started to differentiate. These results suggested that \textit{JMJ1} might be associated with regulating histone demethylation in the parental macronucleus or developing a new macronucleus during conjugation.

\textit{JMJ1} is required for production of viable progeny. From its gene expression and protein localization patterns, we suspected that \textit{JMJ1} might have an essential function in regulating histone methylation during conjugation. However, we were unable to obtain complete germ line \textit{JMJ1}-knockout strains, possibly due to its essential function during conjugation. We thus generated \textit{JMJ1} RNA interference (RNAi)-knockdown strains which expressed \textit{JMJ1} hairpin RNA upon cadmium treatment (31). The efficiency of \textit{JMJ1} RNAi knockdown was verified by Northern hybridization analysis, which showed that the \textit{JMJ1} mRNA was downregulated (Fig. 3B).

To test whether \textit{JMJ1} is required for conjugation, the \textit{JMJ1} RNAi-knockdown strains were crossed with each other, and individual mating pairs were examined for their ability to form progeny. Upon RNAi induction, the progeny production of \textit{JMJ1}-

**FIG 3 Knockdown of \textit{JMJ1} by hairpin RNA.** (A) Schematic representation of the \textit{JMJ1} hairpin RNA construct. The open arrow indicates \textit{JMJ1} mRNA, and double lines represent regions targeted by hairpin RNA. The hairpin cassette was cloned into the rDNA vector and was under the MTT1 promoter control. (B) Northern blot of \textit{JMJ1} expression in knockdown cells. Total RNA samples were extracted from conjugating cells (4, 6, 8, 10, 12, 14, and 16 h postmixing) transformed with the hairpin (hp) RNA construct with or without treatment with 0.05 \( \mu \)g/ml CdCl\(_2\). The approximate size of \textit{JMJ1} mRNA is indicated to the right. The ethidium bromide staining of total rRNA was used as a loading control. (C) Progeny production of \textit{JMJ1}-knockdown cells. Control and \textit{JMJ1} hairpin RNA strains were mated and either untreated or treated with CdCl\(_2\). Individual pairs were cloned into drops of growth medium at 10 h postmixing. For vegetative cells, individual cells were cloned into drops of growth medium with or without 1 \( \mu \)g/ml CdCl\(_2\). Progeny production was determined by the drug resistance phenotype. WT, wild type. (D) Developmental profiles of \textit{JMJ1}-knockdown strains. The conjugation stages of \textit{JMJ1}-knockdown cells were determined by DAPI staining. At least 200 cells were counted for each time point. The stages categorized were single unmated cells (S), meiosis (E), prezygotic (M1), postzygotic (M2), macronuclear development (L1), pair separation (2 Mic/2 Mac) (L2), and micronuclear elimination (1 Mic/2 Mac) (L3).
knockdown cells was severely reduced (Fig. 3C), indicating that JMJ1 is essential for *Tetrahymena* to produce viable progeny. When treated with cadmium, the vegetative growth of JMJ1 RNAi-knockdown strains was not affected (Fig. 3C), which agreed with the conjugation-specific expression profile of JMJ1 mRNA.

To study the role of JMJ1, we examined the progression of conjugation between RNAi-knockdown strains. These strains showed a slight delay in conjugation (Fig. 3D) but were still able to reach the 1 Mic/2 Mac stage (L3), which is the final stage of development. These results suggested that JMJ1 RNAi-knockdown strains could complete most of the nuclear development events.

**JMJ1 is required for H3K27 demethylation in late stages of conjugation.** To determine whether JMJ1 is involved in H3K27 histone demethylation in vivo, protein extracts of JMJ1 RNAi-knockdown or noninduced cells were analyzed by Western blot tests. Consistent with previous reports, noninduced cells showed constant H3K27me3 levels through 6 to 12 h postmixing (Fig. 4A) (42). The level decreased from 15 to 26 h postmixing, which could be due to the elimination of DNA in chromatin that contained the H3K27me3 marks and/or the active turning over of H3K27me3 marks. Compared with noninduced cells, an increase of H3K27me3 level was detected in JMJ1 RNAi-knockdown cells during conjugation.
from 15 to 26 h postmixing, indicating a role for \textit{JMJ1} in regulating the H3K27me3 level during this period.

We used immunofluorescence staining to further study the dynamics of H3K27me3 in conjugating cells. In noninduced cells, H3K27me3 was first detected in the parental macronucleus and meiotic micronucleus. It then appeared in the developing new macronucleus and gradually decreased as conjugation proceeded (Fig. 4B). In \textit{JMJ1} RNAi-knockdown cells, the H3K27me3 signal in both the parental macronucleus and new macronucleus appeared to be not affected in early conjugation but remained very strong in the new macronucleus in late conjugation, clearly different from the situation in noninduced cells (Fig. 4B). The quantitated result of H3K27me3 staining is shown in Fig. S1 in the supplemental material. These results, together with the H3K27me3 dynamics shown in the Western blot analysis, suggested that \textit{JMJ1} actively demethylated H3K27me3 in the new macronucleus during late conjugation stages.

**Partial rescue with \textit{JMJ1}-knockdown phenotype by cosuppression with \textit{EZL2}**. Since RNAi knockdown of \textit{JMJ1} affected H3K27me3 levels in the new macronucleus, we asked whether \textit{JMJ1} could genetically interact with other H3K27 methyltransferases during conjugation. Previous studies have shown that the \textit{Tetrahymena} genome encodes three putative H3K27 methyltransferases, \textit{EZL1}, \textit{EZL2}, and \textit{EZL3}. While \textit{EZL1} is associated with H3K27me3 on DNA elimination elements during conjugation, the function of \textit{EZL2} and \textit{EZL3} is less clear (42). We thus conducted RNAi-knockdown experiments on \textit{EZL2} and \textit{EZL3}, which showed that \textit{EZL2} was critical for H3K27me3 during vegetative growth (Fig. 5A) but \textit{EZL3} was not required. Because \textit{EZL2} is also expressed during conjugation, it may have a role in H3K27me3 regulation during conjugation.

To determine whether \textit{JMJ1} could genetically interact with \textit{EZL1} or \textit{EZL2}, we simultaneously silenced \textit{JMJ1} and \textit{EZL1} or \textit{JMJ1} and \textit{EZL2} during conjugation. To circumvent the problem of residual \textit{Ezl2p} expression during vegetative growth, we constructed a double-knockdown vector to silence \textit{EZL2} or \textit{EZL1} and \textit{JMJ1} simultaneously in both mating partners. The efficiency of \textit{JMJ1}/\textit{EZL2} silencing was confirmed by quantitative PCR and Western blot analysis. The progeny production of \textit{JMJ1}/\textit{EZL2} double-knockdown strains remained low, indicating that the knockdown of \textit{EZL1} could not compensate for the loss of \textit{JMJ1} and vice versa. In contrast, the progeny production of \textit{JMJ1}/\textit{EZL2} double-knockdown strains was partially restored (Fig. 5B) and was similar to that of the \textit{EZL2} single-knockdown cells. This finding demonstrated that \textit{JMJ1} interacts with \textit{EZL2} in the same pathway during conjugation.

To gain further insights into the cross talk between \textit{JMJ1} and \textit{EZL2}, we examined the dynamics of H3K27me3 in \textit{EZL2} single-knockdown and \textit{JMJ1}/\textit{EZL2} double-knockdown strains using Western blot analysis. In \textit{EZL2}-knockdown strains, H3K27me3 was not detected until 6 h postmixing. It then increased from 6 to 12 h postmixing (Fig. 5C), indicating that other H3K27 methyltransferases, such as \textit{EZL1} or \textit{EZL3}, also methylated H3K27 during this time. In contrast to \textit{JMJ1}-knockdown strains, \textit{JMJ1}/\textit{EZL2} double-knockdown strains showed no accumulation of H3K27me3 marks in the late conjugation stages, which is consistent with the partial restoration of progeny production by \textit{JMJ1}/\textit{EZL2} double knockdown. Taken together, these results suggested that \textit{JMJ1} removed \textit{EZL2}-mediated H3K27me3 methylation and the low progeny production of \textit{JMJ1}-knockdown strains could be linked to the accumulation of H3K27me3 in late conjugation stages.

**\textit{JMJ1} knockdown partially inhibits IES elimination**. A previous report has shown that H3K27me3 is required for DNA elim-
FIG 6 IES elimination and chromosome breakage analysis of JMJ1-knockdown cells. (A) Schematic representation of PCR assays to determine DNA elimination and chromosome breakage. White boxes indicate M, R, Cam, or Tlr elements to be eliminated. Black lines indicate sequences retained in the macronucleus. In chromosome breakage analysis, the white box indicates the breakage region, while the black box indicates the telomere region. Arrows indicate the locations of PCR primers. The
ination during *Tetrahymena* conjugation. To further explore the role of *JMJ1* during conjugation, we first assayed the elimination of four different IES elements (the M, R, Cam, and Tlr elements) (5, 34, 75) and the breakage of the chromosome at two sites (Cbs 819 and Cbs 5-2) (28, 78) using pooled genomic DNA collected from *JMJ1*-knockdown strains. The *JMJ1*-knockdown strains showed minor increases in the unprocessed micronuclear forms of the M, R, and Tlr elements (Fig. 6B). In contrast, the Cam, Cbs 819, and Cbs 5-2 elements were processed normally. The minor increases observed in the knockdown strains were unlikely caused by any developmental delay, since DNA samples were extracted at 36 h postmixing, when most conjugating cells already reached the final stage of development.

To further explore the possibility that individual progeny carried different DNA elimination defects, individual sexual progeny were examined. In *JMJ1*-knockdown strains, 30 of 35 (86%) tested progeny accumulated the unprocessed form of the M element, 2 of 35 (5.7%) accumulated the unprocessed form of the R element, and 29 of 35 (83%) accumulated the unprocessed form of Tlr elements, while the Cam element was completely eliminated (Fig. 6C; see Table S1 in the supplemental material). Nevertheless, in noninduced strains, 1 of 10 (10%) tested progeny accumulated the unprocessed form of the M element and 5 of 10 (50%) accumulated the unprocessed form of Tlr elements, while the R and Cam elements were completely eliminated. This result showed that *JMJ1* RNAi knockdown partially inhibited the elimination of IES elements during conjugation and the effect might depend on the sequence or the genomic position of IESs. Differences in elimination efficiency of IESs were observed in several IES elimination mutants, including ∆*EMA1*, ∆*HENA1*, and ∆*WAG1*/∆*CnjB* strains (4, 8, 37).

**JMJ1** is required for efficient H3K27me3 and Pdd1p association with IESs. To examine whether *JMJ1* could regulate histone modifications on IES elements, we performed chromatin immunoprecipitation (ChIP) on different genomic loci. Noninduced cells, *JMJ1*-knockdown cells, and *EZL1*-knockdown cells were processed for ChIP at 10 h postmixing. The relative abundance of two micronuclear IESs, M-mic and R-mic, and one macronuclear gene, *ATU1* (12), was analyzed by quantitative real-time PCR after ChIP. Consistent with previous reports, two heterochromatin markers associated with IES regions, H3K27me3 and Pdd1p (42, 64), were enriched in both IESs in noninduced cells (Fig. 7), while H3K4me2, a euchromatin marker, was enriched in the macronucleus-destined *ATU1* gene (Fig. 7). Interestingly, in both *JMJ1* and *EZL1*-knockdown cells, H3K27me3 and Pdd1p enrichments in both M-mic and R-mic IESs were decreased (Fig. 7), although the enrichments in the R-mic IESs were slightly higher in *JMJ1*-knockdown cells than in *EZL1*-knockdown cells. In contrast, the H3K27me3 enrichment in M-mic and R-mic were not affected in *EZL2*-knockdown cells, indicating that *EZL2* and *EZL1* have different targets (see Fig. S2 in the supplemental material). These results suggested that *JMJ1* was required for efficient H3K27me3 and Pdd1p enrichments in IESs and that *JMJ1* did not directly remove the H3K27me3 modification in the IES chromatin during conjugation.

**JMJ1** knockdown affects the expression of late IES elimination genes. Since H3K27me3 is regarded as a repressive histone mark in other organisms, we speculated that *JMJ1* could regulate *Tetrahymena* development through regulation of gene expression. Because *JMJ1* knockdown partially inhibits IES elimination, we suspected that the expression of IES elimination-related genes was affected. To determine potential gene regulation targets of *JMJ1* in late conjugation stages, we performed gene expression analysis in *JMJ1*-knockdown and noninduced cells. Our analysis revealed that several IES elimination-related genes, including *TPB2*, *PDD3*, *LIA1*, *LIA3*, *LIA5*, and *DIE5*, all showed decreased or delayed expression in *JMJ1*-knockdown cells (Fig. 8A; see Table S2 in the supplemental material). Interestingly, the majority of these genes were activated after 6 h postmixing (when the old macronucleus began to degenerate and the new macronucleus began to form), suggesting that these genes were expressed from the new macronucleus (19, 46, 53, 58, 77). In contrast, the expression of early-expressed IES elimination-related genes, including *TWII* (50), *EZL1* (42), and *GIW1* (55), was not affected (Fig. 8B; see Table S2 in the supplemental material). Interestingly, the expression of *EMA1* and *PDD1* was partially inhibited in *JMJ1*-knockdown cells (Fig. 8B). Since *EMA1* and *PDD1* are expressed from early to late conjugation (4, 43), it is possible that *JMJ1* knockdown inhibited only the late transcription of these genes. The expression of a housekeeping gene, *RPL21* (59), was not affected in *JMJ1*-knockdown cells (Fig. 8B). Thus, general transcription is likely not inhibited in *JMJ1*-knockdown cells. Taken together, these results suggest that *JMJ1* is required for the proper expression of late IES elimination genes but not early-expressed IES elimination genes.

**JMJ1** knockdown affects the H3K27me3 status of late IES elimination genes. To compare enrichment of H3K27me3 marks at genes involved in IES elimination that are expressed late or early, we performed ChIP experiments in *JMJ1*-knockdown and noninduced cells. We observed that H3K27me3 marks on the late IES elimination-related genes, including *TPB2*, *DIE5*, *PDD3*, *LIA1*, and *LIA5*, were removed more slowly or accumulated in *JMJ1*-knockdown cells (Fig. 9A). In contrast, H3K4me2 levels at these repressed loci were relatively low in *JMJ1*-knockdown cells. The H3K27me3 and H3K4me2 levels at early-expressed IES elimination-related genes, including *TWII*, *EZL1*, and *GIW1*, were not affected in *JMJ1*-knockdown cells (Fig. 9B). In conclusion, these results suggest that *JMJ1* is required to regulate the removal of repressive H3K27me3 marks on the late IES elimination-related genes.

**DISCUSSION**

The question addressed by this study was whether the H3K27 demethylase homolog in *Tetrahymena*, *JMJ1*, plays a role in modulating H3K27me3 during conjugation and thus affects hetero-

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chromatin formation or the activation of genes critical for conjugation. We provide evidence that $\text{JMJ1}$ is critical for the proper activation of late DNA elimination genes, thus contributing to the assembly of heterochromatin on IESs, although through an indirect pathway. First, we showed that $\text{JMJ1}$ encodes a JmjC domain protein that is likely an H3K27me3 demethylase. It is localized first in the parental macronucleus and then in the new macronucleus and expressed only during conjugation. Next, using a hairpin RNA-knockdown approach, we showed that $\text{JMJ1}$ expression is critical for progeny production during conjugation and the demethylation of H3K27me3 in late stages of development. Finally, we showed that the expression of late DNA elimination-related genes, such as $\text{TPB2}$ and $\text{PDD3}$, was selectively inhibited in $\text{JMJ1}$-knockdown cells, while the expression of early DNA elimination genes, such as $\text{TWI1}$ and $\text{EZL1}$, was not and in fact was slightly increased. The H3K27me3 marks were also accumulated on late DNA elimination-related genes. Taken together, this study highlights the importance of $\text{JMJ1}$ in selectively regulating both the epigenetic H3K27me3 marks and the expression of late DNA elimination-related genes. To our knowledge, no other gene has been reported to control the expression of late DNA elimination-related genes in the new macronucleus.

Why does Tetrahymena require a specific H3K27 demethylase for conjugation? The EZL1-mediated H3K27 methylation in Tetrahymena has been known to be essential for the completion of conjugation. It is associated with the IES chromatin and essential for IES elimination. Demethylation of these H3K27 marks by a demethylase seemed unnecessary since the whole chromatin was eliminated in this process. The functional significance of $\text{JMJ1}$ demethylase during conjugation thus seems counterintuitive. In other organisms, JMJD3/UTX family members are important developmental regulators and required for proper activation of key development genes such as HOX genes (32, 57). Both JMJD3 and UTX members have been shown to demethylate H3K27 marks mediated by EZH2 homologs. In our experiments, $\text{JMJ1}$/$\text{EZL1}$ double-knockdown cells could not restore the progeny production, while $\text{JMJ1}$/$\text{EZL2}$ cells could, at least partially. Moreover, the ChIP experiment also showed that $\text{JMJ1}$ could not demethylate the IES chromatin. These results indeed suggest that $\text{JMJ1}$ does not demethylate $\text{EZL1}$-mediated H3K27me3 marks, which are related to IES elimination. Consistent with previous reports of JMJD3/UTX homologs, our results suggest that $\text{JMJ1}$ regulates another category of H3K27me3 marks that is carried out by $\text{EZL2}$ and these H3K27me3 marks regulate gene expression. These findings reveal a direct role for $\text{JMJ1}$ in controlling gene expression during new macronucleus differentiation. In this regard, Tetrahymena $\text{JMJ1}$ shares a similar developmental role with other JMJD3/UTX family members in regulating specific gene expression.

During vegetative growth, the micronucleus of Tetrahymena is decorated by the repressive H3K27me3 marks. Although EZL2 is not essential for Tetrahymena, it is likely required for the maintenance of H3K27me3 epigenetic memory. These repressive epige-
netic marks may limit the developmental potential of the newly developed macronucleus and thus need to be removed or reset during conjugation. Recently, one study in *C. elegans* has shown the importance of erasing H3K4me2 methylation marks in the germ line cells to avoid inappropriate transmission of this epigenetic mark to progeny. Mutations of the *C. elegans* H3K4me2 demethylase homolog cause abnormal accumulation of H3K4me2 marks in germ cells and disrupt the normal gene regulation program for sperm development, indicating that the germ line epigenetic marks could have a lasting effect on zygote gene expression (35). We think our finding provided a similar example for the erasing of silencing marks and resetting of the gene expression pattern during sexual reproduction. In our experiments, the partial recovery of the *JMJ1*-knockdown phenotype in *JMJ1*/EZL2 double-knockdown strains demonstrated that *JMJ1* antagonizes EZL2-mediated H3K27me3 during conjugation. The normal micronucleus is heavily decorated by H3K27me3 marks during vegetative growth and at the beginning of conjugation. The new macronucleus is differentiated from a descendant of the micronucleus after postzygotic nuclear division and becomes actively transcribed. We speculate that *JMJ1* is required for the erasure of these repressive H3K27me3 marks inherited from the germ line micronucleus, which are maintained by EZL2. Their removal would prevent inappropriate transmission of these marks into the developing macronucleus and allow the expression of key developmental genes.

Although we observed a correlation between *JMJ1* expression, H3K27me3 demethylation, and increased expression of late DNA elimination-related genes, the exact molecular mechanism that mediates activation of these genes is still unclear. *JMJ1* may directly contribute to the local chromatin configuration via H3K27me3 demethylation of these genes, or it may have an indirect effect by promoting the transcription of other transcription factors. Finally, recent reports have suggested that JMJD3 ho-

**FIG 8** Expression patterns of IES elimination-related genes in *JMJ1*-knockdown cells. (A) Total RNA samples were extracted from conjugating cells (8, 12, 16, and 24 h postmixing) transformed with the *JMJ1* hairpin RNA construct or GFP hairpin RNA construct. Late IES elimination genes (*TPB2, DIE5, PDD3, LIA1, LIA3, and LIA5*) were analyzed by quantitative RT-PCR and normalized using α-tubulin mRNA. RNA samples from cells with the GFP hairpin RNA construct were used as controls. (B) Expression of early IES elimination genes in *JMJ1*-knockdown cells. Total RNA samples were extracted from conjugating cells (8, 12, 16, and 24 h postmixing) transformed with the *JMJ1* hairpin RNA construct or the GFP hairpin RNA construct. The early IES elimination genes (*TWI1, EZL1, PDD1, EMA1, and GIW1*) were quantified by quantitative RT-PCR and normalized with α-tubulin mRNA. The expression of *RPL21* was used as an indicator for the expression of a housekeeping gene.
Methylomes may control transcription through a mechanism unrelated to their histone demethylase activity (25, 47), and JMJ1 may promote transcription through a similar mechanism. Several JmjC domain-containing proteins, such as DMM-1 in *Neurospora crassa*, Epe1 in *Schizosaccharomyces pombe*, and IBM1 in *Arabidopsis thaliana*, have been shown to regulate the boundary of heterochromatin (29, 48, 66, 71). For example, DMM-1 in *Neurospora* preferentially targets the boundary of heterochromatin to inhibit the spreading of heterochromatin marks, including H3K9me3 and DNA methylation, to the euchromatin region (29). However, in JMJ1-knockdown cells, we did not detect significant changes in the size of DNA eliminated. These results indicated that the deletion boundaries of IESs were not significantly affected and, thus, that JMJ1 was probably not involved in determining the boundaries of DNA deletion.

Interestingly, though Jmj1p is present at a low level in the parental macronucleus, the intensity of H3K27me3 in the parental nucleus was unaffected in JMJ1-knockdown strains. This finding suggests that JMJ1 is not required for H3K27me3 demethylation in the parental macronucleus. Note that several nuclear proteins required for the development of the new macronucleus, such as Ez1lp, Pdd1p, and Pdd2p, also appeared first in the parental macronucleus before localizing in the developing macronucleus, but their functions in the old macronucleus have not yet been demonstrated (21, 42, 54).

It is interesting that the intensity of H3K27me3 staining in the degrading parental macronucleus, which decreased normally during conjugation, was unaffected in JMJ1-knockdown strains. This result indicates that other protein factors or mechanisms are involved in demethylation of H3K27me3 in the degrading parental macronucleus. Consistent with this hypothesis, in degrading parental macronucleus, histone H3 has been shown to be subjected to cleavage at the N terminus (40). A recent study has shown that mouse cathepsin L can cleave H3 after residue 21 and also has the ability to

FIG 9 Methylation patterns of IES elimination-related genes in JMJ1-knockdown cells. (A) Methylation patterns of late IES elimination genes in JMJ1-knockdown cells. Conjugating cells transformed with the JMJ1 hairpin RNA construct with or without induction were processed for ChIP assay (9, 12, and 16 h postmixing) using either an H3K27me3 or an H3K4me2 antibody. The pulled down DNA samples from two duplicated experiments were analyzed by quantitative PCR with primers specific to TPB2, DIE5, PDD3, LIA1, and LIA5 and normalized with a nonimmunoprecipitated control. (B) Methylation patterns of early IES elimination genes in JMJ1-knockdown cells. Conjugating cells transformed with the JMJ1 hairpin RNA construct with or without induction were processed for ChIP assay (9, 12, and 16 h postmixing) using either an H3K27me3 or an H3K4me2 antibody. The pulled down DNA samples from two duplicated experiments were analyzed by quantitative PCR with primers specific to TWI1, EZL1, and GIW1 and normalized with a nonimmunoprecipitated control.
gradually cleave the residue between residues 21 and 27 (27). It is possible that a similar histone cleavage activity is responsible for the H3K27 demethylation in the degrading parental macronucleus.

In summary, our findings establish JMJD1 as an H3K27me3 demethylase required for new macronucleus development during *Tetrahymena* conjugation. The regulation of H3K27 methylation by JMJD1 appears to selectively regulate key development genes required for development. Future studies of JMJD1 should provide more insight into the transcriptional regulation during the onset of new macronucleus differentiation.

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