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, JMJ1, in *Tetrahymena*. During conjugation, JMJ1 expression is upregulated and the protein is localized first in the parental macronucleus and then in the new macronucleus. In conjugating cells, knockdown of JMJ1 expression resulted in a severe reduction in the production of progeny, suggesting that JMJ1 is essential for *Tetrahymena* conjugation. Furthermore, knockdown of JMJ1 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 *JMJ1*-knockdown cells and reduced abnormal H3K27me3 accumulation in the new macronucleus. Taken together, these results demonstrate a critical role for JMJ1 in regulating H3K27me3 during conjugation and the importance of JMJ1 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 histone lysine methylation at H3K27, H3K9, and H4K20 is reversible (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, 79). The methylation state of H3K27me3 is also involved in many important cellular processes, including development and 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) (14). H3K27 methylation is recognized by other histone modification reader proteins, which can promote heterochromatin formation (79). The methylation state of H3K27me3 is also related to the formation of heterochromatin and specific residues, the overall accessibility of chromatin and specific histone modifications (13, 36). Among these covalent modifications, the overall accessibility of chromatin and transcriptional activities of genes are affected by these covalent modifications (13, 36). Histone methylation modulators contain a JmjC domain (57). Two recently identified JmjC domain-containing H3K27me3 demethylases, JMJ3 and UTX, revealed the importance of active H3K27me3 demethylases. Both have been shown to remove the repressive H3K27me3 marks and function as transcriptional activators (2, 26, 30, 38, 39). Binding of UTX and JMJ3 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 JMJ3 homolog in *Caenorhabditis elegans* disrupts proper gonadal development and organization (2, 38). In addition to HOX genes, JMJ3 and UTX are also required for the activation of other genes essential for differentiation. For instance, JMJ3 is required for the activation of an H3K27me3-suppressed gene, *brachyury*, during embryonic stem cell differentiation (22). Furthermore, JMJ3 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 JMJ3, UTX is required for the activation of myogenic genes (60). In response to cellular oncogenic stress, JMJ3 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...
removing H3K27me3 to regulate essential gene expression. 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 micronucleus 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 micronuclear 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 micronucleus, 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 cosuppression 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), VI1]], and CU427 [Otx/Otx [cycloheximide sensitive (cy-s), VI1]] 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% proteose 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 JmjD3/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 Masterplus 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 Jmj1p, 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 bistolic transformation of Tetrahymena. Transformants were selected by paromycin and examined for integration at the macronuclear 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 JMJ1/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 pCRH13 vector using two different sets of primers, a forward set with the Pmel-Smal site and a reverse set with the Xhol–Apal site (see Table S4 in the supplemental material for oligonucleotide sequences). The hairpin cassette was cloned into the pBIB ribosomal DNA (rDNA) vector at the Pmel–Apal site. The expression of hairpin RNA was controlled by a Cdc11-inducible metallothionein promoter. Mating cells 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-Nylon+ 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 0.0015 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 postmixing using 0.05 μg/ml of Cdc11. 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 from the wild type.
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 µg/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 2X 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, 1X 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 1X PBS, 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).

FIG 1 Phylogenetic analysis of Tetrahymena JMJ1 and its human homologs. (A) Phylogenetic analysis of the Tetrahymena JmjC domain-containing proteins. The alignment of Tetrahymena JmjC domains and construction of the phylogenetic tree were generated by the neighboring-joining method. Hs, Homo sapiens; Tt, Tetrahymena thermophila. Bar, 0.2 amino acid substitutions per site. The bootstrap values of 1,000 replications are shown on internal branches. The reported demethylase specificity of individual JmjC domain-containing proteins is shown next to the tree. (B) Protein domain structure of the JMJD3/UTX/UTY family members. a.a., amino acids. (C) Sequence alignment of the JMJD3/UTX/UTY family members at the conserved JmjC catalytic domain. Dark gray boxes represent conserved identical amino acid residues, and light gray boxes represent similar residues. Red stars and diamonds indicate the conserved amino acid residues that are important for binding of cofactor Fe(II) and 2-oxoglutarate, respectively, which are required for the catalytic activity of the JmjC enzymes.
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 × 10^7 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 junomion C (JmjC) class enzymes remove tri-methyl 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://tged.ihb.ac.cn/). 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/D/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.
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 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 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 JMJ1 might be associated with regulating histone demethylation in the parental macronucleus or developing a new macronucleus during conjugation.

**JMJ1 is required for production of viable progeny.** From its gene expression and protein localization patterns, we suspected that JMJ1 might have an essential function in regulating histone methylation during conjugation. However, we were unable to obtain complete germ line JMJ1-knockout strains, possibly due to its essential function during conjugation. We thus generated JMJ1 RNA interference (RNAi)-knockdown strains which expressed JMJ1 hairpin RNA strains were mated and either untreated or treated with CdCl2. 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 0.5 g/ml CdCl2. Progeny production was determined by the drug resistance phenotype. WT, wild type. (D) Developmental profiles of JMJ1-knockdown strains. The conjugation stages of 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).

**FIG 3** Knockdown of JMJ1 by hairpin RNA. (A) Schematic representation of the JMJ1 hairpin RNA construct. The open arrow indicates 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 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 g/ml CdCl2. The approximate size of JMJ1 mRNA is indicated to the right. The ethidium bromide staining of total rRNA was used as a loading control. (C) Progeny production of JMJ1-knockdown cells. Control and JMJ1 hairpin RNA strains were mated and either untreated or treated with CdCl2. 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 g/ml CdCl2. Progeny production was determined by the drug resistance phenotype. WT, wild type. (D) Developmental profiles of JMJ1-knockdown strains. The conjugation stages of 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 \textit{JMJ1} is essential for \textit{Tetrahymena} to produce viable progeny. When treated with cadmium, the vegetative growth of \textit{JMJ1} RNAi-knockdown strains was not affected (Fig. 3C), which agreed with the conjugation-specific expression profile of \textit{JMJ1} mRNA.

To study the role of \textit{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 \textit{JMJ1} RNAi-knockdown strains could complete most of the nuclear development events.

\textit{JMJ1} is required for H3K27 demethylation in late stages of conjugation. To determine whether \textit{JMJ1} is involved in H3K27 histone demethylation \textit{in vivo}, protein extracts of \textit{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 \textit{JMJ1} RNAi-knockdown cells.
from 15 to 26 h postmixing, indicating a role for 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 simultaneously in both mating partners. The efficiency of silencing was confirmed by quantitative PCR and Western blot analysis. The progeny production of JMJ1/EZL2 double-knockdown strains remained low, indicating that the knock down of EZL1 could not compensate for the loss of JMJ1 and vice versa. In contrast, the progeny production of JMJ1/EZL2 double-knockdown strains was partially restored (Fig. 5B) and was similar to that of the EZL2 single-knockdown cells. This finding demonstrated that JMJ1 interacts with EZL2 in the same pathway during conjugation.

To gain further insights into the cross talk between JMJ1 and EZL2, we examined the dynamics of H3K27me3 in EZL2 single-knockdown and JMJ1/EZL2 double-knockdown strains using Western blot analysis. 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 EZL1 or EZL3, also methylated H3K27 during this time. In contrast to JMJ1-knockdown strains, JMJ1/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 JMJ1/EZL2 double knockdown. Taken together, these results suggested that JMJ1 removed EZL2-mediated H3K27me3 methylation and the low progeny production of JMJ1-knockdown strains could be linked to the accumulation of H3K27me3 in late conjugation stages.

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
uation 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 Δ*EMA1*, Δ*HEN1*, and Δ*WAG1* 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 chromotkeratin 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 are 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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*Results of DNA deletion and chromosome breakage assays from pooled cells. Genomic DNA was isolated from the indicated matings with or without CdCl2 induction at 36 h postmixing. White arrows indicate the nonrearranged forms, and black arrows indicate the rearranged forms. Signals are quantitated and shown as ratios of the signals (Mic form/Mac form) obtained from ethidium bromide gel staining. (C) Results of DNA deletion assays from isolated pairs. Individual progeny pairs were isolated from the indicated mating with or without CdCl2 induction. These cells were analyzed for IES elimination by single-pair PCR at 24 h postmixing. PCR results of 35 progeny pairs from *JMJ1*-knockdown strains and 10 progeny pairs from untreated strains are shown. Asterisks indicate progeny pairs that contain the nonrearranged form of IES. Progeny pairs isolated from cells with the EZL1 hairpin RNA construct were used as knockdown controls.*
chromatin formation or the activation of genes critical for conjugation. We provide evidence that 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 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 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 TPB2 and PDD3, was selectively inhibited in JMJ1-knockdown cells, while the expression of early DNA elimination genes, such as TWI1 and 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 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 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, JMJ1/EZL1 double-knockdown cells could not restore the progeny production, while JMJ1/EZL2 cells could, at least partially. Moreover, the ChIP experiment also showed that JMJ1 could not demethylate the IES chromatin. These results indeed suggest that JMJ1 does not demethylate EZL1-mediated H3K27me3 marks, which are related to IES elimination. Consistent with previous reports of JMJD3/UTX homologs, our results suggest that JMJ1 regulates another category of H3K27me3 marks that is carried out by EZL2 and these H3K27me3 marks regulate gene expression. These findings reveal a direct role for JMJ1 in controlling gene expression during new macronucleus differentiation. In this regard, Tetrahymena 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-
Histone 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-
Molongos 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 Ezl1p, 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...
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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REFERENCES

29. Matsuda A, Shieh AW, Chalker DL, Forney JD. 2010. The conjugation-


