Extensive DNA elimination occurs as part of macronuclear differentiation during *Tetrahymena* sexual reproduction. The identification of sequences to excise is guided by a specialized RNA interference (RNAi) machinery that targets the methylation of histone H3 lysine 9 (H3K9) and K27 on chromatin associated with these internal eliminated sequences (IESs). This modified chromatin is reorganized into heterochromatic subnuclear foci, which is a hallmark of their subsequent elimination. Here, we demonstrate that Lia4, a chromoshadow domain-containing protein, is an essential component in this DNA elimination pathway. *LIA4* knockout (ΔLIA4) lines fail to excise IESs from their developing somatic genome and arrest at a late stage of conjugation. Lia4 acts after RNAi-guided heterochromatin formation, as both H3K9 and H3K27 methylation are established. Nevertheless, without *LIA4*, these cells fail to form the heterochromatic foci associated with DNA rearrangement, and Lia4 accumulates in the foci, indicating that Lia4 plays a key role in their structure. These data indicate a critical role for Lia4 in organizing the nucleus during *Tetrahymena* macronuclear differentiation.

The organization of the eukaryotic nucleus is critical to achieve proper gene expression and to maintain genome integrity. Individual regions of the genome are partitioned into specific domains, historically called euchromatin and heterochromatin. The DNA of euchromatic domains is relatively dispersed throughout the nucleus, which likely permits greater accessibility to the transcription machinery. In contrast, heterochromatic domains are condensed, a property consistent with its association with transcriptionally silent regions of the genome. This condensed state of heterochromatin also prevents unwanted recombination between the repetitive elements, which can constitute large portions of eukaryotic genomes, thereby increasing genome stability (1, 2). Recent genomewide studies have revealed that euchromatin and heterochromatin actually comprise many distinct chromatin landscapes (3). These landscapes are characterized by specific patterns of histone posttranslational modifications and association with subsets of nonhistone chromosomal proteins. For instance, constitutive heterochromatin is enriched in histone H3 lysine 9 di- and trimethylation (H3K9me2,3) and is bound by heterochromatin protein 1 (HP1), which together spatially organize the modified region within the nucleus and enforce the silent state (4, 5). HP1 forms homodimers via its chromoshadow domain (CSD) to form a critical protein interaction interface that serves as a scaffold to form heterochromatin (6).

Heterochromatic regions are established in early stages of development, forming as early as the two-cell stage in mammalian embryos (7). Nevertheless, heterochromatin is not static and may change as differentiation proceeds. At the level of individual loci, establishment of facultative heterochromatin to enforce gene silencing is necessary for cell type specification. Changes can also lead to global reorganization of the nucleus, as observed during the development of rod photoreceptor cells in nocturnal animals (8). Even after development is completed, heterochromatin remains a dynamic structure. In dividing cells, heterochromatin must disassemble and reassemble in order to be replicated and segregated properly. Also, in response to DNA damage, such as double-strand breaks, this compact chromatin structure is decondensed, providing greater access for the DNA repair machinery (9, 10). As part of the damage response, the histone variant H2AX is phosphorylated and damaged regions are concentrated into subnuclear DNA repair foci (11, 12). Once repaired, the heterochromatic structure must be reestablished on the repaired region and affected loci repositioned within the nucleus.

Differentiation of the somatic genome of the ciliated protozoan *Tetrahymena* provides an opportunity to study de novo formation of heterochromatin during development. Even though ciliates are single-celled organisms, they possess two separate and functionally distinct copies of their genome, maintained in different nuclei. During vegetative growth, the somatic genome, which is polyploid, is carried in the macronucleus, where it serves as the template for all gene expression. The germ line genome, which is diploid, is housed in the transcriptionally silent micronucleus (13). During the sexual reproductive process of conjugation, these nuclei have very different fates. The macronucleus from the previous generation provides all prezygotic gene expression but is eventually silenced and discarded. Meanwhile, the micronucleus undergoes meiosis to produce gametic nuclei that cross-fertilize mating partners and produce zygotic nuclei, which contain a haploid genome copy from each parent and go on to divide and eventually differentiate into a new micronucleus and macronucleus.

Differentiation of the new somatic genome not only requires transcriptional activation of a previously silent genome, but also involves extensive DNA rearrangements. The five micronucleus-derived chromosomes are fragmented at ~180 sites recognized by the presence of a conserved 15-bp chromosome breakage sequence (CBS) (14–16). In addition, DNA segments of various sizes, called internal eliminated sequences (IESs), are eliminated...
from thousands of loci dispersed throughout the genome (17). These sequences include transposons and other repetitive elements (18, 19), which comprise roughly one-third of the micro-nuclear genome (Tetrahymena Comparative Sequencing Project, Broad Institute of Harvard and Massachusetts Institute of Technology [http://www.broadinstitute.org]).

Unlike the conserved chromosomal breakage sequence, there is no conserved sequence that identifies an IES. Instead, these diverse loci are recognized by homologous small RNAs and targeted for removal from the genome by RNA-guided heterochromatin formation (20). The source of these small RNAs is bidirectional transcription of IESs in the meiotic micronucleus, which generates long double-stranded RNAs (dsRNAs) that are processed by a Dicer-like enzyme into 28- to 30-nucleotide (nt) so-called scan RNAs (snRNAs) (21–24). The snRNAs associate with an Argonaute family protein encoded by *Tetrahymena PIWI1* (TWI) and together are transported into the parental micronucleus to be compared to the somatic genome (25). Complexes with snRNAs homologous to sequences present in the parental micronucleus are removed, leaving a pool of Twi1-complexed snRNAs representing sequences found only in the micronucleus (24). These Twi1-snRNA complexes are then imported into the developing macronucleus, where they direct histone H3K9 and H3K27 methylation by Ezl1 on IES chromatin (26, 27). The methylated chromatin domains are bound by the HP1 homologue Pdd1 and, in association with many other proteins, are condensed into distinct sub-nuclear structures called DNA elimination bodies (27). Cleavage events at both sides of the IESs, catalyzed by Tpb2, a domesticated piggyBac transposase, excise the IESs from the developing macronuclear chromosomes, and the resulting double-strand breaks are repaired by a TKU80-dependent DNA repair pathway (28, 29).

The processes necessary to organize IESs into DNA elimination complexes, including the binding of Pdd1 to methylated chromatin, are poorly understood. Once bound, 5,000 to 6,000 Pdd1-bound IESs must be reorganized from dispersed loci throughout the nucleus into condensed DNA elimination foci (30). These condensed heterochromatic foci likely must be maintained by structural proteins present in the foci themselves. In addition, late in conjugation, these structures reorganize from smaller foci into larger but less numerous foci located preferentially at the nuclear periphery (31). Multiple factors that are critical for this dynamic nuclear reorganization have been identified, including the chromatin remodeler Brg1, the double-stranded RNA binding protein Drb2, and the novel proteins Lia1 and Lia5 (32–35). While roles for Brg1 and Drb2 suggest that chromatin remodeling and double-stranded RNA participate in genome reorganization, the fact that several novel proteins with largely unknown functions are essential indicates that much work remains to elucidate the mechanism underlying the formation of DNA elimination bodies and the regulation of DNA rearrangement.

In this study, we investigate the role of a CSD-containing protein in *Tetrahymena*, encoded by *LIA4*. We find that *LIA4* is required for completion of conjugation, as cells lacking this gene, Δ*LIA4*, arrest late in development and fail to excise IESs from developing macronuclei. These mutants do not assemble DNA elimination bodies, even though histone methylation is established in the developing somatic genome. These findings indicate that the CSD protein encoded by *LIA4* has a critical role in the IES excision pathway downstream of histone methylation.

### MATERIALS AND METHODS

**Tetrahymena strains and growth conditions.** Wild-type (WT) *T. thermophila* strains B2086 (II), CU427 (Chxl-1/Chx1-1 [VI, cy-s]), and CU428 (Mpr1-1/Mpr1-1 [VII, mp-s]) and micronucleus-defective strains B*VI* (VI) and B*VII* (VII) were obtained from Peter Bruns (Cornell University, Ithaca, NY). *Tetrahymena* cells were grown in SPP medium (1% proteose peptone, 0.2% dextrose, 0.1% yeast extract, 10 μM FeCl₃) at 30°C and prepared for mating by washing the cells out of the growth medium with 10 mM Tris–HCl (pH 7.4). After starvation by overnight incubation in 10 mM Tris, the cells were mixed to initiate conjugation. To achieve greater synchrony of mating, in some experiments, cells were starved in Dryl’s medium and costimulated with mixing by shaking at 200 rpm for 30 min before allowing pair formation (36). The generation of ΔPDD1, ΔDCL1, and PDD1-CFP strains was described previously (22, 33, 37).

**Alignment of the LIA4 CSD.** The peptide sequence for *LIA4* (Ttherm_00085600) was examined for secondary structure using HHpred (http://toolkit.tuebingen.mpg.de/hhpred) (38). The CSD of *LIA4* was aligned with the CSDs of *Mus musculus* Cbx1 (accession number NP_031648.1); *Homo sapiens* Cbx1 (accession number NP_001120700.1), Cbx3 (accession number NP_057671.2), and Cbx5 (accession number NP_001120794.1); *Drosophila melanogaster* HP1a (accession number NP_723361.1); and *Schizosaccharomyces pombe* Swi6 (accession number NP_593449.1) by using clustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). *T. thermophila* LFL1 (Ttherm_00620940) and LIA4 and LFL1 homologues in other *Tetrahymena* species were identified by BLAST searches of their macronuclear genome sequences (http://www.ciliate.org).

**Generation of ΔLIA4 strains.** Homologous arms for plia4KO were created by amplifying sequences spanning from 1,351 bp upstream of the start codon to the first 9 bp of the gene and from 154 bp to 1,501 bp downstream of the stop codon. Restriction sites were introduced onto the ends of the sequences and cloned into pBS SK(−) at the KpnI and HindIII restriction sites for the upstream arm and at the SacI and SaeI restriction sites for the downstream arm (the primers are listed in Table 1). The NEO3 cassette (39) was excised from pENTR–D–Topo-Mti1/NEO3 (40) and cloned between the two homology arms using Ascl and BsrGI restriction sites that were introduced into the plasmid when the upstream arm was cloned in order to generate plasmid pLIA4KO. The knockout cassette was generated by digesting the plasmid with KpnI and transformed into conjugating WT cells (CU428 × B2086) between 2 and 3 h after mixing by using a PDS–1000/He particle bombardment system (Bio–Rad) as previously described (41, 42). Heterozygous micronuclear transformants were identified by their resistance to 80 μg/ml paromomycin with 1 μg/ml CdCl₂ and 15 μg/ml 6-methylpurine. Successful homologous recombination of the NEO3 cassette into the LIA4 locus was confirmed by PCR screening of the heterozygous micronuclear transformants. Homozygous micronuclear knockout heterokaryons were generated by crossing heterozygous micronuclear transformants with B*VI or B*VII star strains. The resulting homozygous micronuclear knockout heterokaryons were identified by sensitivity to paromomycin–CdCl₂ and by PCR screening of crude cell lysates (the primers are listed in Table 1) (42). The homozygous micronuclear genotype was verified by crossing each strain with CU427, which produced progeny resistant to both cycloheximide (25 μg/ml) and 100 μg/ml paromomycin with 1 μg/ml CdCl₂. The complete (micro- and macronuclear) LIA4 knockout ΔLIA4 11-2 and ΔLIA4 15-3 strains were generated by crossing homozygous micronuclear knockout heterokaryons of different mating types and selecting for progeny resistant to paromomycin–CdCl₂.

**Southern blotting.** *T. thermophila* genomic DNA was isolated using a Wizard genomic DNA purification kit (Promega). The DNA was digested with the appropriate restriction enzyme, size fractionated by agarose gel electrophoresis, and transferred to a nylon membrane (Osmonics, Minnetonka, MI) by downward capillary blotting in 0.5 M NaOH–1.5 M NaCl. The membranes were hybridized at 65°C with radiolabeled probes.
TABLE 1

| Purpose                        | Name                  | Sequence                                                                 |
|-------------------------------|-----------------------|--------------------------------------------------------------------------|
| Creating knockout cassette    | LIA4LARMFKPN          | GATCCGTACCATTTTGGATTTAAGGCTGAACC                                         |
|                               | LIA4LARMHRHIND        | GATCAGGCGCGCGCGCGCCTGTACATCAACATTGGAAGGCAAATAACG                          |
|                               | LIA4RARMSFACII       | GATCCGGGCAACTTTTCTTATTCCTACTTTCTCACA                                      |
|                               | LIA4RARMSFACI        | GATCGAGCTCGGTACCATCGAGAAAACAAATCAGTCTTTGAAAAGCAC                             |
| Screening for knockout lines  | LIA4MTTLR             | AACATCTCAACATTTGTCGACTAAATA                                              |
|                               | LIA4GENLRF            | TTGGTACCTCCCTCTCCTCTCAA                                                   |
|                               | LIA4GENLRR            | GCTTTTTCTTTATTTCCAGGTTCA                                                   |
|                               | LIA4MTTRFR            | CTTCTATCGCCTTGCTGAG                                                       |
|                               | LIA4GENRR             | TCAATAAATGTTGAAATCTTTAGGATTT                                              |
|                               | LIA4GENRF             | CCAATAATAATAATAATAATTGCTGAG                                               |

| RT-PCR to confirm knockout lines | LIA4RT3 | TCATTTCCTATTTTGGCTCACAACATC     |
|                                  | LIA4SEQ3             | ACTGGTAGATGTTAGTGGAGGAAAGGAG                                               |
|                                  | hhp1-RT-Fw           | GGAGCTTCACACTTCAAACAACGG                                                 |
|                                  | hhp1-RT-Rv           | TCGGGGAGAAGCATACCTTAGCA                                                    |

| Detection of circular IES excision products | M_circles_0.9_FW1 | GAAACCCATCCCCCTTTCATTTT         |
|                                             | M_circles_0.9_FW2  | TTGTCTTGAATGTTTACAAAAATG        |

| Assay rearrangement of IESs | M110 | TACGAGTAGTGACGTGAGGCGG           |
|                            | M1194 | GTGGGGGAGGGAAGGATTCAAAC          |
|                            | IES1_MDSL-110 | TGGGGATCTCCTCAGAAGCGGAAT        |
|                            | IES1_MDSL-31  | CCGCTAGACACCCGGTGATCAA          |
|                            | IES7_MDSL-112 | GAGATTGATGGCATTAAAATGGA         |
|                            | IES7_MDSL-158 | AAGCCGACAGATCCGCGATGTC         |
|                            | IES11_MDSR-42   | GGGCCACAATATACAGAGCGAATTTT       |
|                            | IES11_MDSR-34   | GGGCCACCTTGATACCCGATT          |
|                            | R5(168-sense)-2   | AATTATTTCTTTAATCTGACTC          |
|                            | R3(1453-anti)-2   | AAGATAGTTCTAGAATAGAC           |

| Creating the LIA4-mCherry construct | LIA4ENDOCTERM5F | GATCCGGGCGCGCGCTGTGATAGTGCAACAGGTT |
|                                     | LIA4ENDOCTERM5R | GATCGAGCTCCTCCCTCTTATTTTCTTTTATTCTATAGGT |
|                                     | LIA4ENDOCTERM3F | GATCTGCGACAAATAAGTCTATGTTATTTGATTTTATAGT |
|                                     | LIA4ENDOCTERM3R | GATCTCTGAGACCCTTATGAGACCAAAGAC |

in 2× Denhardt’s solution and washed at 65°C in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.5% SDS to remove nonspecific hybridization. Probes were labeled with [α-32P]-dATP, random hexamers, and DNA polymerase I (Klenow fragment). Hybridization was visualized by autoradiography. To examine the LIA4 locus in LIA4 knockouts, isolated genomic DNA was digested with EcoRI and hybridized to a radiolabeled probe corresponding to the upstream homology arm from pLia4KO. Chromosome breakage was measured as previously described (22).

RT-PCR expression analysis. RNA was isolated from growing, starved, and conjugating WT (B2086 × CU428) and ΔLIA4 (ΔLIA4 11-2 × ΔLIA4 15-3) cells at 3 h intervals from 3 h to 12 h postmixing by RNAsol extraction (43). Reverse transcription (RT)-PCR was conducted on WT and ΔLIA4 samples from all time points to show loss of function. Probes were labeled with [32P]-dATP, random hexamers, and DNA polymerase I (Klenow fragment). Hybridization was visualized by autoradiography. To examine the LIA4 locus in LIA4 knockouts, isolated genomic DNA was digested with EcoRI and hybridized to a radiolabeled probe corresponding to the upstream homology arm from pLia4KO. Chromosome breakage was measured as previously described (22).

Determination of percent progeny production. Individual mating pairs from WT (B2086 × CU428) or ΔLIA4 (ΔLIA4 11-2 × ΔLIA4 15-3) crosses were isolated into drops of SPP medium. Conjugants were allowed to complete mating, and viable cells were grown to saturation. To distinguish progeny from pairs that aborted mating, surviving cells were cultured, starved, and mixed with WT strains to assess maturity by the ability to initiate pairing. Cells unable to mate with two different WT strains of different mating types were defined as immature and were counted as true progeny.

IES excision assays. Excision of the B, C, D, M, and R IESs was assayed by PCR in WT (CU428 × B2086), ΔDCL1 (ΔDCL1 1.8.6 × ΔDCL1 4.2.4), and ΔLIA4 (ΔLIA4 11-2 × ΔLIA4 15-3) strains. Genomic DNA was collected at 32 h postmixing, and PCR was conducted with primers flanking the assays IES (the primers are listed in Table 1). The presence of circular IES excision products was assayed by PCR in the WT (CU428 × B2086) and ΔLIA4 (ΔLIA4 11-2 × ΔLIA4 15-3) strains. Genomic DNA was isolated from conjugating cells at 2-h intervals from 10 h to 18 h postmixing, and a nested PCR (two rounds of PCR with 25 cycles each) approach was used to detect circular products (29, 35).

Protein localization and fluorescence microscopy. The developmental stage of mating cells was determined by nuclear configuration. Cells were fixed in 2% paraformaldehyde at 8 h, 12 h, 16 h, and 32 h postmixing and stained with 4’,6-diamidino-2-phenylindole (DAPI) (1 μg/ml) to visualize nuclei. DAPI fluorescence images were captured, and the nuclear morphologies of 100 fixed and DAPI-stained cells were determined and sorted into four categories representing progressive stages of macronuclear development that were previously described: MAC I, MAC II, MAC III, and complete mating (44, 45).

Pdd1-YFP localization. The construction of pBS-ICY-PDD1, a PDD1-yellow fluorescent protein (YFP) expression vector, was described previously (33). pBS-ICY-PDD1 was digested with Bpl and SalI and transformed into starved ΔLIA4 11-2 cells using the PDS-1000/He particle bombardment system (41, 42). Transformants were identified by their

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resistance to 25 μg/ml cycloheximide. Starved transformants were mixed with either starved CU427 (WT mating) or starved ΔLIA4 11-2 (ΔLIA4 mating) cells, and 0.04 μg/ml CdCl2 was added at both 0 h and 4 h postmixing to induce expression of Pdd1-YFP. At 15 h postmixing, cells were harvested by low-speed centrifugation (1,000 × g) and immobilized in 4 to 5 μl 2% methylcellulose. Differential interference contrast (DIC) and YFP fluorescence images were captured using a Qimaging RetigaEX charge-coupled-device camera (Burnaby, British Columbia, Canada) and Openlab software (PerkinElmer). The images were cropped, and their brightness and contrast were adjusted uniformly when necessary using Adobe Photoshop CS5.

**Immunofluorescence analysis.** Conjugating WT (B2086 × CU428), ΔLIA4 (ΔLIA4 11-2 × ΔLIA4 15-3), and ΔPDD1 (ΔPDD1 w3.3 × ΔPDD1 39.1) cells were fixed with Schaudinn’s fixative (2 parts saturated mercuric chloride, 1 part 95% ethanol) in methanol at 9 h, 10 h, and 15 h postmixing to permeabilize the cells. Anti-H3K9me2 [BSA], and 0.01% Tween 20) once and incubated with blocking buffer containing buffer (50 mM Tris-Cl, 150 mM NaCl, 1% bovine serum albumin [BSA], and 0.01% Tween 20) once and incubated with blocking buffer containing blocking buffer and then counterstained with DAPI in blocking buffer.

**RESULTS**

**LIA4 encodes a novel protein with a putative CSD.** The developmental remodeling of the somatic genome involves both the coordinated activation of zygotic gene transcription and the silencing/elimination of repetitive and transposon-derived sequences. Through our efforts to characterize the DNA rearrangement machinery, we identified candidate genes, named localized in macro-nucelar anlagen (LIA), encoding proteins that are expressed exclusively during conjugation and localized within developing macronuclei (47). Although similar in both localization and expression, the Lia proteins did not share homology with one another and possess few conserved domains that might indicate function. We previously demonstrated that Lia1 and Lia3 are each required for IES excision and chromosome fragmentation (34, 35). Similar to the genes encoding these essential proteins, LIA4 expression is rapidly induced starting 6 h into conjugation (47). Unlike the other LIA genes and ~71% of the current gene models (48), the LIA4 coding region contains no introns, despite its relatively large size of 2,428 bp.

**LIA4 is predicted to encode a 991-amino-acid (aa) protein with a molecular mass of 115 kDa.** Previous bioinformatics analyses revealed that the first ~200 residues are enriched in acidic amino acids and that the C-terminal ~700 amino acids are rich in glutamine (19%) (47) but did not identify any obvious conserved domains. Subsequent analyses identified a short region of homology with the CSD of HP1 proteins. An alignment comparing residues 793 to 835 near the C terminus of LIA4 with the CSDs of various HP1 homologues showed that many of the residues important for the structure of the CSD are conserved. In particular, two amino acids required for dimerization of HP1B (Cbx1) (49) are conserved in LIA4 (Fig. 1A).

Using HHpred, a protein homology and structural prediction utility (38), this region of Lia4 is predicted to form a double alpha-helical structure similar to that found in the CSD of HP1 homologues (6) (Fig. 1A). Similarly, a second protein structure prediction program, Phyre2 (50), returned the CSD of *Drosophila* HP1a as the most
similar structure, with a confidence value of 55.7%. Even though the amino acid sequence is only weakly conserved across the entire domain, this similarity of the predicted structures provides further evidence that LIA4 contains a CSD.

Clear homologues can be found in the related species Tetrahymena ellioti, Tetrahymena malacensis, and Tetrahymena borealis, indicating LIA4 is conserved within the genus Tetrahymena. BLAST searches against the T. thermophila genome also revealed that the CSD region of LIA4 shares similarity with another gene (Titherm_00620940), which we have named Lia4-like 1 (LFL1). LFL1 is found in the other Tetrahymena species, as well. An alignment with both genes found in four Tetrahymena species shows the high level of conservation in the putative CSD of LIA4 (Fig. 1B). The conservation between LIA4 and LFL1 extends beyond the alpha-helical region found in LIA4, including an extra 31 residues upstream of its predicted start site.

**LIA4 is required for completion of conjugation.** To determine whether LIA4 is necessary for completion of conjugation, we deleted the LIA4 gene from both the macronucleus and micronucleus. Using biolistics transformation (41, 42), we replaced the WT gene with the NEO3 selectable marker, initially generating heterozygous micronuclear LIA4 knockout lines with both WT and knockout (ΔLIA4) alleles in micronuclei. After using genomic-excision crosses to create strains homozygous for the knockout allele, the resulting lines were crossed together to produce progeny with all LIA4 copies removed from micro- and macronuclei. The ability to generate ΔLIA4 progeny indicated both that LIA4 is not essential for vegetative growth and that zygotic expression is not required for completion of conjugation (data not shown). Southern blot analysis confirmed that all copies of LIA4 were disrupted in these progeny and replaced with the NEO3 cassette (Fig. 2A). RT-PCR analysis verified that WT cells express LIA4 exclusively during conjugation, as previously reported (47), and that ΔLIA4 cells do not produce LIA4 mRNA (Fig. 2B).

As expected, given the lack of LIA4 vegetative expression, we observed no noticeable growth defects for ΔLIA4 strains compared to WT strains (data not shown). In contrast, when we mated ΔLIA4 strains, we found that they failed to produce viable progeny (Fig. 3A). We examined the progression of ΔLIA4 strains through conjugation and showed that they completed early developmental stages normally before arresting at the MAC III stage of conjugation (Fig. 3B and C). MAC III is a very late developmental stage that is characterized by the presence of two macronuclei and two micronuclei (44, 45). WT control cells completed conjugation by eliminating one of these new micronuclei and could divide vegetatively once returned to growth medium (Fig. 3). Thus, Lia4 has an essential role in conjugating cells.

**LIA4 is required for IES excision.** Arrest at the MAC III stage is a common phenotype exhibited by cells (such as ΔPDD1, ΔDCL1, ΔTWI1, and ΔLIA4 cells) that fail to eliminate IESs during development (22, 25, 34, 51). To test whether ΔLIA4 cells can complete DNA rearrangements, we isolated DNA from populations of cells 32 h after initiation of conjugation; this DNA was used as the template for PCR with primers flanking five previously characterized IESs (52–54) to obtain a semiquantitative assessment of excision. For all IESs examined, we observed increased amplification of larger products, indicative of accumulation of the germ line (unrearranged) forms, in the samples recovered from both ΔLIA4 and ΔDCL1 control matings relative to the WT (Fig. 4A). Note that the appearance of the smaller products corresponding to the rearranged loci was expected even if all de novo excision failed, due to the DNA from unmated cells present in the population. Thus, ΔLIA4 cells are unable to perform DNA elimination.

The accumulation of unrearranged DNA in ΔLIA4 matings suggested that IES excision is blocked prior to initiating the double-strand breaks at IES ends; however, it remained possible that some cleavage occurred but that the breaks were not repaired. To determine whether any IES excision was initiated in ΔLIA4 matings, we tested for the presence of circular IES excision products, whose presence is indicative of the formation of double-strand breaks. In WT matings, we observed the presence of the circular products between 10 and 16 h into mating, which roughly corresponds to the timing of IES excision; however, we were unable to detect circular products at any time point in the ΔLIA4 samples (Fig. 4B). This result indicated that ΔLIA4 strains likely arrest before the introduction of double-strand breaks in the IES excision process.

**LIA4 is required for chromosome breakage.** Many mutants that cannot excise IESs also fail to initiate chromosome breakage. The relationship between these two processes is not well understood, and the specific roles that individual proteins play in chromosome breakage have yet to be elucidated. To determine whether chromosome breakage occurs in ΔLIA4 matings, we
monitored processing at the chromosome breakage site located downstream of the LIA1 gene by using Southern blot analysis (22). In this assay, breakage of the micronucleus-derived chromosome is observed by loss of a 10.5-kb EcoRI restriction fragment and detection of a terminal 2.2-kb fragment, which is extended by 300 to 400 nucleotides by telomere addition during growth. The probe also detects an internal 7.8-kb DNA fragment, which serves to control for loading between samples. In the WT matings, we observed a strong band at 2.2 kb and a relatively weak band at 10.5 kb, indicating successful breakage (Fig. 4C). The newly generated 2.2-kb fragment migrated just below an 2.6-kb fragment corresponding to chromosomal termini with fully elongated telomeres derived from the macronuclei of unmated cells in the population. In the ΔLIA4 matings, we did not observe a fragment migrating at 2.2 kb, whereas the 10.5-kb fragment was represented as a relatively strong band, which together indicated a failure of chromosomal breakage (Fig. 4C). This result was comparable to that observed in ΔDCL1 mating strains, which were previously shown to fail to perform chromosome breakage (22). These results indicate that ΔLIA4 strains are unable to complete chromosome breakage.

H3K9me2 and H3K27me3 chromatin marks are established in ΔLIA4 matings. IESs are targeted for elimination by homologous scnRNAs, and their associated chromatin is marked by methylation on histone H3. These modifications guide the association of Pdd1 with IES chromatin, which in turn recruits the machinery to perform excision (26, 37). To determine whether ΔLIA4 cells fail to identify IESs and do not establish one or both modifications, or alternatively, Lia4 acts downstream, we monitored the accumulation of both H3K9me2 and H3K27me3 in nuclei of WT and ΔLIA4 mated cells 9 h postmixing by immunofluorescence (Fig. 5). The levels and patterns observed for both H3K9me2 and H3K27me3, detected with antibodies specific for either modification, were indistinguishable between WT and ΔLIA4 matings (Fig. 5). Thus, Lia4 acts after these modifications are established but before the initiation of IES excision.

### ΔLIA4 lines arrest late in conjugation

![Fig 3](https://example.com/fig3.png)

(A) Progeny production, the percentage of cells that complete conjugation without aborting mating or dying, plotted for both WT and ΔLIA4 matings. (B) The nuclear morphology of DAPI-stained conjugants was used to determine what stage of macronuclear development the cell was undergoing. One hundred individual cells were counted at 8 h, 12 h, 16 h, and 32 h postmixing. Four major postzygotic stages are depicted in the diagram at the top. (C) Representative DAPI staining images from cells late in the conjugation process. WT cells contain only one micronucleus, where ΔLIA4 cells arrest and contain two micronuclei. The micronuclei are indicated by arrows.

**LIA4 is required for formation of DNA elimination foci.** After establishment of chromatin modifications, the completion of IES excision involves extensive nuclear reorganization of the marked loci into increasingly larger foci, a process easily visualized by following the localization of Pdd1 during conjugation (30). To monitor this IES reorganization, we transformed an inducible Pdd1-YFP expression construct into ΔLIA4 strains, which we subsequently crossed to either WT or ΔLIA4 strains, and tracked the localization of Pdd1-YFP throughout development. Large punctate Pdd1-YFP foci were observed in roughly one-third of WT
mating pairs at 15 h (Fig. 6 and data not shown), whereas Pdd1-YFP remained largely diffuse throughout the developing macronucleus in the ΔLIA4 conjugation. Pdd1-YFP foci were never observed in ΔLIA4 mating pairs (Fig. 6), even when monitored as late as 18 h (data not shown), indicating that LIA4 is required for the nuclear reorganization events that take place late in *Tetrahymena* conjugation.

Neither LIA4 nor PDD1 is required for phosphorylation of histone H2AX in developing macronuclei. As we could not detect evidence for IES excision, chromosome breakage, or the nuclear reorganization associated with these events in ΔLIA4 conjugants, we were interested to assess whether other nuclear events were also perturbed. Accumulation of phosphorylated histone H2AX (γH2AX) in the developing macronucleus has been reported (55). Because modification of this conserved histone variant occurs in response to DNA damage, the authors hypothesized that the γH2AX appears in developing macronuclei after double-strand breaks are introduced upon IES excision. If that is the case,
then ΔLIA4 cells should not accumulate γH2AX in developing macronuclei. To investigate this possibility, we monitored γH2AX accumulation by immunofluorescence in WT, ΔLIA4, and ΔPDD1 cells at 10 h and 15 h of conjugation. We detected similar levels of γH2AX in the developing macronuclei of WT, ΔLIA4, and ΔPDD1 cells (Fig. 7). Thus, H2AX phosphorylation occurs without the excision of IESs, and this modification is likely triggered by other events that occur during differentiation of the somatic genome. Accumulation of γH2AX also occurs in TPB2 knockouts (A. Vogt and K. Mochizuki, personal communication), providing further evidence that phosphorylation of this histone variant occurs independently of IES excision.

**Lia4p and Pdd1p are coincident in the developing macronucleus.** We had previously shown that an ectopically expressed green fluorescent protein (GFP)-Lia4 fusion protein localized to developing macronuclei and appeared to colocalize with Pdd1 in DNA elimination foci. To further investigate the relationship between these proteins, we followed the localization of a Lia4-mCherry fusion protein, expressed at normal levels from the endogenous locus and coexpressed with Pdd1-CFP. Unlike Pdd1, Lia4 is not expressed during prezygotic development and is absent from somatic macronuclei (Fig. 8A). During postzygotic development, Lia4-mCherry accumulates in developing macronuclei, and its localization is largely coincident with Pdd1-CFP, although Pdd1 appears to exhibit sharper punctuation than Lia4 (Fig. 8B to D). Late in conjugation, when DNA elimination bodies form, Lia4 localization becomes increasingly concentrated and is strongly coincident with Pdd1 (Fig. 8E and F). As the DNA elimination foci become less numerous, larger, and located preferentially at the macronuclear periphery, both Lia4 and Pdd1 remain closely associated in these structures (Fig. 8G and H). At this stage, Lia4 is generally more diffuse than Pdd1. This could indicate that Lia4 occupies an exterior position in these foci at this stage, or alternatively, that it has a higher off rate than Pdd1 when associated with IES chromatin, giving the appearance that it occupies a larger domain surrounding these structures.

**DISCUSSION**

In this study, we found that Lia4 is required for programmed DNA rearrangements that remodel the developing somatic genome. Mating ΔLIA4 cells failed to initiate IES excision despite establishing wild-type levels of histone H3K9 and H3K27 methylation, which mark these sequences for elimination from developing macronuclei. We also showed that Lia4 colocalizes with Pdd1 in DNA elimination bodies and found that these subnuclear structures do not form in the absence of LIA4, observations that together implicate this novel protein in the assembly and/or subsequent functions of these Pdd1-enriched structures. These data place Lia4 in a
rather enigmatic step between RNA-directed chromatin modification and IES excision.

Analysis of mutants important for genome remodeling allows one to separate the DNA rearrangement pathway into four sequential steps: (i) RNA-directed histone methylation, (ii) formation of DNA elimination complexes, (iii) excision of IESs, and (iv) repair postexcision. The first step is arguably the best understood, as several of the proteins, including Dcl1, Twi1, Giw1, Ema1, and Ezl1, have been identified and many of their mechanistic roles involving the marking of IES chromatin for elimination have been elucidated (21, 22, 25, 27, 56–58). Often, their specific functions have been obvious, as many of the homologues for these proteins have been extensively studied in other eukaryotes. In contrast, the formation of DNA elimination structures is not as well understood at the mechanistic level. Factors, including Lia1, Lia5, Drb2, and Brg1, have been shown to be required for the maturation of DNA elimination foci, but their mechanistic roles have been challenging to define (32–35). The presence of conserved domains in Drb2 and Brg1 suggests that double-stranded RNA binding and chromatin remodeling, respectively, are important for assembly of DNA elimination complexes, whereas other factors, including Lia4, that have been found to act during this step are novel proteins. The assignment of their roles in focus formation has largely been informed by the phenotypes resulting from disruption of their genes.

The proteins involved in the organization of IES chromatin act to set up step 3, excision by the domesticated transposase Tpb2, and the resulting double-strand breaks are repaired. Two proteins, Tku80 and Die5, are known to be required for this terminal repair step (29, 40). Even though the assembly of DNA elimination complexes and the actual excision of IESs can be described as separate steps, the assignment of particular proteins to each cannot be easily made based on the basic phenotypic analysis of mutant strains.

Tpb2 is the only factor that we can unambiguously place in the IES excision step, as it is known to cleave IES ends; however, RNA interference (RNAi) knockdown of TPB2 results in failure to form DNA elimination foci (28). Thus, Tpb2 depletion produces the same phenotype exhibited by ΔLIA4 cells, failure to form Pdd1 foci and to initiate excision of IESs. Mechanistically, Lia4 could participate primarily in the assembly of a higher-order heterochromatic structure at IES loci. Colocalization of Lia4 with Pdd1 clearly shows that it is present in DNA elimination foci (Fig. 6 and 8). Once assembled, Lia4 and other associated proteins would stabilize IES chromatin and perhaps ensure maintenance of the modified histone through a round of DNA replication that precedes IES excision. Nevertheless, we cannot exclude the possibility that Lia4 acts during the IES excision step, potentially by recruiting factors to ends of IESs or otherwise facilitating the cutting process. It is difficult to differentiate between these possibilities because it remains ambiguous whether the organization of IES DNA into elimination foci facilitates their excision or, alternatively, whether the excision precipitates their formation. Colocalization of Lia4 with Pdd1 foci forms in the presence of a non-catalytically active protein as long as the Tpb2 zinc finger domain is functional (59). Thus, DNA elimination structures form in the absence of cleavage. One reason we favor defining formation of DNA elimination complexes and IES excision as separate, albeit linked, steps is that LIA4 (and LIA1 and -5) expression is induced several hours prior to expression of TPB2 and the detection of excised IESs. Future studies are needed to understand the temporal assembly of the proteins that direct accurate excision of the thousands of IESs.

ΔLIA4 cells fail to perform chromosome breakage, which pro-

**FIG 8** Lia4 and Pdd1 colocalize in developing macronuclei. Pdd1-CFP and Lia4-mCherry were expressed in WT mating cells. Fluorescence images from cells at 5 h (A), 10 h (B), 12 h (C and D), 13 h (E and F), and 15 h (G and H) are shown. The boxed regions in panels C, E, and G are magnified in panels D, F, and H, respectively. The scale bars represent 10 μm. DIC, Pdd1-CFP, and Lia4-mCherry images are shown.
vides more support for the idea that this event is coordinated with IES excision. Many mutant strains in addition to ∆LIA4, such as ∆DCL1, ∆LIA5, and ∆PDD1, that fail to excise IESs also fail to fragment chromosomes (22, 33, 35, 51). To our knowledge, no mutants have yet been identified in which IES excision fails and chromosome breakage succeeds. Nonetheless, studies of DIE5 have shown that specific events in these processes can be decoupled, as ∆DIE5 cells form DNA elimination foci yet fail at chromosome breakage (40). This functional coupling could indicate that the two processes share some common machinery, which would include Lia4, or alternatively, that the failure to correctly assemble IES chromatin in the absence of Lia4 has a major impact on the proper organization of the genome, which in turn has pleiotropic effects on differentiation of the nucleus, including the perturbation of chromosome breakage.

Despite confirming that both IES excision and chromosome breakage fail to occur in ∆LIA4 cells, we observed phosphorylation of H2AX (γH2AX) in developing macronuclei (Fig. 7). Detection of γH2AX in developing macronuclei was thought to result from double-strand breaks generated during DNA rearrangement (55). In support of this idea, we reported that ∆LIA5 cells exhibited delayed or decreased accumulation of γH2AX. However, ∆LIA4 cells fail to initiate chromosome breakage or IES excision yet exhibit no decrease in γH2AX accumulation in developing macronuclei. This appears to indicate that other events during nuclear differentiation can trigger H2AX phosphorylation. Alternatively, the phosphorylation of H2AX may simply be developmentally regulated, occurring in preparation for the double-strand breaks that will be introduced by the excision machinery, and does not require active sensing of DNA damage. It is also possible that the appearance of γH2AX occurs in response to a process that does not require Lia4. Our data do not allow us to differentiate between these possibilities. The phosphorylation of H2AX is different in ∆LIA4 and ∆PDD1 mutants and wild-type strains in that the mutants exhibit elevated levels of γH2AX in new micronuclei. This observation indicates an unexpected cross talk between these distinct nuclei as they are differenti-

The putative CSD in Lia4 is its main defining feature, and its presence is consistent with our assertion that the protein is a component of DNA elimination complexes. CSDs are dimerization domains of HP1 proteins that serve as interaction interfaces that stabilize heterochromatic structures (60). Mutations that interfere with HP1 dimerization disrupt the protein’s localization to heterochromatin (6, 61). Similarly, we recently showed that mutation of the Pdd1 CSD severely reduces production of viable progeny and inhibits formation of DNA elimination foci; however, some IES excision still occurred, which suggests that other proteins, possibly including Lia4, contribute to the overall stability of DNA elimination complexes (37). Mutational analysis of the Lia4 CSD could reveal its importance. We had hoped to perform this study by assessing the abilities of both WT and a CSD mutant Lia4 to rescue the developmental lethality of ∆LIA4 cells. Unfortunately, when we expressed WT Lia4 from the parental macronucleus, either induced from an ectopic locus or after reintroducing it into the endogenous locus, the phenotypic rescue that we observed was erratic and insufficiently robust to allow us to draw clear conclusions about the mutants we wanted to test (data not shown). LIA4 is expressed between 6 h and 14 h of conjugation (47, 62), and it is likely that expression of the rescuing construct from the parental macronucleus was insufficient to compensate for normal expression from both parental and zygotic genomes. Consistent with this assumption, we have had difficulty achieving reliable rescue of other mutants for which the genes disrupted have expression profiles similar to that of LIA4 (data not shown).

If the Lia4 CSD indeed serves as an interaction surface recruiting other DNA elimination proteins to the newly established heterochromatin of IESs, it would explain why DNA elimination foci fail to form in its absence. With no other obvious functional domains, we think it is likely that Lia4 is a structural protein linking the Pdd1-bound chromatin to other proteins that functionally reorganize the differentiating genome. For example, the CSD of mammalian HP1α interacts with BRG1, a component of the SWI-SNF chromatin-remodeling complex (63). The activity of BRG1 facilitates HP1α binding to chromatin by creating a more open chromatin state. In addition, HP1α inhibits the chromatin-remodeling activity of BRG1 by preventing it from binding a key region in histone H3 (64). Interestingly, zygotic expression of the Tetrahymena BRG1 homologue, TtBRG1, is required for completion of conjugation and formation of DNA elimination foci, indicating its key role in DNA rearrangement (32). Both LIA4 and TtBRG1 are required after histone methylation, and it would be informative to determine whether either one requires the other to function and cooperate in the DNA rearrangement pathway.

Although both Pdd1 and Lia4 have CSDs, they may interact with different partner proteins. In vertebrates, the CSD of HP1 has been shown to interact with proteins containing a PXVXL motif. Five conserved residues are critical to this interaction: F163, Y164, R167, L168, and W170 in M. musculus HP1β (49). Alignment of this region with Lia4 shows that only F163 and Y164 are conserved identically in the Lia4 CSD (Fig. 1A) and strongly suggests that Lia4 partners possess a different interaction motif. In fact, the sequence of the CSD of Lia4 differs at many residues from those found in vertebrate HP1 proteins. Regardless of this sequence dissimilarity, the Lia4 CSD is predicted to form an alpha-helical structure characteristic of all CSDs. Because Lia4 has no homology to HP1 outside the CSD, it is difficult to determine if this predicted alpha-helical structure is a result of convergent or divergent evolu-

The Lia4 CSD is very similar to a region found in LFL1, a constitutively expressed gene with unknown function. Many of the processes necessary for completion of conjugation are necessary for vegetative growth, as well. For example, the action of TtBrg1, presumably chromatin remodeling, is required for both completion of conjugation and vegetative growth (32). Our results indicate that Lia4 participates in organizing IES loci in the nucleus. Perhaps Llfl1 performs an analogous function during vegetative growth, conjugation, or both on non-IES loci. Future comparative analysis could help to determine how this shared domain has been adapted to roles during different stages of the Tetrahymena life cycle. Further study of Lia4 and Llfl1 could help to identify the mechanistic roles these proteins play in Tetrahymena and thereby reveal why Lia4 is essential for the execution of DNA rearrangement during the differentiation of the somatic genome.

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