Mutations in Pdd1 Reveal Distinct Requirements for Its Chromodomain and Chromoshadow Domain in Directing Histone Methylation and Heterochromatin Elimination

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Pdd1, a specialized HP1-like protein, is required for genome-wide DNA rearrangements that restructure a previously silent germ line genome into an active somatic genome during macronuclear differentiation of *Tetrahymena thermophila*. We deleted or otherwise mutated conserved regions of the protein to investigate how its different domains promote the excision of thousands of internal eliminated sequences (IESs). Previous studies revealed that Pdd1 contributes to recognition of IES loci after they are targeted by small-RNA-guided methylation of histone H3 on lysine 27 (H3K27), subsequently aids the establishment of H3K9 methylation, and recruits proteins that lead to excision. The phenotypes we observed for different Pdd1 alleles showed that each of the two chromodomains and the chromoshadow domain (CSD) have distinct contributions during somatic genome differentiation. Chromodomain 1 (CD1) is essential for conjugation as either its deletion or the substitution of two key aromatic amino acid residues (the W97A W100A mutant) is lethal. These mutations caused mislocalization of a cyan fluorescent protein (CFP)-tagged protein, prevented the establishment of histone H3 dimethylated on K9 (H3K9me2), and abolished IES excision. Nevertheless, the requirement for CD1 could be bypassed by recruiting Pdd1 directly to an IES by addition of a specific DNA binding domain. Chromodomain 2 (CD2) was necessary for producing viable progeny, but low levels of H3K9me2 and IES excision still occurred. A mutation in the chromoshadow domain (CSD) prevented Pdd1 focus formation but still permitted ~17% of conjugants to produce viable progeny. However, this mutant was unable to stimulate excision when recruited to an ectopic IES, indicating that this domain is important for recruitment of excision factors.

HP1 and its homologs are found in all three major branches of the eukaryotic evolutionary tree: protists (e.g., *Hhp1* and *Pdd1*), plants (e.g., *LHP1*), and animals and fungi (e.g., *Swi6* and HP1α, β, and γ). This heterochromatin-associated protein family features two distinguishing conserved domains, the methyllysine-binding chromodomain (CD) near the amino (N) terminus and the dimerization-mediating chromoshadow domain (CSD) at the carboxy (C) terminus separated by a nonconserved hinge region (1). HP1s primarily act to establish and/or maintain silent heterochromatin, a DNA/protein structure that is critical for organizing the genome and ensuring chromosome integrity; however, individual paralogs can fulfill specialized regulatory roles within any given species.

The chromodomain of HP1 typically binds histone H3 di- and trimethylated on lysine 9 (H3K9me2 and H3K9me3, respectively, or H3K9me2/3) (2, 3), allowing these proteins to act as effector molecules by recruiting other regulatory factors to the modified chromatin. Chromatin containing H3K9me2/3 is a major constituent of heterochromatin found at pericentric and telomeric regions in all histone-expressing eukaryotes (4), as well as at the silent mating type locus in *Schizosaccharomyces pombe* (5, 6). More recently, formation of HP1-associated heterochromatin was found to involve RNA interference (RNAi) silencing pathways (7). In *S. pombe*, cells mutant for RNAi components exhibited both chromosome segregation defects (8) and decreased H3K9 methylation at the mating type locus (9). Similar reductions of histone methylation and HP1 mislocalization have been observed in *Drosophila melanogaster* (10), indicating that HP1s have conserved functions.

The RNAi pathway is an evolutionarily conserved mechanism by which double-stranded RNAs trigger the generation of 20- to 30-nucleotide (nt) small RNAs (sRNAs). These sRNAs serve as specificity factors to silence gene expression of homologous sequences, acting to prevent either transcription of genomic elements or, posttranscriptionally, translation through sequestration or destruction of complementary mRNAs (11). One major target of this mechanism is endogenous transposons. For example, in *D. melanogaster* the Argonaut family protein PIWI assists in the silencing of repetitive elements in the germ line genome, including transposons, by associating with sRNAs generated from loci enriched in these elements (12–14). PIWI was also shown to be enriched at H3K9me-containing regions and to interact directly with the HP1a chromoshadow dimer via its PXVXV motif (15).
line-limited loci known as internal eliminated sequences (IESs) (16, 17). These IES elements contain repeats and transposon remnants, and histone modifications target these sequences for elimination from the somatic genome during development. The targeting of these chromatin modifications is directed by 27- to 30-nt sRNAs, called scan RNAs (scanRNAs), that are loaded into complexes with the Tetrahymena PIWI protein, Twi1 (18, 19). The newly formed heterochromatin is assembled with an HP1-like protein encoded by PDD1 (programmed DNA degradation 1) (20) that likely serves as the effector to recruit downstream-acting proteins, including Tpb2, a domesticated piggyBac-like transposase that excises the marked chromatin, resulting in the removal of nearly 50 Mb of the genome (21). Thus, Tetrahymena DNA elimination is an effective means of transposon silencing and serves as an advantageous model with which to study RNAi-directed chromatin modification and HP1-related chromatin regulation (22).

Pdd1 is a highly specialized HP1-like protein that is abundantly and exclusively expressed during Tetrahymena conjugation (20, 23). The protein contains three recognizable domains shared with HP1s (20): an N-terminal chromodomain (chromodomain 1, or CD1), which has affinity for histone H3 tails methylated on either K9 and K27 in vitro (16, 17); a second chromodomain (CD2) immediately adjacent to the first, which has amino acid substitutions at key conserved positions and has undetermined chromatin binding capabilities; and the C-terminal chromoshadow (CSD) domain (24). Consistent with its function as an HP1, Pdd1 binds methylated histone tails and interacts with other proteins important for DNA elimination (25–29). Other proteins recruited to IESs include Pdd3, a chromodomaining-containing protein that preferentially binds trimethylated H3K9 (16, 27). How Pdd1 coordinates the action of the proteins required for IES excision remains poorly understood.

At the initiation of conjugation, PDD1 expression is rapidly induced, and the protein accumulates in both the parental micronuclear and macronuclear during prezygotic development. Its roles in these nuclei have not been carefully explored. In postzygotic development, Pdd1 immediately accumulates within the newly emerged developing somatic macronuclei (i.e., macronuclear anlagen), where it exhibits widespread, but nonuniform, localization before coalescing into several large DNA elimination foci (20, 23). Chromatin immunoprecipitation (ChIP) revealed that Pdd1 is enriched at IES loci but not at the neighboring macronuclear-retained DNA (17). Deletion of PDD1 (∆PDD1) from the parental macronuclear results in a developmental arrest and causes conjugating cells to die shortly after separation from their mating partners (30). In addition to lethality, ∆PDD1 cells display a variety of DNA rearrangement-related abnormalities, including failure to undergo DNA endoreplication, loss of H3K9 methylation in anlagen, and an inability to excise IESs (17, 30).

Evidence suggests that Pdd1’s interaction with specific histone modifications is the basis of its action on IESs and that this protein may bridge two different modifications. H3K9 methylation is detected only in developing macronuclear anlagen (17) and is necessary for DNA elimination (31). This modification is lost in ∆PDD1 cells (17), indicating that Pdd1 is required for establishment of this modification; however, sterically blocking H3K9me by mutation of H3 serine 10 does not impair Pdd1 localization in anlagen (16). In contrast, H3K27me3 can be detected in both vegetative cells (macronucleus) and mating cells (parental macronucleus, micronuclei, and anlagen), showing that this single modification is not restricted to development (17). Cells subjected to a loss of the majority of H3K27me3 in macronuclear anlagen via deletion of the histone methyl transferase EZL1 or components of the IES-directed RNAi machinery (e.g., TWI1) show aberrant Pdd1 localization in both nuclei as well as a total loss of H3K9 methylation (16). The enrichment of these chromatin modifications and Pdd1 at IES loci and the phenotypes observed upon loss of PDD1 strongly support the model that this HP1-like protein acts as an adaptor by recognizing these two chromatin modifications and recruiting the excision machinery to the proper loci (16, 17).

Pdd1 does exhibit unusual properties for an HP1 protein. For example, H3K27me3 is commonly bound by Polycomb-like proteins, which lack the chromoshadow domain and are primarily associated with developmentally regulated gene silencing and maintenance of cell identity (32, 33). To begin to understand how Pdd1 can bridge RNAi-directed H3K27me3 and the subsequent establishment of H3K9 methylation required for heterochromatization and elimination of IESs, we have mutagenized the known domains of PDD1. We hypothesized that each of PDD1’s domains fulfills unique roles in facilitating signal transmission between these modifications and the regulation of this specific form of heterochromatin. Our results reveal critical roles for both chromodomains and also the chromoshadow domain and provide insights into specific events, such as maturation of DNA elimination foci, that occur during somatic genome reorganization.

MATERIALS AND METHODS

Manipulation of Tetrahymena. Wild-type Tetrahymena thermophila strains (B2086, CU427, and CU428) were cultured in liquid medium at 30°C according to standard methods (34). Cells were induced to conjugate by mixing cultures of complementary mating types at equal cell densities (~2.5 × 10⁸ cells/ml) after overnight starvation (>6 h) in 10 mM Tris–HCl (pH 7.4). These strains, or transgens derived from them, were transformed with constructs to create knockout strains or cell lines expressing epitope-tagged proteins by biolistics transformation using a Bio-Rad PDS 1000 He Particle Delivery System for integrative constructs (35) or electroporation (36) for ribosomal DNA (rDNA)-based replicative vectors. Generation of ∆PDD1 strains was as described previously (37); ∆TWI1 strains (19) were provided by K. Mochizuki (Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria).

Generation of modified PDD1 constructs. DNA fragments from the PDD1 locus were amplified by PCR and cloned into pMNBL, a neo3-containing vector (38), by using restriction enzymes and T4 DNA ligase (New England BioLabs, Ipswich, MA) to create an integrative construct for introducing PDD1 alleles into the endogenous locus. See Table S1 in supplemental material for all oligonucleotide primer sequences used (Integrated DNA Technologies, Coralville, IA). The 5′ flanking region was amplified with Phusion polymerase (New England BioLabs, Ipswich, MA) to create an integrative construct for introducing PDD1 alleles into the endogenous locus. See Table S1 in supplemental material for all oligonucleotide primer sequences used (Integrated DNA Technologies, Coralville, IA). The 5′ flanking region was amplified with Phusion polymerase (New England BioLabs, Ipswich, MA) to create an integrative construct for introducing PDD1 alleles into the endogenous locus. See Table S1 in supplemental material for all oligonucleotide primer sequences used (Integrated DNA Technologies, Coralville, IA).
Mutant alleles of *PDD1* were created by mutagenizing a cloned copy of the gene in a pENTR vector (Life Technologies, Grand Island, NY). For the W97A W100A mutant, the N-terminal portion of *PDD1* was amplified with primers 1563 and 3308, and the C-terminal portion was amplified with primers 3309 and 3306. Overlapping fragments were annealed using Phusion polymerase (NEB), with 10 cycles of melting at 98°C for 30 s, and extension at 72°C for 30 s. The annealed product was amplified with primers 1563 and 3306, and cloned using pENTR TOPO (Life Technologies, Grand Island, NY). The ∆CD1 and ∆CD2 deletion mutants were made from a pENTR *PDD1* plasmid, using inverse PCR with primers 2385 and 2386 (∆CD1) and primers 3405 and 3406 (∆CD2) to delete the desired domain. Pdd1/15 CD swap (in which the Pdd1 CD was swapped for that of Pdd3) was made by amplifying the *PDD3* chromodom from genomic DNA with primers 2387 and 2388 and combining this with the pENTR ∆CD1 vector in a QuikChange PCR (Stratagene). The 1504D CSD mutant was also produced by starting with a pENTR *PDD1* plasmid and amplifying it with primers 3615 and 3616 in a QuikChange reaction to mutagenize residue 1504. The truncation mutant lacking the CDS was amplified from genomic DNA with primers 3304 and 3307 and cloned directly into the integrative pMNBL construct.

Mutagenized PDD1 alleles (except for the truncation mutant) were then PCR amplified from their respective pENTR constructs using primers 3304 and 3306 and cloned into the integrative pMNBL vector construct using Hpal and AvrII sites. Constructs were linearized and introduced to ∆*PDD1* cells via biolistics transformation, and transformants were selected in 1× superprotease-peptone (SPP) medium containing 80 μg/ml blasticidin. Gene expression was confirmed by observation of CFP via fluorescence microscopy. The CFP gene of the mutant pMNBL integrative constructs was removed to express untaged versions of the mutants for use in functional assays.

**Fluorescence microscopy.** Mating cells expressing Pdd1-1-CFP were fixed with 2% paraformaldehyde at the appropriate stage of conjugation, rehydrated with water, and mounted for fluorescence microscopy. To visualize histone modifications, cells were collected at 9 h after conjugation initiation, mixed with Schaudinn’s fixative, and applied to glass slides. Samples were air dried for 30 min at room temperature and rehydrated overnight at 4°C in 1× Tris-buffered saline (TBS). Slides were then washed in blocking buffer containing 1× TBS–1.0% bovine serum albumin (BSA) and 0.01% Tween 20 and incubated with rabbit anti-H3K9me2 antibody (lot 27353; Millipore) at a 1:250 dilution for 1 h at room temperature. After samples were washed with 1× TBS five times for 5 min each and reblocked two times for 10 min each, a goat anti-rabbit Alexa-Fluor 488-conjugated secondary antibody (Life Technologies, Grand Island, NY) was applied and incubated for 1 h at room temperature. Washes were repeated, and DNA was stained by the addition of 4′,6-diamidino-2-phenylindole (DAPI) to the final wash. Slides were viewed under a 1×0.6 immersion lens on a Nikon Eclipse E600 upright microscope, and images were captured using a Retiga EX charge-coupled-device (CCD) camera (Q-Imaging) driven by OpenLab software (Improvision).

**Western blotting.** Whole-cell extracts were collected and fractionated on a 12% SDS polyacrylamide gel at 120 V for 5 h. Protein was transferred to a nitrocellulose membrane using a semidry electroblotter at 0.01 mA for 2 h. Membranes were incubated with rabbit anti-PDD1 antibody (ab5338, Abcam, Cambridge, MA), washed, and incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce/Thermo Fisher, Rockford, IL). Images were collected with a digital fluorescent imager (GE Healthcare Biosciences, Pittsburgh, PA).

**LexA tethering and Southern blotting.** LexA-tagged Pdd1 was produced by cloning LexA from pBPHLW1-LexA, a gift of Tom Clandinin (plasmid 26258; Addgene) with primers 3632 and 3633 and fusing it to *PDD1* in place of CD1 in the pMNBL integrative construct, which was transformed into wild-type (CU427 and CU428) cells via biolistics. A DNA fragment containing five different LexA operator binding sites (5×LAop), including cle1-1, reca, lexA-2, wuu, and urD, was synthesized (Integrated DNA Technologies, Coralville, IA) and cloned into the pENTR vector by topoisoasemaser-mediated cloning (Life Technologies, Grand Island, NY). An rDNA plasmid containing M-element border regions flanking a Gateway cassette was used in an LR Clonase II (Invitrogen) reaction to produce the experimental 5×LAop plasmid. Either this or the control M-element rDNA vector was electroproinated into conjugating cells at 9 h postmixing, in either CU427 [CU428] at 37°C, resolved on a 1% agarose gel, and transferred to a nylon membrane. The membrane was prehybridized with salmon sperm DNA for 6 h, and an α-32P-radioabeled probe was generated using a DCLM3 fragment as a template and incubated with the membrane overnight at 65°C (39). The membrane was washed four times with 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS and visualized by phosphorimaging analysis using a Personal FX imager and Quantity One software (Bio-Rad).

**RESULTS**

As the somatic genome differentiates from its germ line origin, it must be organized de novo from a transcriptionally naive state. One-third of the genome, the IESs, is marked for elimination by RNA-guided chromatin modification. Pdd1, a highly specialized HP1-like protein, bridges the initial establishment of H3K27me3 to subsequent chromatin modifications, i.e., H3K9me2/3, and the eventual excision of the marked DNA. *PDD1* has identifiable domains conserved in HP1 proteins including two chromodomains (CD) and a chromoshadow domain (CSD) (Fig. 1A). We mutated these different domains to determine how different regions of *PDD1* participate in its various functions. We deleted one demonstrated and another possible histone methyl-lysine-binding domain, CD1 (∆CD1) and CD2 (∆CD2), and also created a double amino acid substitution of the conserved aromatic cage residues of CD1 (W97A W100A). Disruptions of these residues in HP1 have been shown to impair the protein’s binding to methylated H3K9 (40). In an attempt to alter the protein’s binding specificity, we replaced CD1 with the chromodom of Pdd3 (Pdd1/3 CD swap), another conjugation-specific protein that has been shown to bind H3K9me3, but not H3K27me3, in vitro (16, 17, 27). We also mutated the C-terminal region of the protein, removing the CSD (truncation) or introducing a single point mutation, I504D, known in HP1 proteins to disrupt the assumed dimerization of the CSD (41) (Fig. 1B).

Cells lacking *PDD1* (∆*PDD1*) do not express detectable Pdd1 (see Fig. S1 in the supplemental material) and are unable to produce viable progeny when mated, arresting late in development. To determine whether each domain within *PDD1* performs an essential function, we introduced the untagged, mutated form into two ∆*PDD1* strains of different mating types, integrating the construct back into the neo3-disrupted locus, selecting for transformants by using a linked blasticidin-resistant marker. We then crossed mutant lines and monitored progeny production (Fig. 1C). Upon crossing, cells either produce progeny, die, or abort/back out of conjugation. Upon reintroduction of the wild-type gene, we observed full rescue of the ∆*PDD1* phenotype as 70 to 80% of mating pairs produced viable progeny. In contrast, most *PDD1* mutants tested could not rescue the knockout sufficiently to allow normal development. Two exceptions were the I504D chromoshadow domain and CD swap mutant alleles, which pro-
duced viable progeny but at a severely decreased frequency (~17% and 0.75%, respectively) relative to the wild-type allele (Fig. 1C, see Table S2 in supplemental material). As no viable progeny were recovered for the ΔCD1 mutant, the double CD1 point mutant (W97A W100A), ΔCD2 mutant, or C-terminal truncation mutant (see Table S2 in supplemental material), all of these domains are essential, whereas the chromoshadow domain is important but dispensable.

FIG 1 CD1, CD2, and the CSD have critical functions. Mutations were produced for each of the three PDD1 domains, and resulting progeny levels were assessed. (A) Domain alignment of chromodomains (i) or chromoshadow domains (ii) of Pdd1 and other HP1-like proteins. (B) Schematic illustration of Pdd1 alleles in either the wild-type rescue control (i), CD1 mutants (ii), CD2 mutant (iii), and CSD mutants (iv). Regions deleted are indicated as a dotted box. (C) Progeny production of domain mutants following mutant wild-type crosses (CU427) or mutant mutant crosses. Note the low number of true progeny of wild-type CFP-tagged protein crosses in comparison to the untagged wild type. Accession numbers of sequences used in alignments are as follows: HP1, NP_476755.1; Swi6, CAA50668.1; Pdd1, AAB61684.1; Pdd3, AAF36692.1.

produced viable progeny but at a severely decreased frequency (~17% and 0.75%, respectively) relative to the wild-type allele (Fig. 1C; see Table S2 in supplemental material). As no viable progeny were recovered for the ΔCD1 mutant, the double CD1
Disruption of CD1 or CSD results in mislocalization of Pdd1 both early and late in conjugation. Pdd1 exhibits dynamic reorganization during conjugation, and its assembly into nuclear foci has been proposed to be a visual indication of its role in assembly of heterochromatin that is targeted for elimination (26, 42). To examine the role of Pdd1’s different domains in this nuclear organization, we tagged the C terminus of PDD1 mutants with CFP and examined their localization patterns alone (Fig. 2A to D) or in the presence of wild-type protein (Fig. 2E). Fluorescence microscopy of these tagged mutant proteins revealed that both CD1 and the C-terminal domain are critical for proper Pdd1 localization. Disrupting two aromatic residues of CD1 (W97A and W100A) resulted in a protein that is diffusely distributed, both in the parental macronucleus and the developing anlagen (Fig. 2B). This is consistent with the hypothesis that these residues form a methyl-binding cage that promotes Pdd1-chromatin interaction, which facilitates the proper organization observed with the wild-type CFP-tagged protein (Fig. 2A, top panel). In some W97A W100A matings, approximately 50% of pairs display abnormal early foci, similar to those observed in crosses of ΔCD1 or ΔTWIII mutant strains (see Fig. S2 in supplemental material). These abnormal foci were not detected in mutants lacking the entire chromodomain (ΔCD1), indicating a possible role for this domain in Pdd1 self-association. The appearance of these foci in a mutant expected to abrogate histone binding is consistent with the failure to detect rearrangement-specific H3K27me3 in RNAi mutants and suggests a stochastic aggregation process for unbound Pdd1. Notably, the W97A W100A Pdd1 allele is unable to form 15-h elimination foci when paired with the same untagged mutant but succeeds in the presence of wild-type (CU427) Pdd1 (Fig. 2E), which shows that the mutant protein can assemble with wild-type IES-associated chromatin.

Next, we aimed to determine the importance of the dual histone modification-binding nature of CD1, which has been shown to bind both H3K9me2/3 and H3K27me3. In an attempt to alter the binding specificity of Pdd1, we swapped the CD of PDD3, which only binds H3K9me3 in vitro (16), for PDD1 CD1 and used this mutant to assess how the loss of binding to H3K27me3, but not H3K9me2/3, would affect the protein’s function. Unexpectedly, the domain swap resulted in no aberrant localization in any of the nuclei (Fig. 2B). It is possible that the Pdd3 CD facilitates some binding to H3K27me3, which is present in parental macronuclei and anlagen, when in the context of rest of the Pdd1 coding region.

The function of the second Pdd1 CD has not been previously explored. Deletion of CD2 does not obviously disrupt the local-

**FIG 2** Pdd1 mutations alter nuclear localization. Localization of CFP-tagged mutant proteins in the parental macronucleus in early conjugation (~6 h, top row), anlagen in mid-conjugation (~9 h, second row, and 3-fold enlargement, third row), and late conjugation (15-h foci, fourth and fifth rows) for cells of the wild-type PDD1-CFP (A), CD1 mutant (W97, 100A) (B), ΔCD2-CFP (C), or CFP-tagged CSD mutants (Truncation, ΔCSD) (D). (E) Late-stage conjugation of mutant-CFP x wild-type (CU427) crosses. The specific mutant is labeled at the top of each column. Images in first three rows (Early/Mid-Conjugation) were obtained from same mating; each enlargement depicts one magnified nucleus from the mid-conjugation image shown above (see reference 26 for a full description of Pdd1-CFP localization in mating cells). Scale bar, 10 μm (3.3 μm for the enlarged images).
ization of Pdd1 in early or mid-conjugation (Fig. 2C), suggesting that this domain is not necessary for Pdd1’s initial association with chromatin. However, this mutant protein does not form late (mature) foci even when it is expressed with its untagged mutant form or wild-type Pdd1 (Fig. 2E). It is possible that CD2 forms a critical interface to facilitate association with other proteins or that loss of this internal domain disrupts the folded structure necessary for such interactions.

A dramatic phenotype emerged when we truncated PDD1, removing the C-terminal 45 amino acids. The protein was excluded from both the parental macronucleus and anlagen and instead appeared diffuse throughout the cytoplasm throughout conjugation (Fig. 2D). We conclude that the C terminus likely contains a critical nuclear localization sequence (NLS). The sporadic appearance of the mutant protein in the old macronucleus late in conjugation may be due to a loss of integrity of the membrane of this pycnotic nucleus or a secondary localization sequence within the protein. In addition, truncated Pdd1 often appears concentrated at the fusion junction between mating cells, but the biological significance of this is uncertain. We attempted to assess its functionality in the nucleus by appending the simian virus 40 (SV40) NLS to its C terminus (43) in an effort to direct the truncated protein into nuclei, but this fusion protein could not be detected in mating cells.

In addition to an NLS, the C terminus of PDD1 contains a putative chromoshadow domain, which in HP1 promotes its dimerization and subsequent interaction with other proteins (41). Direct mutagenesis of a single amino acid, residue I504, revealed an important role for this C-terminal domain in DNA rearrangement. Mutation of the corresponding amino acid, L315, in an HP1 wild-type Pdd1, Swi6, renders the protein unable to dimerize (41, 44, 45). Indeed, I504D mutants failed to form foci either in the parental macronucleus or in anlagen (Fig. 2D), indicating the critical nature of the domain and hinting that its function may involve Pdd1 self-association through formation of a dimerization platform, a known feature of CSDs (41). In support of this theory, the protein is unable to form foci in both the presence of untagged I504D and wild-type Pdd1 (Fig. 2D and E).

H3K9me2 but not H3K27me3 is affected in mutants. The distinct localization patterns of the different PDD1 mutants revealed that the different domains of the protein contribute specific functionality to the protein. Each domain may have specific roles in either establishing and/or reading specific histone modifications. During conjugation, H3K27me3 appears in both the parental macronucleus and the macronuclear anlagen, while H3K9me2 is detectable only in anlagen. In PDD1 knockout cells, H3K27me3 is unperturbed, but H3K9me2 is undetectable (16, 17). To determine the contribution of individual Pdd1 domains to the differentiation of IES chromatin, we monitored levels of H3K27me3 or H3K9me2 in mutant strains (using the untagged alleles) by immunofluorescence.

Levels of H3K27me3 staining were unaffected in PDD1 mutants. This was expected as Pdd1 acts downstream of this RNAi-directed modification, which is evident in our control matings of ΔTWI1 cells that failed to accumulate H3K27me3 (Fig. 3A) (16). In contrast, the strains with mutations of individual domains exhibited divergent alterations in levels of H3K9me2. Both the ΔCD1 and CD1 point-mutant cells and a C-terminal truncation mutant that does not enter developing nuclei failed to establish detectable H3K9me2 (Fig. 3B). Other PDD1 mutants had a less severe impact on this modification. In crosses of ΔCD2 and the Pdd1/3 CD swap strains, H3K9me2 levels were significantly reduced but still detectable. Strains containing the I504D mutant showed only a partial reduction of this modification. Thus, CD1, and in particular residues W97 and W100, are absolutely required for the establishment and/or maintenance of H3K9 methylation, but other domains are important to ensure that complete accumulation of this modification is achieved.

Interestingly, the Pdd1/3 CD swap mutant largely mimicked the localization of wild-type Pdd1-CFP in parental macronuclei, suggesting that it might be able to bind H3K27me3 in vivo; however, this mutant only partially facilitated H3K9me2 accumulation (Fig. 3B). This may reflect the importance of the dual-binding affinity of Pdd1 CD1 to mediate the transition from the H3K27me3 modification chromatin to the doubly modified state. The affinity of the Pdd3 CD for one particular lysine modification relative to the other may alter the stability of the chimeric protein on H3K27me3 relative to that of the wild-type Pdd1 and, in turn, make this mutant less effective at establishing H3K9me2-modified chromatin. Alternatively, the Pdd1 CD1 may facilitate interactions with chromatin or other proteins that are involved in establishing H3K9me2 which the Pdd3 CD does not. Either way, it is clear that the Pdd3 CD cannot fully replace the function of Pdd1 CD1.

A decrease in H3K9me2 in ΔCD2 mutants (Fig. 3B) was unexpected, considering that this protein appears to localize normally and that the second chromodomain is significantly divergent from CD1, lacking the critical aromatic-cage residues. It is possible that CD2 plays an auxiliary role in either stabilizing the protein or helping recruit other factors that increase the efficiency of modification. Interestingly, the strongest methylation signal was seen in the I504D chromoshadow domain mutant, lending support to the hypothesis that this mutant successfully interacts with chromatin via its N-terminal chromodomain, while the C terminus fulfills a separate role, possibly mediating critical protein-protein interactions.

The severity of the developmental arrest corresponds to the altered localization and chromatin modifications observed in mutants. Cells lacking Pdd1 (ΔPDD1) can enter conjugation and initiate macronuclear differentiation but arrest at the end of development with a total of four nuclei: two macronuclei and two micronuclei (two-macronucleus/one-micronucleus stage). Wild-type cells complete development by eliminating one of these two micronuclei and are then poised to divide once provided food. To determine the severity of the developmental arrest, we examined the nuclear morphology of each mutant at its conjugation endpoint. Nuclei of mated strains expressing untagged mutant alleles (n ≈ 200 for each cross) (see Table S3 in supplemental material) were stained with DAPI at ~24 h after cells were mixed to induce conjugation. ΔPDD1 cells rescued by introduction of a wild-type construct proceed to the two-macronucleus/one-micronucleus stage approximately 93% of the time. This was in stark contrast to the point mutant, ΔCD1, and Pdd1 truncation cells, none of which ever progressed beyond the two-macronucleus/one-micronucleus-arrest stage (Fig. 4). A combination of arrested and terminal endpoint phenotypes was observed in crosses of the CD swap, ΔCD2, and I504D mutant strains as 51%, 21%, and 73% of mated pairs reached the terminal the two-macronucleus/one-micronucleus stage, respectively (Fig. 4). Notably, I504D mutants proceed to the end of conjugation with no indication of Pdd1
self-organization, suggesting that absorption of the second micro-
nucleus does not require a downstream signal dependent upon
focus formation. However, despite the relatively successful pro-
gression to the normal nuclear phenotype, the I504D mutant still
produces progeny only 17% of the time, indicating that its dys-
function may be most problematic in later stages of conjugation.

M element requires domains for rearrangement different
from those of IES C. As some Pdd1 mutants proceeded beyond
the developmental arrest and established detectable levels of
H3K9me2, it is possible that they also directed some IES excision.
To assess which PDD1 mutants are able to promote excision, we
examined the rearrangement of two different IES regions. The M
element is a well-characterized IES that exhibits alternative rear-
rangement, eliminating either 0.6 kb (∆M0.6 kb) or 0.9 kb (∆M0.9 kb)
from the germ line-derived locus. The unrearranged and two re-
arranged forms can be detected in a single PCR using oligonucle-
otide primers flanking the eliminated region. The parental macro-
nuclei of all of the mutant strains possessed only the ∆M0.9-kb form;

FIG 3 CD1 is required for H3K9me2. Histone methylation staining of cells expressing untagged, mutant PDD1 alleles at 10 h postmixing. (A) Mated cells
immunostained for histone modifications show that H3K27me3 is present in anlagen of all Pdd1 mutants but not in ∆TW11 cells. (B) H3K9me2 levels are either
absent (W97A W100A, ∆CD1, truncation, and knockout [ΔPDD1] mutants) or reduced (CD swap, ∆CD2, or I504D mutant) in Pdd1 mutant cells. Each mutant
pair was assigned a qualitative rating of staining (− to +++ based on intensity and prevalence of immunofluorescence in all cells viewed compared to wild-type
Pdd1. Scale bar, 10 µm. (C) H3K9me2 levels measured by intensity relative to cytoplasmic background fluorescence.
thus, after mating, any appearance of the 0.6-kb form was indicative of successful rearrangement in the developing macronuclei. Of all of these mutants, only the I504D mutant was able to complete de novo rearrangement of the M element (Fig. 5A).

As different IESs may have distinct rearrangement requirements or efficiencies, we monitored the rearrangement of a second IES that exhibits alternative rearrangement, IES C, in each mutant cross (46). All parent lines have a single rearranged form; therefore, appearance of a slightly larger, ~180-bp, band reveals de novo rearrangement. As expected, in wild-type rescued strains and the I504D mutants, a rearranged form of IES C was detectable (Fig. 5B). Even though neither the ΔCD2 nor CD swap mutant produced detectable rearrangement of the M element, these same mutants did rearrange the IES C to some degree. Both of these mutants showed evidence of H3K9me2 establishment; thus, their ability to carry out some rearrangement is consistent with the severity of other phenotypes observed (Fig. 2 to 4).

**Phosphorylation of Pdd1 mutants.** Phosphorylation of the heterochromatin protein Swi6 has been shown to be critical for protein function by regulating the recruitment of the SHREC silencing complex (47), while in Drosophila, HP1 phosphorylation is critical for heterochromatin binding (48). Pdd1 is also phosphorylated during conjugation, which is evident in Western blot analyses as a slower migrating protein that appears around 6 h and peaks at 8 h (20, 29). The purpose of this modification is not known, but its transient appearance suggests a mid-conjugation function; and its reduction corresponds to the maturation of elimination foci. As several of the domain mutants fail to form foci (Fig. 2), we investigated the possibility of a link between these mislocalization phenotypes and the phosphorylation state of the protein. Whole-cell protein extracts from mated cells of each mutant type were collected at 1-h intervals, fractionated by SDS-PAGE, and detected by Western blotting with an anti-Pdd1 antibody to assess the emergence of larger protein forms.

We were unable to determine the phosphorylation state of the truncation mutant because the protein did not accumulate to sufficient levels to allow detection, likely due to its failure to enter the nucleus (see Fig. S3 in supplemental material). For all but possibly one of the mutants, ΔCD2, phosphorylation was not obviously perturbed (see Fig. S3). For the ΔCD2 mutants, the ratio between faster-migrating (unmodified) and slower-migrating (phosphorylated) proteins appeared to be biased toward the unmodified form (see Fig. S3). This result may suggest either that CD2 itself is phosphorylated or, alternatively, that the lack of the second chromodomain alters the protein structure, reducing the accessibility of phosphorylated residues to their kinase. A search for phosphorylatable residues in CD2 using the NetPhos (version 2.0) prediction software (49) did not yield any potential candidates for modification, which would favor the second alternative.

**CSD mutant cannot recruit necessary excision factors.** A previous study showed that a LexA-Pdd1 fusion protein is sufficient to drive DNA elimination when it is tethered to a target sequence (containing LexA binding sites), likely in the absence of upstream chromatin modifications (17). We adopted this assay to assess the different roles of the various domains of *PDD1*. CD1 of Pdd1 is postulated to recruit the protein to H3K9- and H3K27-methylated histones. To determine whether Pdd1 recruitment through chromatin interaction is both the primary and essential role of this domain, we replaced CD1 with the LexA DNA binding domain (Fig. 6A), added a C-terminal CFP tag to confirm expression and correct localization, and integrated this fusion construct into the *PDD1* locus. Only a portion of the macronuclear copies of *PDD1* were replaced, allowing us to express both the wild-type and fusion proteins in the same transformants. Into strains expressing this fusion we introduced a chimeric IES, 5×LAop, consisting of five different LexA operator binding sites (50) flanked by M-element boundary sequences, carried on an rDNA-based replicating vector (Fig. 6A). In wild-type cells, neither homologous scnRNAs
nor the LexA binding domain is present to direct Pdd1 to this artificial IES; thus, it should fail to rearrange. The resulting transformants were analyzed by Southern blotting for evidence of rearrangement. This chimeric IES shows no detectable rearrangement when introduced into conjugating wild-type strains (CU427/H11003 CU428) (see Fig. S4 in supplemental material); however, in cells expressing LexA-Pdd1, excision of the 5/H11003 LAop was easily detected (Fig. 6C). Thus, CD1 is required for recruitment of Pdd1 to IES chromatin but is not needed to otherwise facilitate excision.

We next tested the role of CD2 and the chromoshadow domain in IES excision by either deleting CD2 from or introducing the I504D mutation into LexA-Pdd1 (indicated as LexA-CD2 and LexA-I504D, respectively). Cells expressing these altered fusions were mated, and the 5/H11003 LAop plasmid was introduced. The LexA-CD2 mutant was able to perform excision of the chimeric IES, which is consistent with the idea that one role of this domain may be to help recruit or stabilize Pdd1 on the modified chromatin. It is not essential for the recruitment of the remainder of the IES excision machinery. It remains possible that this LexA-ΔCD2 mutant may heterodimerize with the wild-type Pdd1 still expressed in these transgenic cells, and this functional Pdd1 is sufficient to facilitate rearrangement. In contrast, the LexA-I504D mutant did not support rearrangement of the 5×LAop plasmid (Fig. 6C). This further supports the idea that the I504D mutant is unable to interact with its wild-type counterpart, and alone it is unable to recruit the necessary downstream excision machinery to the LexA-IES.

**DISCUSSION**

By generating mutants in the various domains of *PDD1*, we have gained new insights into how this specialized HP1-like protein contributes to developmental remodeling of the somatic genome. An atypical feature of *PDD1* is the presence of two chromodomains. We found that the first chromodomain is absolutely required for the establishment of H3K9 methylation (Fig. 3) but is dispensable for IES excision if the remainder of the protein is recruited to the eliminated sequence, tethered by a LexA DNA binding domain (Fig. 5A). Cells expressing these altered fusions were mated, and the 5×LAop plasmid was introduced. The LexA-ΔCD2 mutant was able to perform excision of the chimeric IES, which is consistent with the idea that one role of this domain may be to help recruit or stabilize Pdd1 on the modified chromatin. It is not essential for the recruitment of the remainder of the IES excision machinery. It remains possible that this LexA-ΔCD2 mutant may heterodimerize with the wild-type Pdd1 still expressed in these transgenic cells, and this functional Pdd1 is sufficient to facilitate rearrangement. In contrast, the LexA-I504D mutant did not support rearrangement of the 5×LAop plasmid (Fig. 6C). This further supports the idea that the I504D mutant is unable to interact with its wild-type counterpart, and alone it is unable to recruit the necessary downstream excision machinery to the LexA-IES.

**Fig 5** CD1, but not CD2 and the CSD, is required for IES excision. PCR assessment of IES rearrangement of genomic DNA in cells expressing untagged, mutant *PDD1* alleles. (A) M-element rearrangement of Pdd1 mutants. Unrearranged DNA results in a 1.2-kb product (top arrow) while all parent cells contain the 0.3-kb fragment exclusively (bottom arrow). Emergence of the 0.6-kb fragment (middle arrows) in 24-h postmating samples indicates new rearrangement. For all, P1 and P2 lanes contain genomic DNA isolated from the two starved, unmated parental cell lines; genomic DNA isolated from the pool of mated cells 24 h after the two parent cell lines were mixed is represented in the other lanes. (B) Rearrangement of IES C. The parental rearranged form is ~150 bp, with new rearrangement visible as an ~180-bp band. The asterisk (*) indicates a nonspecific amplification product. P1 and P2 represent the parental rearranged forms for all mutants; labeled mutant lanes contain genomic DNA isolated from the specified mutant × mutant crosses 24-h after mixing.
The establishment of this modification is an essential function of Pdd1. Even though CD2 and chromoshadow domain mutants supported some IES excision, neither Pdd1 mutant protein assembled into mature DNA elimination foci, even when expressed together with wild-type Pdd1 (Fig. 2E). Mutations in CD1 are able to assemble into these foci in the presence of wild-type protein (Fig. 2E); thus, it is the other domains that have important roles in the formation of mature Pdd1 foci. Nevertheless, the fact that they support some rearrangement suggests that the nuclear reorganization evident by focus formation is less critical than the establishment of the chromatin modifications in the first place.

Previous data has indicated that Pdd1 bridges RNAi-directed H3K27 methylation and the establishment of H3K9 methylation on IESs. In an effort to determine whether the ability of CD1 to bind either of these modifications was required for Pdd1 to mediate this transition, we replaced the dual-specificity CD1 of PDD1 with the CD of PDD3, which was shown to bind only H3K9me2 and not H3K27me3 in vitro (16, 17). Somewhat unexpectedly, this chimeric protein exhibited wild-type Pdd1 localization in parental macronuclei, where only H3K27me3 occurs, and formed mature foci in anlagen (Fig. 2B). Importantly, this chimera rescued some H3K9 methylation, albeit to a lesser degree than the wild type. Furthermore, approximately half the exconjugant population escaped the developmental arrest of the parent ΔPDD1 strains, reaching the two-macronucleus/one-micronucleus endpoint although cells expressing this PDD1/3 chimera produced few viable progeny. Together, these results suggest that the Pdd3 CD, in the context of Pdd1, may be able to bind H3K27me3 in vivo. The altered binding of the Pdd3 CD may be due to assistance from other Pdd1-histone interactions; even so, only 1 out of 132 pairs produced viable progeny, indicating that it cannot efficiently substitute for CD1.

The enigmatic second chromodomain of PDD1 clearly performs an important function as its loss renders the cells unable to produce progeny or efficiently rearrange DNA. Nevertheless, the localization of the ΔCD2 mutant protein was largely indistinguishable from that of the wild type until late conjugation, when elimination foci failed to form. We did observe an apparent reduction of the phosphorylated form of this mutant despite its lack of obvious phosphorylation sites (see Fig. S3 in supplemental material). It is possible that the reduction in phosphorylation in these mutants is an impediment to Pdd1 function.

Mutations engineered at the C terminus of PDD1 clearly perform an important function as its loss renders the cells unable to produce progeny or efficiently rearrange DNA. Nevertheless, the localization of the ΔCD2 mutant protein was largely indistinguishable from that of the wild type until late conjugation, when elimination foci failed to form. We did observe an apparent reduction of the phosphorylated form of this mutant despite its lack of obvious phosphorylation sites (see Fig. S3 in supplemental material). It is possible that the reduction in phosphorylation in these mutants is an impediment to Pdd1 function.
ing the SV40 NLS to this mutant resulted in failure of expression, but modifying this approach may allow a more thorough investigation of possible additional functions specified by the C terminus. The phenotype of the I504D mutant was consistent with this putative chromoshadow domain promoting dimerization. While this mutant was able to facilitate some IES rearrangement in the genome and produce progeny at a very low rate, it failed to coalesce into DNA elimination foci. This suggests that efficient dimerization is likely required for the maturation of DNA elimination foci but also indicates that Pdd1 focus formation is not critical for completion of conjugation. In these mutants, at least some of the H3K9me2 that accumulated must be targeted to IESs, but this has not been directly measured. It is worth noting that the low level of progeny recovered from I504D matings is comparable to what is observed when C-terminal CFP-tagged Pdd1 cells are mated (Fig. 1C), suggesting that the CFP tag may be a hindrance to the function of the CSD, possibly interrupting protein-protein contacts. Indeed, in the absence of untagged Pdd1, no mature CFP foci are observed, with the proteins instead forming an aberrant filamentous pattern in the nucleus (see Fig. S5 in supplemental material). These observations suggest that CSD-mediated dimerization plays an important role in Tetrahymena genome rearrangement and may help recruit the downstream excision-end-rejoining machinery. This hypothesis is supported by data from the LexA tethering experiment, in which the I504D mutant could produce little or no rearrangement when recruited to our test IES.

Our finding that the I504D mutation supported some IES excision from genomic sites but not when it is tethered to the LexA-IES in the plasmid-based assay is a bit paradoxical. This could simply reflect the fact that that rearrangement efficiency of plasmid-based IESs is routinely low relative to that of genomic IESs (note that the majority of the plasmid-based M element failed to be rearranged, even in wild-type matings) (see Fig. S4 in supplemental material). The difference in efficiencies may result from the long time period that genomic IESs are available to interact with Pdd1 and the rest of rearrangement machinery, relative to the plasmid copies that are transiently introduced late into conjugation. Alternatively, local concentration of Pdd1 may be higher at plasmid copies that are transiently introduced late into conjugation but recruiting the downstream components of the excision machinery (note that the majority of the plasmid-based M element failed to note that the majority of the plasmid-based M element failed to rearrange, even in wild-type matings) (see Fig. S4 in supplemental material). These observations suggest that CSD-mediated dimerization plays an important role in Tetrahymena genome rearrangement and may help recruit the downstream excision-end-rejoining machinery. This hypothesis is supported by data from the LexA tethering experiment, in which the I504D mutant could produce little or no rearrangement when recruited to our test IES.

In studying the phenotypes of these mutants, we have focused on the failure of postzygotic events of chromatin modification, focus formation, and DNA elimination. It is possible that some phenotypes we observed are due to the absence of wild-type Pdd1 from prezygotic (parental) micro- and/or macronuclei. Perhaps the simplest explanation for Pdd1’s presence in parental macronuclei is to fulfill the function shared by all HP1 homologs in downregulating transcription by promoting heterochromatin formation (reviewed in reference 51). As conjugation progresses in Tetrahymena, the old parental macronucleus becomes pycnotic while the newly emerged anlagen rapidly transition into functional somatic nuclei. This massive conversion to zygotic expression would require a significant reallocation of transcriptional resources, and it is possible that PDD1 is highly expressed early in conjugation to reduce genome-wide activity from the parental macronucleus in preparation for development of the next generation. The mutants that we have generated in this study should be useful in investigating Pdd1 function in these prezygotic stages where its roles remain elusive.

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