Proteins containing a double-stranded RNA (dsRNA) binding motif (DSRM) participate in diverse biological pathways in a wide range of organisms. This motif was first identified in the developmentally essential gene Staufen of *Drosophila melanogaster* and has since been recognized to be encoded in the genomes in all three domains of living organisms, as well as in viruses (63; reviewed in references 20 and 67). DSRM proteins commonly act in developmental pathways (e.g., RNA localization by the Staufen family and developmental transcriptional regulation by the DIP1 family) (5, 18, 62, 68) but also have ubiquitous roles in transcriptional and translational regulation (e.g., PKR family and PKR-associated proteins) (26, 45, 55, 58). Proteins vital for RNA interference (RNAi) also contain DSRMs. These include members of the RNase III family (e.g., PKR family and PKR-associated proteins) (26, 45, 55, 58). Proteins vital for RNA interference (RNAi) also contain DSRMs. These include members of the RNase III family (e.g., PKR family and PKR-associated proteins) (26, 45, 55, 58).

In the ciliate *Tetrahymena thermophila*, the DSRM-containing protein *Dicer*-like 1 (*DCL1*) has been shown to play a pivotal role in a process linking RNAi to heterochromatin formation and developmentally regulated DNA elimination (42, 49). Like all ciliates, *T. thermophila* is unicellular yet contains two distinct types of nuclei, the somatic macro nucleus and the germ line micronucleus (reviewed in references 46 and 56). The polyploid micronucleus (∼50C) acts as a transcriptionally active somatic nucleus during vegetative growth, while the diploid, germ line micronucleus is transcriptionally silent (19, 70; reviewed in references 46 and 56). Under optimal growth conditions *T. thermophila* undergoes asexual, binary fission; however, when starved *T. thermophila* reproduces through the sexual process of conjugation, generating new micronuclei and macronuclei from the parental germ line micronucleus (reviewed in references 46 and 56). During the maturation of the zygotic macronucleus, the macronuclear chromosomes are fragmented at ∼180 sites, lose ∼15% of their overall genomic content, and are amplified to ∼50C (1, 7, 14, 19, 29, 69, 70). The loss of genome complexity is the result of programmed DNA rearrangements that remove specific DNA sequences, called internal eliminated sequences (IESs), from thousands of chromosomal sites (46, 56).

DNA elimination has been shown to be guided by an RNAi-related mechanism (11, 42, 47, 49). Bidirectional transcription of the germ line genome in meiotic micronuclei provides an abundant source of IES-specific dsRNA (11, 44). The resulting noncoding RNAs (ncRNAs) are processed into 27- to 30-nucleotide (nt) sRNA species, called scan RNAs (scsnRNAs), by Dcl1p in the meiotic micronucleus (42, 49). These scsnRNAs are exported into the cytoplasm, where they are bound by a PIWI homologue, Twi1p (47). Twi1p/scnRNA complexes are transported into the parental macronucleus, where these complexes scan macronuclear ncRNAs, and possibly mRNAs. The Twi1p/scnRNA complexes homologous to the parental macro nucleus are removed from the pool of active complexes, and the remaining complexes are transported to the zygotic macro nuclei upon their emergence, where they guide H3K9 and H3K27 methylation of IES-associated histones by the E(z) homologue Ezl1p (38, 47, 48). Methy lated histones in zygotic macronuclear are bound by the chromo domain-containing proteins Pdd1p and Pdd3p, which along with other associated
proteins form large nuclear structures called DNA elimination bodies late in conjugation (38, 40, 51, 66). DNA elimination in these bodies is catalyzed by the domesticated PiggyBac transposase Tbp2p, resulting in removal of IESs from zygotic macronuclei (13).

A second endogenous RNAi pathway that acts to silence genes and/or pseudogenes is evidenced by a class of 23- to 24-nt sRNAs that accumulate during vegetative growth (33). These sRNAs are homologous to loci clustered at ~12 genomic positions and exhibit biased polarity, mapping to only one strand. They are produced by the essential Dicer protein Dcr2p in a coupled reaction with an RNA-dependent RNA polymerase, Rdr1p (34). This coupling likely accounts for the strand specificity observed.

As dsRNA has clear roles in regulating genome structure and activity, we characterized the two putative tandem DSRM-containing proteins, double-stranded RNA binding proteins 1 and 2 (Drb1p and Drb2p), encoded in the T. thermophila genome (21, 64). We show that both are nuclear proteins that exhibit distinct subnuclear organization. By knocking out the gene for each, we found that Drb2p is essential both during vegetative growth and also late in conjugation, where it facilitates DNA elimination body formation and subsequent RNAi-dependent DNA elimination. Drb1p, in contrast, is dispensable but is nonetheless important for efficient prezygotic development. Our data do not support that either protein acts as an essential Dicer partner protein as do tandem DSRM proteins in other eukaryotes, but instead our data suggest that these proteins have diverse roles during the T. thermophila life cycle and expose a role for dsRNA late in macronuclear development (4, 12, 17, 23, 27, 37, 57, 65).

MATERIALS AND METHODS

Tetrahymena strains and growth conditions. Standard wild-type, laboratory T. thermophila strains CUA27 (Chc/Chc [VI, cy-s]), CUA28 (Mpr/Mpr [VII, mp-s]), B2086 (II), and micronucleus-defective strains B*VI (VI) and B*VII (VII) were originally obtained from Peter Bruns (Cornell University, Ithaca, NY). BT1 [CUG/CUA27, Chc/Chc [VII, cy-s]] was generated through genomic exclusion mating between CU242 and B*VII. These strains or their transformed progeny were used for expression studies, biolistic transformations, and subsequent analyses. Drb1 heterozygous knockout strains were described earlier (42). C2 cells were grown and maintained as previously described (25, 52). Strains were starved to synchronize their cell cycle and expose a role for dsRNA late in macronuclear development (4, 12, 17, 23, 27, 37, 57, 65).

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Identification of DRB1 and DRB2 sequences. DRB1 (TITHERM_00078870; NCBI Gene ID 783703) and DRB2 (TITHERM_00082551; NCBI Gene ID 7836999) sequences were identified by BLAST search of the T. thermophila macronuclear genome (http://www.ciliate.org) and the D. melanogaster R2D2 accession number CG7138 (6, 8). Heterozygous micronuclear knockout strains were described earlier (42).

For Pdd1-YFP localization in DRB2 mic knockout strains, pENTR-D-PDD1 was recombined with pSB2-ICG-GTWT by using LR clonase II (Invitrogen) to create pSB2-ICG-PDD1. Bell- and Sall-digested pSB1-ICG-PDD1 was transformed into starved, homogenous micronuclear DRB2 knockout strains (B*VII, accession numbers CG6866 and 6 and B*VII, accession numbers CG6867 and 7) and by using the PDS-1000/He particle bombardment system (Bio-Rad) as previously described (6, 8). Heterozygous micronuclear transformants were identified by their resistance to 25 µg/ml cycloheximide. To visualize localization, starved transformed cells were mixed to begin conjugation in 0.08 µg/ml CdCl2 to induce expression of the fusion protein. Live cells were harvested by low-speed centrifugation (1,000 × g at 4 h, 10 h, and 14 h postmixing, with 4.6-diamidino-2-phenylindole (DAPI; 1 µg/ml) and immobilized in 5 µl 2% methylethelenc. DIC, FCP fluorescence, YFP fluorescence, and DAPI fluorescence images were captured using a Qimaging RetigaEX charge-coupled-device camera (Burnaby, British Columbia, Canada) and Openlab software (PerkinElmer). Images were cropped and their brightness and contrast uniformly adjusted using Adobe Photoshop CS3.

Generation of DRB1, DRB2, and PDD1 knockout strains. Genomic sequences upstream and downstream of each gene’s coding region were amplified by PCR and recombined into pDONR-P41-R1 (upstream) and pDONR-P2R3 (downstream) by using BP Clonase (Invitrogen) (Table 1). The resulting donor plasmids containing up- and downstream regions were mixed with equal amounts of pENTR-D-MT1/NEO3 and the multisite destination vector pDEST-R4-R3, along with LR Clonase Plus II (Invitrogen) to create the DRB knockout plasmids pDEST-B4-DRB1Up-B1-MT1/NEO3-B2-DRB1Down-B3 and pDEST-B4-DRB2Up-N1-B1-MT1/NEO3-B2-DRB2Down-B3. DRB1 and DRB2 knockout constructs were linearized by digestion with KpnI and transformed into conjugating wild-type cells (CU427) by monitoring segregation of paromomycin resistance conferred by pENTR-D-PDD1 and by screening for progeny resistant to 50 µg/ml paromycin/CdCl2 and 15 µg/ml 6-methylpurine. Heterozygous micronuclear transformants were verified by sequencing with primers (Table 1). The resulting donor plasmids containing up- and downstream regions were mixed with equal amounts of pENTR-D-MT1/NEO3 and the multisite destination vector pDEST-R4-R3, along with LR Clonase Plus II (Invitrogen) to create the DRB knockout plasmids pDEST-B4-DRB1Up-B1-MT1/NEO3-B2-DRB1Down-B3 and pDEST-B4-DRB2Up-N1-B1-MT1/NEO3-B2-DRB2Down-B3. DRB1 and DRB2 knockout constructs were linearized by digestion with KpnI and transformed into conjugating wild-type cells (CU427) by monitoring segregation of paromomycin resistance conferred by pENTR-D-PDD1 and by screening for progeny resistant to 50 µg/ml paromycin/CdCl2, and verifying by PCR detection of the knockout allele (10).

RT-PCR expression analysis. RNA was isolated from growing, starved, and conjugating T. thermophila (CU428 × B2086) at 2-h intervals from 2 h to 14 h by RNAseol extraction (22). RNA isolation from DRB1 knockout and DRB2 mi-
| Primer purpose and name | Sequence (5’–3’) |
|------------------------|-----------------|
| **For RT-PCR to determine expression** | |
| DRB1 | |
| 1688-Loq1-836 | CGAAAAGGGGGTTAGGTTTTCTAGC |
| 1689-Loq1-1325r | CCCCATTCCATGTTTTCAG |
| DRB2 | |
| 1692-Loq3-1875 | GCAATAGCCAACACAAAGAGTGAGC |
| 1879-Loq3-2230r | GCATCAATAAGGCCATACACCAC |
| ATU1 | |
| 3364-ATU1-2391r | GTGCGCAATAGAAGCGGTTGACA |
| 3365-ATU1-1997 | TGCTCGATAACGAGGCATC |
| **For gene amplification of coding sequence** | |
| DRB1 | |
| 1701-Loq1X | CACCCTCGAGAAAATGAATTCTTAGCAAG |
| 1732-Loq1rH-Short | AAGCTTTAGACTTATACTTTTCATGAAAG |
| DRB2 | |
| 1887-Loq3X-Modified | CACCCTCGAGAAAATGGCGCAATCTTTTAGATTTATAG |
| 1911-Loq3P-Full length | CTGCAGCCCATTACAAATTTAAAGTTATGATCATAAGC |
| **For knockout cassette generation** | |
| DRB1 upstream | |
| 1761-Loq1-2321AattB4 | GGGGACAACTTTGTATAAGGTCGATTACATAAGATTGTATTCC |
| 1762-Loq1-3366rattB1 Downstream | GGGGACTGCTTTTTGTACAAACTTGCACTTTTAGGAAATAATGAATGTGTCAC |
| DRB2 upstream | |
| 2370-DRB2-1226AattB4 | GGGGACAACTTTGTATAGAAAAGTGGTACCGAAAGCCTATGGGAGAGCAAG |
| 2372-DRB2-2595rattB1 | GGGGACTGCTTTTTGTACAAACTTGCACTTTTAGGAAATAATGAATGTGTCAC |
| DRB2 downstream | |
| 2322-DRB2-6530attB2 | GGGGACAGCTTTCTTGTACAAAGTGGGTGTGTTTAAAAAGAAGGTGTGTGTATTAG |
| 2323-DRB2-7708ArattB3Ext | GGGGACAACTTTGTATAAGGTAACCTTAAACCGCACCCAG |
| **For knockout PCR screening** | |
| DRB1 | |
| 5’ | 1679-MTT1-11484r | ATTTGGAATTAAGTACTTTCCAAAC |
| 1946-DRB1-1086 | CGCGCACTTTTGATTGATAGTGC |
| 3’ | 1866-Neo KO 2 | CTTGATATTGCTGAAGAGCTTG |
| 1867-Loq1-5353 | CAGGGGAAGATATTTTATGAAGC |
| 1868-Loq1-5764r | GGGGGCATTAATGAGCTTG |
| DRB2 | |
| 5’ | 2477-DRB2-2195 | CAATTTATCTATTTATAAATAATCCCTTTACTAC |
| 2478-DRB2-2805r | AAAATCTGTTTATGAGCTTG |
| 3’ | 3001-LIA4MTTLR | AACATTCAACATTTGTCGACTAAATA |
| 2391-DRB2-6323 | GCTTAGATGATATTAGCTAAGTCTT |
| 2392-DRB2-6721r | AAAAGAGAGTGAGTTTTTCTTTGG |
| **For assay rearrangement of IESs** | |
| M | |
| 1439-M808 | ATATTGATGCTGATAATAGGTTTGTCGATAG |
| 3111-M602 | AGCTTAAACAAATGCGCATAATGAG |
| 3114-M1194 | GTGGGGAGGGAAGAGATTACAC |
| B | |
| 3246-IES7_MDSL-112 | GGATTGATGCTGACTAAATGG |
| 3247-IES7_MDSR-158 | AAGCCCAAGATACCCGAGATCC |
Conjugation stage of each mating at 30 h was determined by comparison of images with previously described wild-type stages of conjugation (44). Images were cropped and their brightness and contrast uniformly adjusted using Adobe Photoshop CS3.

**Results**

The *T. thermophila* macronuclear genome encodes two proteins with tandem DSRMs. For optimal sRNA production and protein localization, Dicer and Drosha homologues in *C. elegans*, *D. melanogaster*, and *H. sapiens* require association with a tandem DSRM-containing protein (4, 12, 17, 23, 27, 37, 53, 57, 65). Bioinformatic analysis (BLAST, Pfam, and ClustalW) of the *T. thermophila* macronuclear genome identified two genes, *DRB1* and *DRB2*, encoding tandem DSRM-containing proteins (Fig. 1A). Alignment of their putative DSRMs with other DSRM-containing proteins indicated conservation in the regions where key residues known to be important for DSRM structure and function are located. The homologies of these proteins with other tandem DSRM proteins did not extend beyond these domains (Fig. 1A and data not shown). However, alignment of full-length *DRB1* and *DRB2* revealed additional regions of similarity outside the DSRMs; one in the N-terminal region (NTR) and two in the C-terminal regions (CT1 and CT2) of each protein (data not shown). In the ciliate *Paramecium*, only *DRB1* homologues are evident, which suggests that the duplication and diversification of these proteins occurred after these two ciliates diverged.

RT-PCR and Northern blot analysis demonstrated that *DRB1* and *DRB2* are both expressed throughout much of the
*T. thermophila* life cycle (Fig. 1C and D and data not shown). *DRB1* mRNA levels are low in growing and starved cells but increase significantly during meiosis (2 to 4 h into conjugation, when scnRNA production occurs) and again after the appearance of the zygotic macronuclei (8 h), a pattern that parallels *DCL1* expression (Fig. 1C) (42, 49). Its decrease in expression at 6 h coincides with the drop in *ATU1* RNA levels, which may simply reflect the switch between parental and zygotic expression.

*DRB2* expression is higher during vegetative growth but also shows less dramatic induction during conjugation relative to *DRB1*. After decreased expression during starvation, *DRB2* is induced starting at 2 h of conjugation and peaks at 8 h, shortly after the appearance of the zygotic macronuclei (Fig. 1D). This profile suggests possible roles for Drb2p during both growth and development.

**DRB1 and DRB2 encode nuclear proteins that localize to distinct structures.** Ectopic expression of Drb1p and Drb2p tagged with YFP or CFP, respectively, on their C termini showed that both are nuclear proteins visible in small foci throughout the macronucleus during vegetative growth (data not shown), whereas green fluorescent protein (GFP) alone expressed in cells is uniformly distributed (42, 43). During early conjugation, both proteins localize to the parental macronucleus in distinct foci (Fig. 2). Later, at the beginning of zygotic macronuclear differentiation (10 h), all Drb1p-YFP and most Drb2p-CFP disappeared from the parental macronucleus and then appeared in zygotic macronuclei (Fig. 2A and B, middle rows). Whether the foci seen in the parental macronucleus are functionally related to those observed in zygotic macronuclei could not be determined (Fig. 2A and B, compare top and middle rows). Near completion of zygotic macronuclear development (14 h into conjugation), Drb1p-YFP localization was primarily diffuse (Fig. 2A, bottom row). In contrast, the small Drb2p-CFP foci coalesced into larger foci, although low-level diffuse localization remained throughout the zygotic macronucleus as well (Fig. 2B, bottom row).

Upon initial inspection, the size and number of nuclear foci of Drb1p and Drb2p in parental macronuclei appeared rather different. To better compare their localizations, Drb1p-YFP and Drb2p-CFP were coexpressed and visualized 4 h into conjugation. Their nuclear foci were distinct, with only a small degree of overlapping localization (Fig. 2C). *DRB1* and *DRB2*...
were best reciprocal hits in a BLASTp analysis of the CT2 regions, which could explain the small overlap in localization through partially redundant protein function. Despite this, it seems that both Drb1p and Drb2p have distinct primary functions based on their localizations and divergent protein sequences outside their DSRMs and CT2.

In addition to its abundant macronuclear localization, Drb1p-YFP also localized to the micronucleus just prior to and during crescent formation (prophase meiosis I) (Fig. 2A, top row, and data not shown). Drb1p-YFP was observed specifically at the poles of these nuclei, at either one or both ends depending on the developmental stage. This micronuclear localization pattern is quite distinct from that of Dcl1p, which is found throughout the nucleoplasm of the crescent micronucleus, and suggests that Drb1p may not be a critical Dcl1p protein partner (42, 49). Point localization of Drb1p-YFP was seen early in conjugation once the micronucleus began to elongate at one end, and later, after the crescent micronucleus fully elongated, it was seen at both ends of the micronucleus (Fig. 2A, top row, and data not shown). Upon anaphase of meiosis I, Drb1p-YFP micronuclear localization is lost. While it is likely that DRB1 and DRB2 arose from an ancient gene duplication, differential localization and expression patterns indicate that each DSRM-containing protein has specific cellular roles.

**DRB2, but not DRB1, is essential for growth and development.** We created strains lacking each gene to establish whether and when each protein functions during the *T. thermophila* life cycle. Constructs containing the *NEO3* selectable marker, flanked by up- and downstream homology regions to either DRB1 or DRB2, were bio synthetically transformed into cells during conjugation to generate heterozygous macronuclear/micronuclear knockout strains. By taking advantage of the random assortment of alleles during amitotic macronuclear division, we obtained strains for which all wild-type DRB1 gene copies in the macronucleus were replaced with the knockout allele, which revealed that Drb1p is not required for vegetative growth (Fig. 3A and B and data not shown).

To further verify that DRB1 is not essential, homoygous micronuclear knockout strains were crossed to produce complete DRB1 knockout cell lines. Southern blot analysis of genomic DNA isolated from these strains detected only the DRB1 knockout allele (Fig. 3A). RT-PCR of the DRB1 knockout strains during conjugation confirmed loss of all DRB1 expression (Fig. 3B). While these complete DRB1 knockout strains showed no growth defects, matings between two DRB1 knockout strains generated progeny at a reduced rate relative to crosses of wild-type strains (Table 2). The DRB1 knockout cells that were able to complete conjugation arrested with two new macronuclei and a single micronucleus, as do wild-type conjugants, until they were returned to growth medium and started vegetative growth (Fig. 4). The observation that only a fraction of mated DRB1 knockout cells progressed to zygotic development suggests that Drb1p is important, but not essential, for prezygotic development. The lack of Drb1p during this stage(s) of early conjugation resulted in substantial premature abortion of conjugation (data not shown).

Unlike our experience with DRB1, we were unable to identify strains in which all macronuclear copies of DRB2 were disrupted, which indicates that vegetative DRB2 expression is essential (Fig. 3C and D). To verify this, we first performed genomic exclusion crosses between the original heterozygous micronuclear knockout strains and “star” strains (B’VI and B’VII) to create strains homoygous for the knockout cassette in the micronucleus while maintaining wild-type copies of DRB2 in the macronucleus to support growth (see Materials and Methods for details). These homoygous micronuclear knockout strains were then crossed in an attempt to generate strains homoygous for the knockout cassette in both the micro- and macronucleus, thus eliminating all wild-type DRB2 gene copies. Despite each individual DRB2 micronuclear knockout strain being able to produce progeny when complemented by crossing to wild-type strains, when these lines were crossed to each other no viable progeny emerged (Table 3).

Further analysis revealed that DRB2 micronuclear knockout strains are unable to reach the terminal stage of conjugation with 2 macronuclei and 1 micronucleus even 30 h after pairing, but instead arrest with 2 macronuclei and 2 micronuclei (Fig. 4). Thus, not only is DRB2 expression necessary for vegetative growth, but zygotic DRB2 expression is essential for completion of conjugation as well (Fig. 3C and 4). As observed in other mutants that arrest at the 2-macronuclei, 2-micronuclei stage, conjugating DRB2 mic knockouts underamplified their macronuclear DNA relative to zygotic macronuclei of wild-type conjugants at their terminal stage prior to refeeding (15, 42, 47, 49). Although DRB2 mic knockout strains only lack zygotic expression of DRB2, the majority of conjugants arrest at the 2-macronuclei, 2-micronuclei stage, while the remainder arrest after elimination of one of the remaining micronuclei (Fig. 4, bottom). RT-PCR analysis of DRB2 mic knockout matings showed reduced, but not complete loss of, expression after 12 h of conjugation relative to wild-type cells, when zygotic DRB2 expression normally should predominate (Fig. 3D). Unmated cells as well as parentally expressed DRB2 mRNA in the DRB2 mic knockout mating population accounted for the DRB2 mRNA detected. The residual, parentally expressed DRB2 transcripts may enable a fraction of cells to proceed further into conjugation and eliminate one micronucleus.

**DRB2 mic knockouts fail to remodel chromosomes late in conjugation.** The DRB2 conjugation arrest phenotype is commonly observed in knockouts of genes necessary for genome rearrangement in *T. thermophila*, including DCL1, TWI1, and PDD1 (15, 42, 47, 49). To determine whether the DRB2 mic knockout arrest is accompanied by failure of RNA-directed DNA elimination or due to some other perturbation during conjugation, we monitored the rearrangements of several IESs. Genomic DNA was isolated from mated cell populations 30 h after initiating conjugation, when all genome reorganization should be completed in wild-type cells. PCR using primers able to detect both the unrarranged (micronuclear form of the locus) and rearranged (macronuclear form) IESs allowed assessment of the level of excision. Whereas DNA from wild-type mating populations showed predominantly the rearranged locus for each IES, DRB2 mic knockout or control DCL1 knock out matings exhibited accumulation of the unrearranged form of both IES B and the M IES (Fig. 4 and data not shown). IES B is a 327-bp IES found within the *LIA2* gene, and the M IES is a well-studied intergenic IES that undergoes alternative rearrangement that removes either 0.6 kb or the complete 0.9-kb
IES (2, 22a). PCR analysis of IES B clearly showed that the 597-bp product indicative of the micronuclear locus was overrepresented in the DCL1 and DRB2 mic knockout matings relative to wild type (Fig. 5A). It is important to note that the cell populations monitored included some percentage of unmated cells, whose DNA likely contributed much of the template for the 270-bp product representing the rearranged form in the mutant cell lines. The PCR analysis of the M IES utilized three primers for PCR, which we have found provides a more quantitative assessment of its rearrangement. Two bands at 1,192 bp and 386 bp resulted from amplification of micronuclear DNA containing the IES, while two other bands at 592 bp and 292 bp were the products of removal of either 0.6 kb or 0.9 kb of the M IES locus. As observed for IES B, the unrearranged form of the M IES was overrepresented in the DCL1 and DRB2 mic knockout mating populations relative to wild-type matings (Fig. 5B). This difference was less apparent in DRB2 mic knockout matings than in the DCL1 mutant, which may have been due to persistence of parental Drb2p. Analysis of other IESs further demonstrated that these mutants exhibit

![Diagram](image_url)

**FIG. 3.** Generation of DRB1 complete and DRB2 mic knockout strains, based on Southern blot analysis of knockout strain genomic DNA. (A) Genomic DNA isolated from wild-type (WT) CU428, four DRB1 macronuclear/micronuclear knockout strains (DRB1 KO), and four micronuclear strains (B*VI and B*VII[A3;A5]) was digested with XmnI prior to gel electrophoresis. (C) Genomic DNA from DRB2 mic knockout heterozygous strains (DRB2 mic KO het) was digested with ClaI and SacI prior to analysis. The diagram of WT (DRB1/2) and KO (MTT1/NEO3) alleles is shown on the right of each panel. Black arrowhead, band expected for the knockout allele; white arrowhead, band expected for the wild-type fragment. (B and D) RT-PCR expression analysis of DRB KO strain matings. RNA isolated 4 h (parental expression) and 12 h (zygotic expression) into conjugation was converted to cDNA (RT+), and PCR using gene-specific primers was used to assess loss/reduction of expression. A control reaction with reverse transcriptase omitted (RT-) is also shown. Primers specific to the α-tubulin gene (ATU1) provided a normalization control between samples. In panel B, the star marks a nonspecific RT-PCR band detected with DRB1 primers. Diagrams of the DRB1/2 and ATU1 loci; the relative locations of forward and reverse PCR primers (black arrows) are shown on the right.
TABLE 2. Progeny production of DRB1 knockouts in wild-type and knockout matings

| Cross | % pair survival (S/N) | % progeny production (P/N) |
|-------|-----------------------|---------------------------|
| CU427 × DRB1 KO 5.1.3 | 97.2 (171/176) | 96.5 (165/171) |
| CU427 × DRB1 KO 6.1.6 | 98.8 (87/88) | 98.9 (86/87) |
| CU427 × DRB1 KO 6.1.12.1 | 98.0 (169/176) | 98.2 (166/169) |
| CU427 × DRB1 KO 6.1.12.2 | 98.9 (174/176) | 97.1 (169/174) |
| CU427 × DRB1 KO 7.1 | 97.7 (129/132) | 98.4 (127/129) |
| CU427 × DRB1 KO 7.7.2 | 99.2 (131/132) | 94.7 (124/131) |
| DRB1 KO 5.1.3 × 6.1.12.1 | 93.5 (247/264) | 33.3 (6/18) |
| DRB1 KO 5.1.3 × 6.1.12.2 | 94.7 (250/264) | 51.5 (35/68) |

a The pair survival is the percentage of pairs alive (S) of the total pairs (N) isolated.

b Progeny production is the percentage of surviving pairs (S) that successfully completed conjugation and made new macronuclei (P).

substantial failure of RNA-directed DNA elimination (data not shown).

Assessment of chromosome breakage near the LIA1 locus also showed that DRB2 mic knockout progeny fail to properly fragment chromosomes (Fig. 5C). Before the completion of conjugation, the chromosomes in the zygotic macronuclei, which contain 5 chromosomes amplified to between 4 and 8 copies, are fragmented at approximately 180 chromosome breakage sites (CBSs) to produce the shortened macronuclear chromosomes. In knockouts of genes essential for genome rearrangement, including DCL1 and TWI1, chromosome breakage fails, as does IES elimination (42, 47). In a Southern blot assay of wild-type progeny, chromosome breakage at the LIA1 locus resulted in a band of approximately 2.5 kb in the zygotic macronuclei. The copies of this chromosome from the parental macronucleus were visible as a 2.6-kb band, as they have longer telomeres relative to newly fragmented ends. Unbroken micronuclear chromosomes were detected as a 10.5-kb band. The probe also detected a 7.8-kb fragment present in all nuclei. Due to the increased copy number of the locus in the macronucleus in the progeny of wild-type crosses, the 2.5-kb and 2.6-kb fragments are more intense than the larger 10.5-kb micronucleus-specific fragment. As in the control matings of DCL1 knockout cells, the postconjugation populations of DRB2 mic knockout crosses have increased levels of the 10.5-kb unarranged fragment and lack the 2.5-kb fragment indicative of de novo chromosome breakage (Fig. 4C). A previous report on chromosome breakage in a somatic knockout of PDD1 showed that chromosome fragmentation was able to occur (15). Here we report that crosses of homozygous PDD1 knockout strains showed failure of chromosome breakage, as observed with DCL1 and DRB2 mic knockouts, emphasizing the importance of zygotic expression of PDD1 and DRB2 in chromosome breakage (Fig. 5C).

DRB2 colocalizes with Pdd1p in DNA elimination bodies. Failure of DNA elimination and chromosome breakage in DRB2 mic knockout strain matings indicated that the conjugation arrest phenotype described earlier was a result of failure to complete RNA-directed DNA elimination. The localization of Drb2p-CFP into large foci 14 h into conjugation, which is when DNA elimination normally occurs, prompted us to ascertain whether Drb2p-CFP was localized into DNA elimination bodies. These nuclear structures are enriched for the essential DNA elimination, chromodomain-containing protein Pdd1p and are the putative sites of IES removal. Strains expressing Drb2p-CFP or Pdd1p-YFP were mated, and localization of both proteins was monitored at 8 h into conjugation, very early in zygotic macronuclear differentiation, and later at 14 h into conjugation, when DNA elimination occurs (Fig. 6). As was previously reported, Pdd1p-YFP was diffusely localized in the zygotic macronuclei at 8 h, and as conjugation proceeded toward DNA elimination around 14 h, Pdd1p-YFP localization gradually became unevenly dispersed, forming first small foci and then finally large foci (Fig. 6) (40, 41). Localization of Drb2p-CFP in the zygotic macronuclei at 8 h into conjugation was not markedly different from Pdd1p-YFP localization, with small Drb2p-CFP foci throughout the nucleus (Fig. 5). However, at 14 h into conjugation Drb2p-CFP foci aggregated into larger foci, which colocalized with the Pdd1p-

![FIG. 4. Zygotic expression of DRB2 is necessary for completion of conjugation. (Top) Terminal arrest phenotype of wild-type (WT), ΔDCL1, ΔDRB1, and ΔDRB2 mic cells 30 h into conjugation. WT, ΔDCL1, ΔDRB1, and ΔDRB2 mic cells were mated and harvested after 30 h into conjugation. Cells were then DAPI stained, and DIC (left) and DAPI (right) images were obtained. White arrowheads, micronuclei; white arrows, zygotic macronuclei. (Bottom) Cells with the indicated terminal arrest phenotype of WT, ΔDCL1, ΔDRB1, and ΔDRB2 mic 30 h into conjugation.](Image)
TABLE 3. Progeny production of DRB2 mic knockouts in wild-type and knockout matings

| Cross                      | % pair survival (S/N)* | % progeny production (P/S)* |
|----------------------------|------------------------|----------------------------|
| B*VII427 x B*VI9D2/AD2    | 99.6 (263/264)         | 98.8 (260/263)             |
| B*VII427 x B*VI9D2/AD2    | 99.2 (262/264)         | 99.2 (260/262)             |
| CU427 x B*VI9D2/AD2       | 95.1 (251/264)         | 99.6 (250/253)             |
| CU427 x B*VI9D2/AD2       | 97.3 (257/264)         | 100 (257/257)              |
| B*VII9D2/AD2 x B*VII9D2/AD2| 2.8 (5/176)            | 0.0 (0/5)                  |
| B*VII9D2/AD2 x B*VII9D2/AD2| 1.7 (3/176)            | 0.0 (0/3)                  |
| B*VII9D2/AD2 x B*VII9D2/AD2| 0.0 (0/176)            | 0.0 (0/0)                  |
| B*VII9D2/AD2 x B*VII9D2/AD2| 1.1 (2/176)            | 0.0 (0/2)                  |

* Pair survival is the percentage of pairs alive (% S/N) isolated.
* Progeny production is the percentage of surviving pairs (% P/S) that successfully completed conjugation and made new macronuclei (%).

YFP-containing DNA elimination bodies, indicating a possible interaction with each other in zygotic macronuclei.

Localization of Pdd1-YFP and Drb2p-CFP is not exclusive to the zygotic macronuclei. Residual localization of both proteins was seen in the parental macronucleus as well. At 8 h into conjugation, both proteins formed strong, distinct foci in the parental macronucleus, with Pdd1p-YFP foci localized to the nuclear periphery and Drb2p-CFP foci found in the nuclear interior. During DNA elimination at 14 h into conjugation, remaining Pdd1p-YFP was found throughout the parental macronucleus as well. At 8 h into conjugation during DNA elimination in DRB2 mic knockouts, we sought to determine how Pdd1p localization was affected in DRB2 mic knockout strain matings. DRB2 mic knockout strains were transformed with an inducible Pdd1p-YFP expression construct, and the resulting transformants were mated and their Pdd1p-YFP localization was examined. At 10 h into conjugation during zygotic macronuclear differentiation, Pdd1p-YFP localization in both DRB2 mic knockouts crossed to wild-type strains, which rescues loss of DRB2 from the mating partner, and DRB2 mic knockout matings appeared mottled throughout the developing zygotic macronucleus without obvious defects (Fig. 7A). However, late in conjugation (14 h), Pdd1p-YFP failed to form DNA elimination bodies in zygotic macronuclei in DRB2 mic knockout matings (Fig. 7B). Thus, Pdd1p-YFP foci fail to mature into DNA elimination bodies without a zygotic DRB2 expression. These data indicate that DRB2 participates in the maturation of DNA elimination bodies and implicates a possible role for uncharacterized dsRNAs in genome reorganization.

DISCUSSION

Our analyses of DRB1 and DRB2 have revealed that each has unique and important functions. While both are predominantly nuclear proteins, they localize into distinct subnuclear foci. Furthermore, disruption of the each gene showed that Drb2p has essential functions during both growth and development, while Drb1p appears to be important for prezygotic development. The similarities of these two proteins outside their predicted DSRMs suggest that they may have arisen from an ancestral gene duplication. If that is the case, they have significantly diverged in function since the duplication event.

FIG. 5. DNA rearrangements of IESs and chromosome breakage are impaired in DRB2 mic knockouts. (A and B) Rearrangements of IES B (A) and M IES (B) were assessed by two- or three-primer PCR, respectively, in genomic DNA isolated from wild-type (WT), ΔDCL1, and ΔDRB2 mic cells postconjugation. White arrowheads, the unrearranged/micronuclear form; black arrowheads, the rearranged/macronuclear form; unlabeled bands, nonspecific products. Diagrams of each IES locus are shown below the gel image. IES, white and dark boxes) sequence. The expected PCR product size is provide beside arrows. The M IES undergoes alternative rearrangement through elimination of the 0.6-kb (white box) or the 0.9-kb (white and dark gray boxes) sequence. The expected PCR product size is provide beside each form. (C) Chromosome breakage fails in DRB2 mic knockouts. (Left) Southern blot hybridization of total genomic DNA isolated from WT or mutant cells postconjugation. White arrowheads, the unrearranged/micronuclear form; black arrowheads, the rearranged/macronuclear form; unlabeled bands, nonspecific products. Diagrams of each IES locus are shown below the gel image. IES, white and dark gray boxes; flanking DNA, gray boxes; PCR primers, black and gray arrows. The M IES undergoes alternative rearrangement through elimination of the 0.6-kb (white box) or the 0.9-kb (white and dark gray boxes) sequence. The expected PCR product size is provide beside each form. (Right) Diagram of CBS near the LIA1 locus in the micro- and macronuclei. Southern blot band sizes are listed next to each locus diagram. White circle, CBS; white arrow, LIA1 gene; Tel, telomere.
Upon initial recognition that the *T. thermophila* genome encodes two DSRM-containing proteins, we looked for evidence that would connect them as protein partners for the Dicer homologues encoded by *DCL1* and *DCR2* (42, 49). Tandem DSRM-containing partner proteins for Dicer and Drosha family proteins, including R2D2, Loqs, and Pasha in *D. melanogaster*, RDE-4 in *C. elegans*, and TRBP2 and DGCR8 in *H. sapiens* and other mammals, play vital roles in RNAi by ensuring proper sRNA delivery and in many cases cleavage of sRNA precursors (12, 17, 23, 27, 37, 57, 65). Our analyses provided little support that Drb1p or Drb2p serve as major Dicer partners. Neither protein showed abundant localization in meiotic micronuclei, where Dcl1p acts (Fig. 2A and B, top rows) (42, 49). We also did not find defects in scnRNA accumulation in complete *DRB1* knockouts (data not shown). As Drb2p is essential for growth, we were unable to generate full knockouts with which to examine scnRNA accumulation upon its loss. The *T. thermophila* Dicer protein, Dcr2p, is also essential for growth, but a previously published characterization of Dcr2p complexes did not find Drb2p to be an interacting protein (34, 35).

While we did not find evidence that these proteins act with Dcl1p, we uncovered a critical role for Drb2p in the RNAi-directed DNA elimination pathway. Loss of zygotic expression was sufficient to block DNA rearrangement; thus, Drb2p is needed well downstream of scRNA biogenesis by Dcl1p (Fig. 2A and B). Colocalization of Drb2p with Pdd1p-containing DNA elimination bodies and loss of these DNA elimination bodies in *DRB2* mic knockouts implicate zygotically expressed Drb2p in promoting development or stabilizing these large nucleoprotein structures (Fig. 6 and 7). This may indicate that Drb2p/RNA complexes mediate the formation of mature DNA elimination bodies through facilitating protein-RNA or protein-protein interactions within these structures. Although the exact mechanism of Drb2p action remains to be discovered, its importance in late stages of genome reorganization suggests an unrecognized role for dsRNA in RNAi-directed DNA elimination.

Drb2p is also required for vegetative growth, as we were unable to replace all wild-type *DRB2* gene copies with the disrupted allele. We tried extensively to assort *DRB2* out of the macronucleus without success (data not shown). Furthermore, when *DRB2* partial knockout strains were grown in nonselective medium (without paromomycin), the remaining wild-type *DRB2* copies rapidly replaced the *DRB2* knockout allele (data not shown). As both Drb2p and Dcr2p are essential for growth, it remains possible that they act in the same pathway (34, 35). We cannot rule out the possibility that these proteins transiently interact, as do RDE-4 and DCR-1 in *C. elegans* (65). Further investigation of the function of Drb2p during growth may provide key insights into the role of this protein during both growth and genome reorganization.

While Drb1p is predominantly a macronuclear protein, it also localizes to one or both ends of the crescent micronucleus during the prophase of meiosis I (Fig. 2 and data not shown). Further investigation of this micronuclear point localization indicated that colocalization of Drb1p with cenH3, the centromeric histone H3 (unpublished data) (9, 16, 39). Knockouts of *DBR1* were able to complete conjugation, yet a significant percentage of pairs aborted mating without forming new macronuclei. Together, the localization of Drb1p near centromeres and possibly with telomeres and the reduction in knockout

![Figure 6](image.png)
cells completing prezygotic stages of development are consistent with a role for Drb1p in maintaining micronuclear chromosomal structure (Fig. 2 and data not shown). Thus, the analysis of both of these DSRM-containing proteins strongly suggests that they perform critical chromosomal functions.

Although many tandem DSRM-containing proteins have been found to interact with Dicer and Drosha family proteins, this is by no means the only job that these proteins containing DSRMs undertake (12, 17, 23, 27, 37, 57, 65; reviewed in references 20 and 67). Roles for these proteins include cleavage of long noncoding RNAs into sRNAs by RNase III family members, RNA editing by the ADAR family, translation inhibition in response to viruses by PKR family members, and developmental RNA localization by the Staufen family (3, 4, 26, 28, 30, 36, 45, 50, 55, 62, 63). Besides the partner proteins for the Dicer and Drosha families, at least one other protein family, the NFAT family, also encodes tandem DSRMs. The NFAT family proteins, which contain a DZF protein domain in addition to tandem DSRMs, are putative nuclear, nucleotide transferases that participate in DNA repair and RNA transport (32, 59, 60, 73; reviewed in reference 31). Further study of DRB1 and DRB2 in *T. thermophila* may reveal new roles for tandem DSRM-containing proteins. The great evolutionary distance between ciliates and other eukaryotes could also facilitate understanding of how DSRM-containing proteins evolved within the eukaryotic lineage (54). Much remains to be gleaned about the roles of DSRM-containing proteins in eukaryotes, and we expect further investigation of Drb1p and Drb2p functions will provide greater understanding of RNAi-directed DNA elimination and roles for dsRNA in regulating chromosome structure.

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