Two GW Repeat Proteins Interact with *Tetrahymena thermophila* Argonaute and Promote Genome Rearrangement^{∇†}

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In conjugating *Tetrahymena thermophila*, massive DNA elimination occurs upon the development of the new somatic genome from the germ line genome. Small, ~28-nucleotide scan RNAs (scnRNAs) and Twi1p, an Argonaute family member, mediate H3K27me3 and H3K9me3 histone H3 modifications, which lead to heterochromatin formation and the excision of the heterochromatinized germ line-limited sequences. In our search for new factors involved in developmental DNA rearrangement, we identified two Twi1p-interacting proteins, Wag1p and CnjBp. Both proteins contain GW (glycine and tryptophan) repeats, which are characteristic of several Argonaute-interacting proteins in other organisms. Wag1p and CnjBp colocalize with Twi1p in the parental macronucleus early in conjugation and in the new developing macronucleus during later developmental stages. Around the time DNA elimination occurs, Wag1p forms multiple nuclear bodies in the developing macronuclei that do not colocalize with heterochromatic DNA elimination structures. Analyses of $\Delta WAG1$, $\Delta CnjB$, and double $\Delta WAG1 \Delta CnjB$ knockout strains revealed that *WAG1* and *CnjB* genes need to be deleted together to inhibit the downregulation of specific scnRNAs, the formation of DNA elimination structures, and DNA excision. Thus, Wag1p and CnjBp are two novel players with overlapping functions in RNA interference-mediated genome rearrangement in *Tetrahymena*.

Small RNA-directed gene silencing, also called RNA interference (RNAi), is a conserved process that can occur post-transcriptionally by direct interaction with viral and cellular RNAs, as well as at the transcriptional (chromatin) level through targeting histone and/or DNA methylation (3, 51). A central role in RNAi belongs to Argonaute family proteins, which bind to small RNAs that target them to complementary nucleic acid sequences (15). Developmentally regulated genome rearrangement in the ciliated protozoan *Tetrahymena thermophila* is considered to be the ultimate form of transcriptional gene silencing, since the heterochromatinization of the small RNA-targeted sequences leads to their deletion from the genome (4, 33). The Argonaute family protein Twi1p (*Tetrahymena* Piwi), which is associated with ~28-nucleotide (nt) scan RNAs (scnRNAs), plays a key role in *T. thermophila* transcriptional gene silencing (18, 34).

Like most ciliated protozoa, *T. thermophila* contains two nuclei, a germ line micronucleus (Mic) and a somatic macronucleus (Mac). In growing cells, the Mic is transcriptionally silent and undergoes mitotic divisions, while all gene expression occurs from the Mac, which divides amitotically (8, 16). During sexual conjugation, which occurs between two starved strains of different mating types, cells pair and the Mic undergoes meiosis. One of the four meiotic products gives rise to two

haploid pronuclei. One of the pronuclei in each cell migrates to the other pair member, where it fuses with another, stationary pronucleus to form a diploid zygotic nucleus. Two new Mics and two new Macs develop from the zygotic nucleus after two rounds of mitosis (designated the 2 Mic/2 Mac stage). Upon the formation of the new Macs, gene expression switches to them from the parental Mac, which eventually is degraded. The two cells separate, forming exconjugants in which one of the two Mics is resorbed. The remaining Mic divides mitotically, and the first vegetative division yields two progeny cells, each with a new Mic and a new Mac. The entire developmental program takes ~24 h to complete.

Despite their shared zygotic origin, the Mic and Mac are characterized by distinct genomes. The diploid micronuclear genome consists of five pairs of metacentric chromosomes. During macronuclear differentiation, the germ line-derived chromosomes are subjected to breakage, followed by de novo telomere addition and endoreplication. As a result, the macronuclear genome contains ~225 chromosomes present in ~45 copies. In addition, ~15% of the germ line DNA is eliminated from the developing macronuclear genome upon the deletion of ~6,000 internal eliminated sequences (IESs) and the rejoining of the flanking macronuclear-destined sequences (MDSs).

One fundamental issue is how the developing progeny cells discriminate between DNA to be eliminated and genomic regions destined to form the macronuclear genome. It has been shown that two histone H3 modifications, H3K27me3 and H3K9me3, are associated specifically with the IESs in the developing macronuclei (24, 45). Both of these epigenetic marks are dependent on *Tetrahymena thermophila* Ezl1p, a homolog

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of *Drosophila melanogaster* histone lysine methyltransferase E(z) (24). Chromodomain proteins Pdd1p, which interacts with H3K27me3 and H3K9me3 in vitro, and Pdd3p, which binds H3K9me3 in vitro (24, 45), also are associated specifically with IESs in vivo (37, 44). H3K9me3, H3K27me3, Pdd1p, Pdd3p, and germ line-limited DNA all are found in specific doughnut-shaped structures called DNA elimination bodies, which assemble at the periphery of the new Mac at the time of IES elimination and disappear after genome rearrangement is completed (24–26, 45).

Substantial evidence indicates that the deposition of histone H3 methylation marks on the germ line-limited sequences in the developing *T. thermophila* Macs is specified by an RNAi-related mechanism. Early in conjugation, the bidirectional transcription of the micronuclear genome (7) is thought to produce double-stranded RNAs that then are diced by a *T. thermophila* Dicer-like RNase, Dcl1p, into ~28-nt scnRNAs (27, 36). scnRNAs then form complexes with Twi1p, which is localized first to the parental Mac and then to the new Mac (34, 35). While both IES-specific and MDS-specific scnRNAs are transcribed from the micronuclear genome (7), the scnRNA pool becomes enriched in IES-specific sequences by the time the new Mac develops (35). Significantly, the parental micronuclear genome can directly influence IES elimination, since introducing an IES into the parental Mac inhibits its excision from the developing new Mac (5, 6). Based on these observations, it was proposed that scnRNAs are scanned against parental Mac genome sequences, scnRNAs having homology to the parental Mac sequences are degraded, and the remaining micronucleus-specific sequences target IESs for elimination (6, 34).

Phenotypic analyses of knockouts of *T. thermophila* genes encoding proteins involved in the RNAi pathway establish a link between RNAi and chromatin modification/DNA elimination. In *Twil* knockouts, scnRNA levels are decreased compared to those of the wild-type cells, H3K27me3 and H3K9me3 levels are greatly reduced in the new Macs, and IES elimination does not occur (23, 24, 34). Likewise, *DCL1* is required for scnRNA production, H3K27me3 modification, IES elimination, and the specific association of histone H3 methylated at K9 with the IES sequences (24, 27, 36). Also, disruption of RNAi pathway genes (*Twil* and *DCL1*) or mutations that affect chromatin modification (*EZL1* knockout and histone H3 K9Q substitution) lead to similar developmental arrest at the 2 Mic/2 Mac stage at the end of conjugation, prior to the resorption of one of the Mics (27, 34, 36).

To learn more about how Twi1p/scnRNA complexes target chromatin modifications and DNA elimination, we used tandem affinity purification and mass spectrometry to identify proteins copurifying with Twi1p in the new developing Macs. Here, we present the analysis of the expression and localization of two novel Twi1p-associated proteins, Wag1p and CnjBp. We find that *T. thermophila* mutants in which both *WAG1* and *CNJB* genes are disrupted are deficient in the downregulation of specific scnRNAs and in DNA elimination. Interestingly, these two proteins possess GW (glycine and tryptophan) repeats, which also are present in a number of Argonaute-associated proteins in other organisms.

MATERIALS AND METHODS

***Tetrahymena* culture conditions.** All *T. thermophila* strains were grown in super proteose peptone medium (13). For conjugation, cells were starved in 10 mM Tris, pH 7.4, for 16 to 24 h at 30°C. To ensure synchronous pairing, the on/off shake method was used: cells of two different mating types were mixed, incubated for 30 min without shaking, incubated for 30 min with shaking at ~180 rpm, incubated for 30 min without shaking, and then incubated for 30 min with shaking. Shaking prevents pair formation, and no cell pairs were detected immediately after the final shaking was terminated; this time was counted as 0 h postmixing, the start of the mating time point. *Tetrahymena* species strain construction and viability testing are described in the supplemental material.

Indirect immunofluorescence. For the cellular localization of FLAG-hemagglutinin-Wag1p (FLAG-HA-Wag1p) shown in Fig. 2, cells were fixed using partial Shaudin's fixative (50). HA-CnjBp-expressing cells were fixed using Lavdovsky's solution (ethanol-formalin-acetic acid-water; 50:10:1:39). For the visualization of DNA elimination structures and intranuclear structure analysis (see Fig. 5 and 6), cells were washed with phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde in PBS for 10 min at room temperature, gently pelleted (300 × g for 1 min), and permeabilized with 0.4% Triton X-100 in PBS for 3 min at room temperature. After being washed with ice-cold PBS three times, cells were dropped onto polylysine-coated coverslips. Anti-Twi1p rabbit polyclonal antibody (1) was used at a 1:500 dilution for immunostaining. Rabbit anti-Pdd1p was provided by C. David Allis (The Rockefeller University, New York, NY) and used at a 1:100 dilution. Mouse anti-HA antibody (Sigma-Aldrich) was used at 1:300 for HA-CnjBp staining and at 1:1,000 for FLAG-HA-Wag1p staining. Mouse anti-V5 (Invitrogen) was used at a 1:300 dilution. Anti-H3K27me3 (ab5338; 1:500; Abcam) and anti-H3K9me3 (07-442; 1:25; Upstate) both were rabbit polyclonal antibodies. Secondary antibodies were fluorescein isothiocyanate anti-rabbit immunoglobulin G (1:150; Sigma) and Alexa Fluor 568 anti-mouse immunoglobulin G (1:500; Molecular Probes). Digital images were collected using an Olympus BH-2 fluorescence microscope or a Leica TCS SP5 confocal microscope.

Northern blotting and reverse transcription-PCR (RT-PCR) analysis. Total *T. thermophila* RNA was extracted using TRIzol reagent (Invitrogen). For the Northern blotting of *WAG1* and *CNJB* mRNA, 10 µg of total RNA per lane was loaded onto a formaldehyde-agarose denaturing gel. For the PCR amplification of the ~0.8-kb DNA fragment used as a *WAG1* probe, 5'-GCTAAGTTGTGA GTAATTGG-3' and 5'-CCATTGCCATTTGAAGAAC-3' primers and genomic DNA were utilized. For making the ~1.8-kb *CNJB* probe, 5'-TACGC TCCAATTGCATAATCAACTC-3' and 5'-CAAGTCTTCGCTTGATCTAT TTAC-3' primers were used.

For RT-PCR, total RNA (10 µg) from each time point was treated with DNase using a Turbo DNA-free kit (Ambion). Approximately 1 µg of RNA was used for the first-strand cDNA synthesis (SuperScript III reverse transcriptase; Invitrogen) using a poly(dT) primer. PCR primer sequences for *WAG1* were 5'-GTC AATTCCTTTGCGAACCT-3' and 5'-CAATAGCAGTTTCATAGTCT-3'. PCR primer sequences for *CNJB* were 5'-GGATCCAGATCTATCGATCTTG TATAACAGATGTCCC-3' and 5'-ATATCCCAGAGTGTGTTAGATGC-3'. One microgram of DNase-treated wild-type RNA (4 h postmixing) was used as a negative control. The Northern blotting of scnRNAs was performed as described previously (1), except that the carbodiimide cross-linking method (39) was used. Oligonucleotide sequence probes for Mi-9, Tlr1-1, and Mi-3 are listed in reference 1. Northern blotting signals were quantified using the Personal Molecular Imager system (Bio-Rad).

IES elimination assays. Mating pairs were deposited into drops of super proteose peptone medium at 10 h postmixing. A single exconjugant from each pair was lysed, and IES elimination assays were performed as described previously (1). Wild-type and Δ *CnjB* exconjugants completed their development and started to divide at 24 to 28 h postmixing, and the exconjugants were collected at 24 h postmixing. Since most Δ *WAG1* exconjugants and all the Δ *WAG1* Δ *CnjB* exconjugants did not undergo cellular division 24 h after being mixed, exconjugants were collected at 32 h postmixing to allow sufficient time for DNA elimination in case it was delayed.

Nucleotide sequence accession number. The *WAG1* cDNA sequence was deposited in GenBank under accession number FJ416495.

RESULTS

Two GW repeat-containing proteins associate with Twi1p.

In a recent report (1), we described the tandem affinity purification of FLAG-HA-tagged Twi1p from conjugating *Tetrahymena*

FIG. 1. Wag1p and CnjBp associate with Twi1p. (A) Schematic representation of Wag1p and CnjBp domains. Numbers indicate amino acid residue positions in the protein. GW, glycine/tryptophan repeat domains; Tex, Tex domain; Zn, Zn finger domain. (B and C) Western blot analysis of the proteins coimmunoprecipitated from mating cells containing the epitope-tagged proteins as indicated at the top of the panels. HA-CnjBp, Twi1p, and V5-Wag1p were detected by Western blotting as indicated on the right. (B) Anti-HA IP, proteins coimmunoprecipitating with HA-CnjBp. (C) Anti-V5 IP, proteins coimmunoprecipitating with V5-Wag1p.

mena cells and characterized a putative DExH box helicase, Ema1p, which copurified with Twi1p. Peptides corresponding to two other proteins were identified by mass spectrometry in the same affinity purified samples (see Fig. S1A and S2A in the supplementary material for amino acid sequences and matched peptides). One was a 1,127-amino acid (~123-kDa) protein, which corresponds to the THERM_00299870 gene product in the *Tetrahymena* Genome Database (www.ciliate.org). Since a substantial portion of its amino acid sequence (53%) is occupied by a GW-rich domain containing GW, WG, and GWG repeats, we called this protein Wag1p (for W and G motifs). The GW repeat domain of Wag1p (residues 156 to 758) is predicted to be unstructured, whereas its N-terminal and C-terminal portions are expected to contain helical regions (Fig. 1A; also see Fig. S1B in the supplemental material).

The second Twi1p-associated protein identified was a ~200-kDa protein encoded by THERM_01091290, or *CnjB* (29). In the NCBI database, this protein corresponds to *T. thermophila* Zinc knuckle family protein XP_001025435 (gi 146182859). The N-terminal region of CnjBp (Fig. 1A) contains a Tex domain similar to that found in transcription factors related to *Saccharomyces cerevisiae* Spt6 (11, 41). The C-terminal portion of CnjBp contains two regions that are similar to the GW repeat domain of Wag1p by sequence but shorter in length. Also, there are seven CCHC zinc fingers (46) located between the two GW-rich regions. The C-terminal CnjBp region, which comprises about a third of the protein sequence, is predicted to be unstructured (see Fig. S2B in the supplemental material). Apart from Wag1p and CnjBp, no other proteins with multiple GW repeats were found in the *T. thermophila* genome by similarity search. The closest heterologous proteins with similarity to Wag1p and CnjBp GW repeat domains are Nowa1p and Nowa2p from the ciliate *Paramecium tetraurelia*, which are

involved in IES elimination during developmentally regulated macronuclear genome rearrangement (38).

Wag1p and CnjBp coimmunoprecipitate with Twi1p and interact with each other. To analyze the interaction of CnjBp and Wag1p with Twi1p by reciprocal coimmunoprecipitation (co-IP), we made *T. thermophila* strains that expressed either N-terminally tagged V5-Wag1p or N-terminally tagged HA-CnjBp (see Fig. S3 in the supplemental material for diagrams of DNA constructs). The HA-CnjBp strain was mated with the V5-WAG1 strain, and whole-cell lysates were made 5 h after cell mixing, when Twi1p localizes to the parental Mac, and 9 h postmixing, when Twi1p is in the new Mac (34). In *T. thermophila* mating pairs, protein exchange between the two cells through the conjugation junction (31) should allow the formation of HA-CnjBp/V5-Wag1p complexes if these two proteins interact. Cell lysates made from each of the tagged strains mated with the wild-type cells were used as negative controls for V5-Wag1p IP or for HA-CnjBp IP, respectively.

When HA-CnjBp was immunoprecipitated with anti-HA agarose, Twi1p specifically copurified with it (Fig. 1B), whether the cell lysates were made at 5 h or at 9 h postmixing. Twi1p levels were comparable in all extracts used in this experiment (see Fig. S4 in the supplemental material). Western blotting using anti-V5 antibody revealed that V5-Wag1p coimmunoprecipitated with HA-CnjBp. Twi1p also coimmunoprecipitated with V5-Wag1p on anti-V5 agarose (Fig. 1C), whereas HA-CnjBp was enriched only twofold compared to the non-specific binding control. Thus, CnjBp and Wag1p interact with each other either directly or through the other proteins (e.g., Twi1p).

WAG1 and CnjB gene expression and protein localization. Northern blots of RNAs isolated from growing, starved, and conjugating cells (Fig. 2A) revealed that both *WAG1* and *CnjB* are upregulated early in conjugation (2 to 4 h postmixing), when cells form pairs and the Mic acquires a crescent shape (see reference 28 for *CnjB* expression analysis). *TWII* mRNA shows a similar expression pattern (34). RT-PCR analysis, which allows the detection of low levels of mRNA, indicates that *WAG1* and *CnjB* also are expressed in growing and starved cells (Fig. 2B). We tested whether we could detect Wag1p and CnjBp from growing and starved cells by IP and Western blotting. Approximately 10^8 growing or starved *T. thermophila* cells were required to detect very small amounts of HA-CnjBp, while the same amount of growing or starved cells was not sufficient for V5-Wag1p visualization (data not shown). Approximately 30 times less cell material was required to detect both proteins from the conjugating cells (data not shown). Thus, CnjBp is present throughout the life cycle, but it is not clear whether Wag1p is produced during growth and starvation.

During *T. thermophila* conjugation, gene expression switches from somatic (parental Mac) to zygotic (new Mac) starting ~7 to 8 h postmixing, when the new Mac begins developing. To test whether *WAG1* and *CnjB* are expressed both parentally and zygotically, we mated two strains in which all the macronuclear (somatic) copies of both genes were knocked out (see below), so that no *WAG1* or *CnjB* expression could occur from the parental Mac. No *WAG1*- or *CnjB*-specific RT-PCR products could be detected at 4 h in these somatic *WAG1Δ CnjBΔ* (designated somΔ*WAG1*somΔ*CnjB*) mutants (Fig. 2B). How-

FIG. 2. *WAG1* and *CnjB* expression and localization during conjugation. (A) *WAG1* and *CnjB* mRNA levels analyzed by Northern blotting. (B) RT-PCR analysis of *WAG1* and *CnjB* expression. WT, wild-type; *somΔWAG1 somΔCnjB*, a mutant strain in which all of the somatic copies of both genes were deleted. (C to H) HA-Wag1p localization; (I to N) HA-CnjBp localization. In each experiment, a wild-type strain was mated to either a FLAG-HA-*WAG1* or HA-*CnjB* strain; immunofluorescence staining in both cells is due to the transfer of the tagged proteins between the conjugants. The cell stages are early micronuclear crescent (C and I), late crescent stages of micronuclear meiotic prophase (D and J), pronuclear exchange and fusion (E and K), postzygotic mitosis (F and L), development of the new macronucleus (G and M), and pair separation (H and N). Mics can be distinguished from the Macs by their smaller size (C, E to I, and K to N) or by their elongated shape (C, D, I, and J). In panels G to H and M to N, arrows and asterisks indicate old/parental Macs and new Macs, respectively.

ever, in both cases, RT-PCR products appeared at 10 h post-mixing, presumably because the newly developed Macs expressed wild-type copies of *WAG1* and *CnjB* inherited from the Mics. Thus, *WAG1* and *CnjB* are expressed from the parental as well as the zygotic Macs.

We used N-terminally tagged FLAG-HA-*WAG1* and HA-*CnjB* strains to localize tagged Wag1p and CnjBp during conjugation by indirect immunofluorescence. Neither protein was

detected using anti-HA antibody in log-phase or starved cells (data not shown). FLAG-HA-Wag1p was detected in the parental Mac starting from the pair formation stage (Fig. 2C) through the micronuclear crescent stage (Fig. 2D), micronuclear meiosis, fertilization (Fig. 2E), and postzygotic mitosis (Fig. 2F). FLAG-HA-Wag1p staining was lost in the parental Macs and appeared in the new Macs when the new Macs developed (Fig. 2G). Thus, FLAG-HA-Wag1p localization is similar to that of its interacting partner, Twi1p (34). However, HA-Twi1p was reported to disappear from the new Macs at the pair separation stage (34), which occurs at ~12 h of conjugation, whereas FLAG-HA-Wag1p staining did not decrease until 18 h of conjugation, and it was detectable at a low level in most separated cells between 18 and 24 h (Fig. 2H and data not shown). V5-Wag1p localization exhibited the same pattern (data not shown).

HA-tagged CnjBp was first detected at the pair formation and crescent stages and localized to both Mics and Macs (Fig. 2I and J). Micronuclear fluorescence decreased during meiotic divisions and disappeared by the end of the second meiosis (Fig. 2K). Similarly to HA-Wag1p and Twi1p, HA-CnjBp disappeared from the parental Macs (Fig. 2L) and appeared in the new Macs (Fig. 2M and N) upon macronuclear development. HA-CnjBp staining disappeared from the new Macs at or just after the pair separation stage (12 h postmixing) (Fig. 2N and data not shown). Since the strains used in this study contained FLAG-HA-*WAG1* or HA-*CnjB* in the Macs and wild-type copies of the genes in the Mics, the localization of the zygotically expressed *WAG1* and *CnjB* gene products could not be investigated.

Phenotypes of the $\Delta WAG1$, $\Delta CnjB$, and $\Delta WAG1 \Delta CnjB$ knockout strains. Since Wag1p and CnjBp interact with Twi1p and *WAG1* and *CnjB* genes are highly upregulated in conjugating cells, we expected these two proteins to have a function in conjugation, probably in Twi1p/scnRNA-mediated DNA elimination. To study the function of *WAG1* and *CnjB* gene products, we made knockout strains (see the materials and methods in the supplemental material for details) by disrupting these genes with the *neo3* cassette (43), which confers resistance to paromomycin.

Two *WAG1* knockout homozygous homokaryon strains of different mating types were made by replacing the entire *WAG1* coding region with *neo3* (see Fig. S5 in the supplemental material). In these strains, both micronuclear copies and all of the macronuclear copies of *WAG1* were deleted, and no *WAG1* sequence could be detected by Southern blotting (see Fig. S5 in the supplemental material) or by PCR (data not shown). Thus, in $\Delta WAG1$ strains, no *WAG1* expression can occur either from the parental Mac early in conjugation or from the new Macs late in conjugation.

$\Delta WAG1$ cells grew normally and, upon starvation and mixing, formed pairs and proceeded through conjugation. Similarly to wild-type cells, all $\Delta WAG1$ cells underwent the transition from the 2 Mic/2 Mac stage to the 1 Mic/2 Mac stage at the end of conjugation (16 to 24 h) (Fig. 3A). To test whether $\Delta WAG1$ strains give rise to viable progeny, we isolated single pairs of conjugating $\Delta WAG1$ cells into drops of medium. We found that most (98 to 99%) of the $\Delta WAG1$ exconjugants did not undergo micronuclear and cellular division upon feeding and eventually died. Only 1 to 2% of the $\Delta WAG1$ progeny,

FIG. 3. Phenotypes of $\Delta WAG1$, $\Delta CnjB$, and $\Delta WAG1 \Delta CnjB$ strains. (A) Schematic representation of the final stages of conjugation. After cell separation and old Mac elimination, *Tetrahymena* species contain two Mics and two Macs. In the wild-type, $\Delta CnjB$, and 1 to 2% of $\Delta WAG1$ cells, one Mic is resorbed and the remaining Mic undergoes mitosis only after feeding. By 24 h postmixing, two live progeny cells are obtained from each pair member. $\Delta TWI1$ and $\Delta WAG1 \Delta CnjB$ cells fail to resorb one Mic, whereas the majority of the $\Delta WAG1$ progeny arrest at the 1 Mic/2 Mac stage. (B) Progeny viability from $\Delta WAG1$ matings. Wild-type B2086 and CU428 strains were used as controls. (C) $\Delta WAG1$ cells have normal Mics, whereas $\Delta WAG1$ progeny acquire small Mics after ~60 fissions. Arrows indicate Mics. (D) Progeny viability from $\Delta CnjB$ and $\Delta WAG1 \Delta CnjB$ matings.

which were selected by paromomycin resistance (see the materials and methods in the supplemental material for details), survived, compared to 66% viability for the wild-type progeny (Fig. 3B). The surviving $\Delta WAG1$ progeny showed defects during vegetative growth: upon microscopic examination, we observed abnormal cells in which cell division occurred prior to the completion of the micronuclear and/or macronuclear division (see Fig. S6 in the supplemental material). After ~60 cell fissions, all $\Delta WAG1$ progeny had small Mics (Fig. 3C). Similar phenotypes were not observed in growing $\Delta WAG1$ somatic knockout strains. Thus, $WAG1$ expression during conjugation is essential for the production of normal progeny.

When a $\Delta WAG1$ strain was mated to a wild-type strain, wild-type levels of normal progeny were obtained (Fig. 3B), indicating that, in each hybrid pair, the wild-type cell provided sufficient $Wag1p$ to compensate for the loss of $Wag1p$ in the mutant pair member. Similar results were obtained when either the FLAG-HA- $WAG1$ or V5- $WAG1$ strain was used in

place of the wild-type strain in a similar experiment (Fig. 3B), indicating that neither tag affected $Wag1p$ function.

Homozygous homokaryon $\Delta CnjB$ strains of two different mating types were generated by replacing the N-terminal portion of the *CnjB* coding region with a *neo3* cassette (see Fig. S7 in the supplemental material). In growing $\Delta CnjB$ cultures, some cells (10 to 50%) had Mics that looked smaller than the wild-type Mics. Due to the micronuclear defects, many, but not all, conjugating $\Delta CnjB$ cells aborted development prior to new Mac formation: mating partners separated at 6 to 8 h postmixing, retaining their parental Macs. We isolated single-cell $\Delta CnjB$ clones with small Mics and used a genomic exclusion cross with a wild-type strain to obtain cells with normal-size, rejuvenated Mics (16). These rejuvenated cells were not able to maintain their normal Mics during vegetative growth: after ~20 to 60 fissions, we could observe cells with small Mics (data not shown). These results suggest that vegetatively expressed *CnjBp* is important for micronuclear chromosome maintenance. In contrast to $\Delta CnjB$ strains, HA-*CnjB* cells did not show any micronuclear abnormalities, indicating that HA-*CnjBp* was functional.

For the analysis of *CnjBp* function in conjugating cells, we isolated single cells from growing $\Delta CnjB$ cultures and selected subcultures in which most Mics looked normal. These strains, in which at least 90% of the pairs developed new Macs upon mating, were used in all subsequent experiments. By isolating single pairs into drops of medium, we found that $\Delta CnjB$ cells produced viable progeny at the wild-type level (Fig. 3D). Since $\Delta CnjB$ progeny could not be distinguished from the parental $\Delta CnjB$ cells by antibiotic resistance, we used two methods to confirm that mating $\Delta CnjB$ cells actually completed conjugation instead of aborting their development upon feeding. First, cells from all drops were tested for their ability to mate with each other. This test is based on the fact that newly generated *T. thermophila* progeny are sexually immature, and ~60 cell fissions are required for them to become competent for mating. Indeed, all cell colonies tested in our experiment were unable to form pairs when mixed with each other, but they could pair after they matured. Second, we isolated one exconjugant from each of the 12 drops at 24 h postmixing and examined it cytologically, while the other exconjugant was allowed to divide and form a clonal line. In all 12 cases, we observed cells that had developed two new Macs. Therefore, the data presented in Fig. 3D represent the survival rate for true progeny. We conclude that *CnjBp* expression in conjugating cells is not required for progeny viability.

Our analysis indicated that the developmental effects of *WAG1* or *CnjB* deletion were not as strong as those of a *TW11* knockout: $\Delta TW11$ progeny arrest at the 2 Mic/2 Mac stage and do not survive (34). We reasoned that deleting both *WAG1* and *CnjB* genes might have a stronger impact on *Tetrahymena* development than deleting each gene separately. Hence, we made strains in which all of the micronuclear and macronuclear copies of both genes were replaced by *neo3*. Similarly to $\Delta CnjB$ cells, some of these $\Delta WAG1 \Delta CnjB$ cells had small Mics and aborted development upon mating. Therefore, we isolated single-cell $\Delta WAG1 \Delta CnjB$ subcultures that did not display these abnormalities. Upon mating, $\Delta WAG1 \Delta CnjB$ strains gave rise to progeny that arrested at the 2 Mic/2 Mac stage, persisted at this stage up to 48 h postmixing, and even-

tually died (Fig. 3A). No viable $\Delta WAG1 \Delta CnjB$ progeny were obtained (Fig. 3D).

Since Wag1p and CnjBp interact with Twi1p, we tested whether the absence of these two proteins in $\Delta WAG1 \Delta CnjB$ cells leads to mislocalization of Twi1p. Immunofluorescence with anti-Twi1p antibody revealed that Twi1p localized to the parental Mac at early stages of conjugation and then to the newly formed Macs in both wild-type and $\Delta WAG1 \Delta CnjB$ cells (see Fig. S8 in the supplemental material). Thus, the nuclear localization of Twi1p is not dependent on GW repeat proteins.

Downregulation of specific scnRNAs is inhibited in $\Delta WAG1 \Delta CnjB$ cells. In conjugating *T. thermophila* cells, scnRNAs of ~28-nt appear at approximately 2 h postmixing and persist until the end of conjugation (34). scnRNA levels are greatly reduced in *TWI1* knockouts, presumably because scnRNAs need to be complexed with Twi1p to be protected from degradation (34). We used the ethidium bromide staining of RNA separated on denaturing polyacrylamide-urea gels (34) to demonstrate that bulk scnRNA levels were not affected significantly in any of the knockout strains used in this study (see Fig. S9 in the supplemental material). However, the levels of specific scnRNAs (Mi-9, Tlr1-1, and others) can vary at different time points and between different mutant strains, as was recently demonstrated by Northern hybridization using different 50-base oligonucleotides as probes (1). Mi-9 is a 50-nt DNA sequence that corresponds to a part of an ~190-bp MI repeat present in multiple copies in the somatic genome and in an ~0.9-kbp M element IES (1). Levels of Mi-9-specific scnRNAs have been shown to drop significantly in wild-type cells between 4 and 6 h postmixing (1). This is consistent with the scanning model, according to which scnRNAs homologous to sequences in the parental Mac are reduced or eliminated from the scnRNA pool (34, 35). In contrast, scnRNAs detected by hybridization with the Tlr1-1 IES-specific Tlr1-1 probe persist from 2 to 12 h in conjugation, presumably because they are not scanned against parental genome sequences (1).

We quantified Mi-9-specific and Tlr1-1-specific scnRNA levels at different time points in wild-type cells using a carbodiimide-mediated RNA cross-linking method that allows the sensitive detection of small RNAs (39). Using this procedure, we also were able to detect scnRNAs homologous to a 50-nt Mi-3 probe, which corresponds to a germ line-specific portion of the M element IES outside the MI repeat (1). scnRNA levels peaked at 4 h postmixing for all three probes used, and scnRNA levels at other time points were calculated as a percentage of the value at 4 h (Fig. 4A and B). We observed a significant difference between the levels of Mi-9-specific scnRNAs and IES (Tlr1-1 and Mi-3)-specific scnRNAs between 4 and 8 h postmixing, when the Twi1p/scnRNA complex is in the parental Mac (Fig. 4A and B). Mi-9-specific scnRNA levels decreased nearly to background levels by 8 h postmixing (Fig. 4A and B). Interestingly, Tlr1-1- and Mi-3-specific scnRNA levels also declined, although only by about 40%, between 4 and 8 h (Fig. 4B). By searching the *Tetrahymena* macronuclear genome using Tlr1-1 and Mi-3 sequences as queries, we were able to identify 28-nt sequence stretches that were at least 80% identical to Tlr1-1 and Mi-3. Therefore, an ~40% decrease in the levels of Tlr1-1- and Mi-3-specific scnRNAs also could be explained by the elimination of scnRNAs that have some homology to these parental Mac genome sequences.

FIG. 4. Northern blot analysis of scnRNAs. (A) Northern hybridization of scnRNAs from the wild-type cells using Mi-9, Tlr1-1, and Mi-3 oligonucleotides as probes. RNA was isolated at different time points postmixing. (B) Quantitation of the wild-type scnRNA levels using Mi-9, Tlr1-1, and Mi-3 probes. (C) Northern hybridization of scnRNAs from $\Delta WAG1$, $\Delta CnjB$, and $\Delta WAG1 \Delta CnjB$ strains using Mi-9 as a probe. (D) Quantitation of the Northern blots using an Mi-9 probe. (E) Quantitation of the Northern blots using a Tlr1-1 probe. (F) Quantitation of the Northern blots using an Mi-3 probe. For every Northern blot, the signal at 4 h postmixing was chosen to be 100%, and the relative signals at different times were determined. Error bars represent the standard deviations of duplicate measurements.

We next tested whether the relative levels of specific scnRNAs are altered in the absence of GW repeat protein expression. In $\Delta WAG1$ and $\Delta CnjB$ single knockouts, Mi-9 scnRNA levels declined at a rate similar to that in the wild-type strain (Fig. 4C and D). Significantly, in $\Delta WAG1 \Delta CnjB$ cells the levels of Mi-9-specific scnRNAs decreased from 100% to only ~60% between 4 and 8 h (Fig. 4C and D).

Northern hybridization using Tlr1-1 as a probe revealed that in $\Delta WAG1 \Delta CnjB$ conjugating cells, Tlr1-1-specific scnRNA levels do not decline significantly between 4 and 8 h, when Twi1p/scnRNA is in the parental Mac (Fig. 4E; also see Fig. S10 in the supplemental material for the Northern blot). However, Tlr1-1 scnRNAs in $\Delta WAG1 \Delta CnjB$ knockouts were downregulated between 8 and 12 h, when Twi1p/scnRNA is present in the new Mac (Fig. 4E). This delay in Tlr1-1 level decline is not due to a delay

in progression through the conjugation stages in $\Delta WAG1 \Delta CnjB$ strains, as all cells used in our experiments were examined cytologically and found to develop new Macs at the same time (data not shown). The reasons for the Tlr1-1 scnRNA decrease between 8 and 12 h, which occurs in the wild-type as well as mutant cells, are unknown. This could reflect the failure of certain Twi1p/scnRNA complexes to be transferred from the parental to the new Macs, the instability of scnRNAs in the new Macs, the degradation of scnRNAs after they served as guides for histone modifications, or their extension by RNA-directed RNA polymerase (17).

Mi-3-specific scnRNA levels did not decrease significantly between 4 and 12 h in $\Delta WAG1 \Delta CnjB$ cells (Fig. 4F; also see Fig. S10 in the supplemental material). Thus, the downregulation of three different scnRNAs (Mi-9, Tlr1-1, and Mi-3) was inhibited in $\Delta WAG1 \Delta CnjB$ cells compared to their levels in the wild-type, $\Delta WAG1$, and $\Delta CnjB$ cells.

Twi1p interacts with the ncRNA transcripts in $\Delta WAG1 \Delta CnjB$ knockouts. In *Tetrahymena* cells lacking *EMA1*, a gene encoding a putative RNA helicase, Mi-9 scnRNA levels do not decrease as much after 4 h postmixing as they do in the wild-type cells (1). Ema1p is associated with Twi1p and is required for the interaction of Twi1p/scnRNA complexes with the non-coding RNAs (ncRNAs) transcribed in the parental Mac between 4 and 8 h postmixing and in the new Mac between 8 and 12 h postmixing (1). It was proposed that the interaction of Twi1p/scnRNA with the complementary transcripts in the parental Mac is a basis for scnRNA scanning against parental Mac sequences. In $\Delta EMA1$ cells, the lack of Mi-9 scnRNA downregulation could be due to the inability of Twi1p/scnRNA to interact with homologous ncRNAs (1). The interaction of Twi1p/scnRNA complexes with the complementary ncRNAs in the new Mac also could serve to specify DNA sequences to be eliminated during macronuclear development. To test whether Wag1p and CnjBp are required for the interaction of Twi1p with ncRNAs, we immunoprecipitated Twi1p from conjugating wild-type and $\Delta WAG1 \Delta CnjB$ knockouts lysed at either 5 or 9 h postmixing, using anti-Twi1p antibody as described previously (1). To detect ncRNAs transcribed in the parental Mac, RT-PCR was performed using primers specific for the M element or Tlr1-1 element MDS (Fig. 5A). We found that MDS-specific transcripts coimmunoprecipitated with Twi1p at 5 h postmixing in $\Delta WAG1 \Delta CnjB$ knockouts, although smaller amounts of ncRNA were present in a $\Delta WAG1 \Delta CnjB$ IP fraction than in the wild-type IP fraction (Fig. 5A). Primers complementary to the M element or Tlr1-1 IES were used to detect ncRNAs transcribed in the new Mac at 9 h postmixing (Fig. 5B). Similar amounts of IES-specific ncRNAs coimmunoprecipitated with Twi1p in the wild type and in $\Delta WAG1 \Delta CnjB$ cells (Fig. 5B). These results indicate that in $\Delta WAG1 \Delta CnjB$ cells, the interaction between Twi1p/scnRNA complexes and ncRNAs is inhibited in the parental Mac but not in the new Mac.

IES elimination is inhibited in $\Delta WAG1 \Delta CnjB$ double knockout cells. In conjugating *T. thermophila* cells, developmentally programmed DNA rearrangement occurs at 14 to 16 h postmixing. *Twil* knockouts are deficient in DNA elimination: all four of the IESs tested, namely the Tlr1 element, the Cal element, and the closely linked M and R elements, are not processed in mating $\Delta TWII$ cells (1, 34). In addition, chromosome breakage/telomere formation is partially inhibited in *Twil* knockouts, as

FIG. 5. Co-IP of ncRNA with Twi1p. Wild-type (W) or $\Delta WAG1 \Delta CnjB$ (Δ) conjugating cells were lysed at either 5 or 9 h postmixing, and Twi1p was immunoprecipitated with anti-Twi1p antibody attached to protein A agarose. Protein A agarose beads with no anti-Twi1p antibody were used as a negative control. Input, RNA isolated from crude lysates. (A) IP using lysates at 5 h. ncRNAs were detected by RT-PCR using primers specific for M element MDS or the Tlr1-1 element MDS. (B) IP using lysates at 9 h. ncRNAs were detected by RT-PCR using primers complementary to the M element or Tlr1-1 element IES.

determined using the Tt819 DNA sequence (34). We used the four previously characterized IESs and the Tt819 site to determine whether DNA rearrangement is affected in conjugating $\Delta WAG1$, $\Delta CnjB$, and $\Delta WAG1 \Delta CnjB$ cells.

Mating pairs were isolated into drops of medium at 10 h postmixing. Exconjugants were collected at 24 h postmixing or at later time points (see Materials and Methods) and analyzed by single-cell PCR using primers flanking the excised sequences (see Fig. S11 in the supplemental material for detailed M element analysis). We failed to detect any defects in DNA rearrangement in $\Delta WAG1$ and $\Delta CnjB$ mating cells (Table 1). All 41 $\Delta WAG1 \Delta CnjB$ exconjugants tested contained processed forms of the Cal element. In contrast, M, R, Tlr1, and Tt819 element rearrangements occurred in only some $\Delta WAG1 \Delta CnjB$ cells (Table 1). Thus, DNA rearrangement is inhibited but not abolished in the developing macronuclei of $\Delta WAG1 \Delta CnjB$ cells, and the degree of such inhibition may be sequence and/or position specific.

DNA elimination structures are not formed in $\Delta WAG1 \Delta CnjB$ cells. *Tetrahymena* genome rearrangement is associated with heterochromatic DNA elimination bodies, which appear at the periphery of the new developing Macs at ~14 h postmixing (24, 26). We used antibodies specific for H3K27me3, H3K9me3, or Pdd1p to test whether DNA elimination structures are formed in GW repeat mutants. We were able to visualize doughnut-shaped structures in the wild-type, $\Delta WAG1$, and $\Delta CnjB$ cells (Fig. 6). In $\Delta WAG1 \Delta CnjB$ cells, histone H3K9me3- and H3K27me3-specific staining was present in the developing Macs, but the characteristic doughnuts were never formed (Fig. 6A and B), and the staining disappeared by 16 h postmixing in most cells (data not shown). Pdd1p also was found in the Macs of the double knockouts, mostly at the nuclear periphery (Fig. 6C). This is different from developing Macs in *Twil* knockout cells, where histone H3 modified by K9me3 or K27me3 cannot be detected and Pdd1p forms large aggregates (23, 24).

Wag1p is found in nuclear foci that are distinct from DNA elimination structures. Argonaute family and GW repeat pro-

TABLE 1. PCR analysis of IES excision and chromosome breakage/telomere addition in $\Delta WAG1$, $\Delta CnjB$, and $\Delta WAG1 \Delta CnjB$ mating cells

Sequence element and function	$\Delta WAG1$			$\Delta CnjB$			$\Delta WAG1 \Delta CnjB$		
	Re ^a	NRe ^b	%Re ^c	Re	NRe	%Re	Re	NRe	%Re
IES excision									
M	36	0	100	36	0	100	5	50	9
R	34	0	100	38	0	100	6	30	17
Cal	46	0	100	31	0	100	41	0	100
Tlr1	29	0	100	35	0	100	10	28	26
Chromosome breakage/telomere addition									
Tt819	16	0	100	16	0	100	6	10	38

^a Re, the number of cells containing rearranged DNA elements.
^b NRe, the number of cells in which only the unrearranged forms of the same elements were detected.
^c %Re, percentage of cells containing rearranged elements.

teins often are associated with specific nuclear or cytoplasmic bodies. For example, four mammalian Argonaute proteins (Ago1 through Ago4) and GW repeat protein GW182 are localized to the cytoplasmic P bodies (22, 42). AGO4 protein, a key effector in

RNA-directed DNA methylation in *Arabidopsis thaliana*, colocalizes either with Cajal bodies (21) or with GW repeat-containing protein NRPE1 in AB bodies (20). Therefore, we analyzed the subnuclear localization of Twi1p, Wag1p, and CnjBp in the developing Macs in *Tetrahymena* species.

We utilized somatically tagged FLAG-HA-*WAG1* and HA-*CnjB* strains to examine the localization of GW repeat proteins as well as Twi1p in paired (11 h postmixing) and in separated (14 to 16 h) cells. Using anti-Twi1p and anti-HA antibodies, we found that Twi1p and HA-CnjBp were diffusely distributed throughout the nucleoplasm in developing Macs (Fig. 7A) until they disappeared shortly after cell separation (data not shown).

We next studied the localization of FLAG-HA-Wag1p. In paired *T. thermophila* cells, FLAG-HA-Wag1p-specific staining exhibited a diffuse pattern, and H3K27me3 staining was not condensed into any distinct structures at this conjugation time point (Fig. 7B). Upon cell separation and prior to the appearance of DNA elimination structures, FLAG-HA-Wag1p started to form speckles, many of which were found at the macronuclear periphery (Fig. 7C). At this time, H3K27me3 staining was clustered closer to the macronuclear periphery as well. However, FLAG-HA-Wag1p and H3K27me3 did not colocalize (Fig. 7C). At the time of DNA elimination, FLAG-HA-Wag1p was found in multiple nuclear granules, which were distinct from H3K27me3-containing structures (Fig. 7D). Similarly, little or no overlap between DNA elimination structures and FLAG-HA-Wag1p-containing granules was observed when anti-Pdd1p antibody was used to visualize DNA elimination doughnuts (Fig. 7E). FLAG-HA-Wag1p also did not colocalize with the nucleolar marker fibrillarin (9), which was concentrated at the periphery of the new Macs at the 2 Mic/2 Mac (see Fig. S12A in the supplemental material) and 1 Mic/2 Mac (see Fig. S12B in the supplemental material) stages. Thus, Wag1p-containing macronuclear bodies constitute novel conjugation-specific macronuclear structures that are distinct from both DNA elimination structures and nucleoli.

FIG. 6. DNA elimination structures are not formed in $\Delta WAG1 \Delta CnjB$ conjugating cells. Wild-type (WT), $\Delta WAG1$, $\Delta CnjB$, or $\Delta WAG1 \Delta CnjB$ cells were fixed at 14 h postmixing and immunostained with anti-H3K27me3, anti-H3K9me3, or anti-Pdd1p antibody (shown in green), and DNA was counterstained with 4',6'-diamidino-2-phenylindole (DAPI) (blue). Confocal images are shown. The cells are at the 2 Mic/2 Mac stage, except one $\Delta WAG1 \Delta CnjB$ cell stained with anti-H3K9me3, which also contains an old Mac. Bar, 5 μ m.

DISCUSSION

In *T. thermophila*, DNA sequences to be eliminated from the developing macronuclear genome likely are determined by their homology to scnRNAs and targeted by a Twi1p-dependent pathway, similarly to Argonaute-dependent heterochromatic silencing in other organisms. The detailed mechanism by

FIG. 7. Localization of Twi1p, HA-CnjBp, H3K27me3, HA-Wag1p, and Pdd1p in developing macronuclei at the indicated times postmixing. Cells were stained with anti-Twi1p (shown in green) plus anti-HA (red) (A), anti-H3K27me3 (green) plus anti-HA (red) (B to D), or anti-Pdd1p (green) plus anti-HA (red) (E) and counterstained with DAPI (blue). Confocal images are shown. Asterisks indicate old Macs in paired (A and B) or separated (C to E) cells. Bar, 5 μ m.

which scnRNAs acquire their specificity for IES sequences in the parental Mac and then target these DNA sequences for elimination in the new Mac is not understood. Here, we identify two GW repeat proteins, Wag1p and CnjBp, that associate with Twi1p and function in *T. thermophila* DNA elimination, possibly by regulating the interactions between Twi1p/scnRNA complexes and other proteins/nucleic acids.

We detected the interaction of Wag1p and CnjBp with Twi1p at 5 h postmixing, when all three proteins colocalize in the parental Mac. At this time in conjugation, scnRNAs become enriched in IES-specific sequences, most likely by scanning against parental macronuclear genomic sequences, which likely occurs by the interaction of Twi1p/scnRNA complexes with ncRNAs (1, 34, 35). We demonstrated that the interaction between Twi1p/scnRNA complexes and ncRNAs is inhibited in parental Macs of double $\Delta WAG1 \Delta CnjB$ knockouts (Fig. 5). However, not all of the scnRNA quantitation results with the

wild-type and $\Delta WAG1 \Delta CnjB$ cells (Fig. 4) can be explained solely by the existing scanning model. For example, we found that scnRNA levels decrease between 4 and 8 h postmixing not only in the case of MDS-specific Mi-9 scnRNAs ($\sim 80\%$ reduction) but also in the case of IES-specific Tlr1-1 and Mi-3 scnRNAs ($\sim 40\%$ reduction). This observation could be explained by the scanning model by assuming that some of the scnRNAs detected by Tlr1-1- and Mi-3-specific probes are eliminated because of their homology to the parental Mac sequences. Also, according to the scanning model, the accumulation and reduction profiles of the IES-specific and MDS-specific scnRNAs should be similar in mutants characterized by weakened interaction between Twi1p/scnRNA complexes and ncRNAs. Surprisingly, although this interaction is weakened in conjugants at 5 h in double $\Delta WAG1 \Delta CnjB$ knockouts, we observe an even greater stabilization of Tlr1-1- and Mi-3-specific scnRNA levels (little or no downregulation between 4

and 8 h) than in the Mi-9 scnRNA profile (~60% reduction between 4 and 8 h) (Fig. 4D to F). This could be explained by different degradation rates of specific scnRNAs if Twi1p/scnRNA complexes are stabilized in $\Delta WAG1 \Delta CnjB$ cells. Alternatively, different scnRNAs could undergo selective secondary amplification in $\Delta WAG1 \Delta CnjB$ knockouts. Another possibility is that Wag1p and CnjBp promote interaction between ncRNAs and imperfectly matched scnRNAs. In the absence of these two GW repeat proteins, scnRNAs that are well matched with their targets (e.g., Mi-9) might be preferentially eliminated from the scnRNA pool, whereas scnRNAs having weak homology to the parental Mac sequences (such as some of the Tlr1-1 and Mi-3 scnRNAs) are not degraded. Clearly, there are aspects of the regulation of scnRNA biosynthesis and accumulation that remain to be elucidated in order to distinguish among these possibilities.

Upon macronuclear differentiation, which starts at 7 to 8 h postmixing, Twi1p/scnRNA, Wag1p, and CnjBp localize to the new Mac. Wag1p and CnjBp form complexes with Twi1p at 9 h postmixing, and these interactions must be important for subsequent genome rearrangement, since the formation of DNA elimination structures, IES processing, and chromosome breakage are severely inhibited in $\Delta WAG1 \Delta CnjB$ knockouts (Fig. 6 and Table 1). We were able to rule out the possibility that Wag1p and CnjBp are required for the recruitment of Twi1p/scnRNA complexes to the IES-specific ncRNAs (Fig. 5). Hence, these two GW repeat proteins could participate in downstream events, such as the recruitment of the Ezl1p complexes or the formation of DNA elimination structures.

It generally is assumed that developmental arrest and progeny lethality caused by mutations in the *T. thermophila* DNA elimination pathway are due to the failure to complete genome rearrangement. $\Delta WAG1 \Delta CnjB$ mating cells arrest at the 2 Mic/2 Mac stage, which is typical of other *T. thermophila* mutants defective in genome rearrangement ($\Delta TWI1$, $\Delta PDD1$, $\Delta EMA1$, $\Delta EZL1$, and others). Conjugating $\Delta CnjB$ mutants were able to excise four different IESs, process a Tt819 element, and generate viable progeny (Table 1 and Fig. 3D). However, arrest at the 1 Mic/2 Mac stage in $\Delta WAG1$ knockouts, which form DNA elimination structures and rearrange DNA, is puzzling. One possibility is that $\Delta WAG1$ cells display defects in the rearrangement of IESs or chromosome breakage elements that were not tested in our single-cell PCR assays. Alternatively, the developmental arrest and lethality of $\Delta WAG1$ progeny may be due to other Wag1p functions that are not related to macronuclear genome reorganization (see below).

The examination of GW repeat protein expression and localization suggests Twi1p-independent functions for Wag1p and CnjBp. First, low levels of *WAG1* and *CnjB* transcripts are detected in vegetative and starved cells by RT-PCR (Fig. 2B), whereas *TWI1* expression is conjugation specific (34). While growing $\Delta WAG1$ cells do not exhibit any obvious phenotype, growing $\Delta CnjB$ cells show a high frequency of cells with small, defective Mics, raising the possibility that CnjBp is involved in micronuclear chromosome maintenance. Early in conjugation (micronuclear crescent stages), when scnRNA precursors are transcribed and diced by Dcl1p, CnjBp colocalizes with Dcl1p in the Mic (Fig. 2I and J) (27, 36). Considering the presence of nucleic acid binding domains in CnjBp (Fig. 1A), it is tempting

to speculate that the micronuclear function of CnjBp is related to scnRNA formation and/or export from the Mic prior to Twi1p/scnRNA complex assembly. However, any function CnjBp performs in the Mic is not essential for IES elimination or for the successful completion of conjugation.

Wag1p localization in macronuclear speckles (Fig. 7C to E) suggests a Twi1p-independent function in late development after pair separation (~12 h postmixing). The appearance of these Wag1 bodies is accompanied by Twi1p disappearance from the new Mac and the formation of nucleoli, which are marked by fibrillarin and Nopp52 (32, 44). DNA elimination structures and Nopp52 do not colocalize in the new Macs (44), and we show that Wag1p bodies do not colocalize with nucleoli or with DNA elimination structures (Fig. 7; also see Fig. S12 in the supplemental material). Thus, Wag1p macronuclear bodies represent a novel class of macronuclear structures in conjugating *T. thermophila*. Since Twi1p is only 1 of 12 *T. thermophila* Argonaute family proteins, it will be interesting to test whether any other Argonautes are able to interact with the GW-rich domain of Wag1p and localize to the Wag1p-containing bodies in the developing Macs.

Recent studies provide other examples of GW repeat proteins involved in RNAi-mediated processes. Metazoan GW182 family members, which interact with Argonaute-containing miRISCs (microRNA-induced silencing complex), localize to cytoplasmic RNA-processing bodies (P bodies), and play an essential role in microRNA-mediated gene silencing (10). In *A. thaliana*, two proteins involved in RNA-directed DNA methylation, RNA polymerase V subunit NRPE1 and the KOW domain-containing protein KTF1, interact with the Argonaute family protein AGO4 through their GW repeat domains (2, 12, 14, 21, 30). Although *Schizosaccharomyces pombe* RNA-induced transcriptional silencing complex component Tas3 (49) contains only a couple of WG motifs within its ~70-residue region homologous to a human GW family protein TNRC6B, mutating one of these motifs is sufficient to abolish the interaction of Tas3 with Ago1 (40, 48). The results of domain swapping between *A. thaliana* NRPD1b and human GW182 (12, 47) argue that the interaction between GW-rich domains and Argonaute proteins is evolutionarily conserved. This idea is supported by the identification of Wag1p and CnjBp, two *T. thermophila* GW repeat proteins that interact with Twi1p and function in small RNA-dependent DNA elimination. In another ciliate, *P. tetraurelia*, two RNA binding GW repeat proteins, Nowa1p and Nowa2p, also have been shown to be involved in epigenetically regulated genome rearrangements (38). It would be interesting to determine whether Nowa proteins interact with *P. tetraurelia* Argonaute(s) and whether they are involved in small RNA scanning (19) in this organism.

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