An essential role for the DNA breakage-repair protein Ku80 in programmed DNA rearrangements in *Tetrahymena thermophila*

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ABSTRACT Programmed DNA rearrangements are important processes present in many organisms. In the ciliated protozoan *Tetrahymena thermophila*, DNA rearrangements occur during the sexual conjugation process and lead to the deletion of thousands of specific DNA segments and fragmentation of the chromosomes. In this study, we found that the *Ku80* homologue, a conserved component of the nonhomologous end-joining process of DNA repair, was essential for these two processes. During conjugation, *TKU80* was highly expressed and localized to the new macronucleus, where DNA rearrangements occur. Homokaryon *TKU80*-knockout mutants are unable to complete conjugation and produce progeny and are arrested at the two-micronuclei/two-macronuclei stage. Analysis of their DNA revealed failure to complete DNA deletion. However, the DNA-cutting step appeared to have occurred, as evidenced by the presence of circularized excised DNA. Moreover, chromosome breakage or de novo telomere addition was affected. The mutant appears to accumulate free DNA ends detectable by terminal deoxynucleotidyl transferase dUTP nick end labeling assays that led to the degradation of most DNA in the developing macronucleus. These findings suggest that Tku80p may serve an end-protective role after DNA cleavage has occurred. Unexpectedly, the large heterochromatin structures that normally associate with DNA rearrangements failed to form without *TKU80*. Together the results suggest multiple roles for Tku80p and indicate that a Ku-dependent DNA-repair pathway is involved in programmed DNA rearrangements in *Tetrahymena*.

INTRODUCTION

Programmed DNA rearrangements have been found in both prokaryotes and eukaryotes. Through the alteration of specific genomic sequences, they have crucial influences on gene expression and cell differentiation. It is intriguing that programmed DNA rearrangements occur at not just one or a few loci but on a genome-wide scale in most ciliated protozoa and several metazoans (Borst and Greaves, 1987; Yao *et al.*, 2002; Smith *et al.*, 2009).

In ciliates, it has been proposed that this phenomenon may serve a genome surveillance role (Yao and Chao, 2005; Chalker and Yao, 2011). However, the molecular mechanisms are not fully understood.

The ciliate *Tetrahymena thermophila* is a unicellular eukaryote with two distinct nuclei: a silent germline nucleus (micronucleus) and a transcriptionally active somatic nucleus (macronucleus). The macronucleus is renewed through the sexual conjugation process in which the parental micronuclei of paired cells undergo meiosis, gametic nucleus exchange, and nuclear fusion to generate zygotic nuclei that further divide and differentiate into new micronuclei and macronuclei. The parental macronuclei are degraded at the end of this process. Programmed DNA rearrangements have been found to occur in the new, developing macronuclei. Thousands of specific DNA elements (internal eliminated sequences [IESs]) are deleted, which comprise ~34% of the germline genome (Yao and Gorovsky, 1974; *Tetrahymena* Comparative Sequencing

Project, Eli and Edythe L. Broad Institute of Harvard and MIT, www.broadinstitute.org). In addition, chromosome breakage coupled with de novo telomere addition occurs at ~200 specific sites (Yao, 1989). Subsequently the macronuclear chromosomes endo-duplicate to ~45 times of the haploid genome (Yao and Chao, 2005).

The DNA deletion process in *Tetrahymena* is guided by a mechanism related to RNA interference (RNAi; Mochizuki *et al.*, 2002; Yao *et al.*, 2003). Double-stranded transcripts (dsRNAs) of IESs are generated in early stages of conjugation and are processed into 27- to 30-nucleotide small RNAs by the Dicer-like protein Dcl1p (Chalker and Yao, 2001; Malone *et al.*, 2005; Mochizuki and Gorovsky, 2005). The small RNAs associate with Argonaute protein Twi1p (*Tetrahymena* PAZ-Piwi domain protein) and direct heterochromatin-associated histone modifications (H3K27me3 and H3K9me3) at corresponding genomic sites in the developing macronucleus (Mochizuki *et al.*, 2002; Mochizuki, 2004; Taverna *et al.*, 2002; Liu *et al.*, 2007). The chromodomain proteins Pdd1p and Pdd3p (programmed DNA degradation proteins) are recruited to heterochromatic regions and later aggregated into large subnuclear foci (Madireddi *et al.*, 1996; Taverna *et al.*, 2002; Liu *et al.*, 2007; Yao *et al.*, 2007). Recent studies revealed that a domesticated *piggyBac* transposase Tpb2p is involved in the assembly of the Pdd1p-containing heterochromatic structures and is likely responsible for DNA cleavage in the DNA deletion process (Cheng *et al.*, 2010; Chalker and Yao, 2011). After deletion, these excised DNA elements are believed to be degraded rapidly through linear or circular intermediates, and the remaining flanking sequences are rejoined together without leaving new ends (Austerberry *et al.*, 1984; Saveliev and Cox, 1994, 1995, 1996; Yao and Yao, 1994). However, the molecular mechanism that rejoins these thousands of chromosome ends in *Tetrahymena* remains to be discovered.

The V(D)J recombination of immunoglobulin genes is a well-known example of programmed DNA rearrangements. A recombination-activating gene (RAG) protein complex recognizes the recombination signal sequences and induces double-strand DNA breaks (DSBs). Subsequently, the broken ends are rejoined by the nonhomologous end-joining (NHEJ) pathway (Gellert, 2002). Unlike the other DSB repair mechanism—homologous recombination—NHEJ is homologous-template independent and can result in variable outcomes, including insertion or deletion of sequences at the junction. In *Tetrahymena*, DNA deletion happens precisely and can produce junctions with only infrequent (a few percent) and minor (<10 base pairs) variations, such as in the M, R, and mse2.9 elements (Austerberry and Yao, 1987; Austerberry *et al.*, 1989; Li and Pearlman, 1996). However, some elements are deleted with highly variable junctions (tens and hundreds of base pairs in most events), like the Tlr1 and rdn elements (Patil *et al.*, 1997; Yao *et al.*, 2003). Moreover, DNA deletion induced by injection of dsRNAs also generated highly variable rejoining junctions (Yao *et al.*, 2003). It has been suggested that IESs in ciliates may be derived from transposable elements or foreign DNA elements. Some IESs with sequence features of transposons were found in *Euplotes*, *Oxytricha*, and *Tetrahymena* (Doak *et al.*, 1994; Klobutcher and Herrick, 1997; Patil *et al.*, 1997; Wuitschick *et al.*, 2002). The DNA deletion mechanism also resembles the excision of some transposons. In *Drosophila*, transposition of P element adopts a cut-and-paste mechanism. After excision of the P element, the broken ends at the donor site can be repaired through the NHEJ pathway (Rio, 2002). Recently a study revealed that DNA-Ligase-IV-XRCC4, the core component of NHEJ, was required for genome rearrangement in another ciliate, *Paramecium tetraurelia* (Kapusta *et al.*, 2011). Thus it is possible that the rejoining process in programmed DNA deletion in *Tetrahymena* relies on the NHEJ pathway or a related mechanism.

Ku protein is an important NHEJ component and is evolutionarily conserved in eukaryotes. Ku forms a heterodimeric protein complex consisting of two subunits of ~70 and 80 kDa, referred to as Ku70 and Ku80, respectively. Ku70 and Ku80 form an asymmetric, ring-like structure with sequence-independent DNA-binding ability (Downs and Jackson, 2004). In the NHEJ process, Ku is the first protein that binds to DSB sites and is believed to hold the DNA ends in proximity. In addition, Ku may contribute to DNA end protection and can serve as a recruitment platform for repair factors, including polymerases and ligases (Downs and Jackson, 2004; Lieber, 2010). Mammalian cells or mice deficient in Ku70 or Ku80 displayed increased sensitivity to DSBs and were defective in V(D)J recombination (Jeggo *et al.*, 1995; Ferguson and Alt, 2001). Studies in other organisms, such as bacteria, yeasts, flies, and plants, also showed the conserved role of Ku in DSB repair through NHEJ (Downs and Jackson, 2004). Furthermore, Ku has been shown to be physically associated with telomeres in many organisms and has an important role in regulating telomere addition and telomere maintenance (Riha *et al.*, 2006). In *Tetrahymena*, de novo telomere addition occurs after chromosome breakage during DNA rearrangements. Whether Ku is involved in de novo telomere addition in *Tetrahymena* remains unknown.

In this study, we focus on the role of *Tetrahymena thermophila* Ku80 (*TKU80*) during conjugation. Our results show that *TKU80* is essential for programmed DNA deletion. In the absence of *TKU80*, DNA deletion fails to complete, suggesting a role for NHEJ in this process. However, the step of DNA cutting still occurs, together with the failure of de novo telomere addition, resulting in developmental arrest and the degradation of the new macronucleus. Moreover, our results also suggest that Tku80p played an unexpected role in heterochromatin structure formation.

RESULTS

Identification of *T. thermophila* Ku70/Ku80 (*TKU70/80*) orthologues

To understand the role of Ku proteins in programmed DNA deletion, we searched for *Ku* orthologues in the *Tetrahymena* genome database (TGD Wiki, http://ciliate.org). Three *Ku* orthologues were found, including two genes for *Ku70* and one for *Ku80* (TTHERM_00684440, TTHERM_00561799, and TTHERM_00492460, named *TKU70-1*, *TKU70-2*, and *TKU80*, respectively). All three predicted *Tetrahymena* Ku proteins contain the three conserved domains in eukaryotes: a Ku70/Ku80 N-terminal α/β domain (von Willebrand factor type A domain [vWFA]), a central Ku-core domain, and a divergent C-terminal region (Figure 1A). Compared to Tku-70-1p, Tku70-2p has an additional C-terminal SAP (SAF-A/B, Acinus, and PIAS) domain, which is a putative DNA/RNA–binding domain. In Tku80p, the C-terminal domain contains a region for binding DNA-dependent protein kinase catalytic subunit (DNA-PK$_{CS}$). The related ciliate *P. tetraurelia* also contains multiple *Ku* orthologues, including two *Ku70* and three *Ku80* (Kapusta *et al.*, 2011). On the basis of the phylogenetic tree, Tku70-2p is more similar to the two Ku70 of *Paramecium* than to Tku70-1p (Figure 1B). This suggested that *TKU70-1* might be derived from a gene duplication before the evolutionary branching of *Tetrahymena* and *Paramecium* and was subsequently lost from *Paramecium*. Multiple sequence alignments of the central Ku-core domains in Tku70-1p, Tku70-2p, and Tku80p revealed that the KU70 core domains contain 44.5% similarity and the KU80 core domain contains 45.7% similarity compared with the other species listed in Supplemental Figure S1. Expression profiles
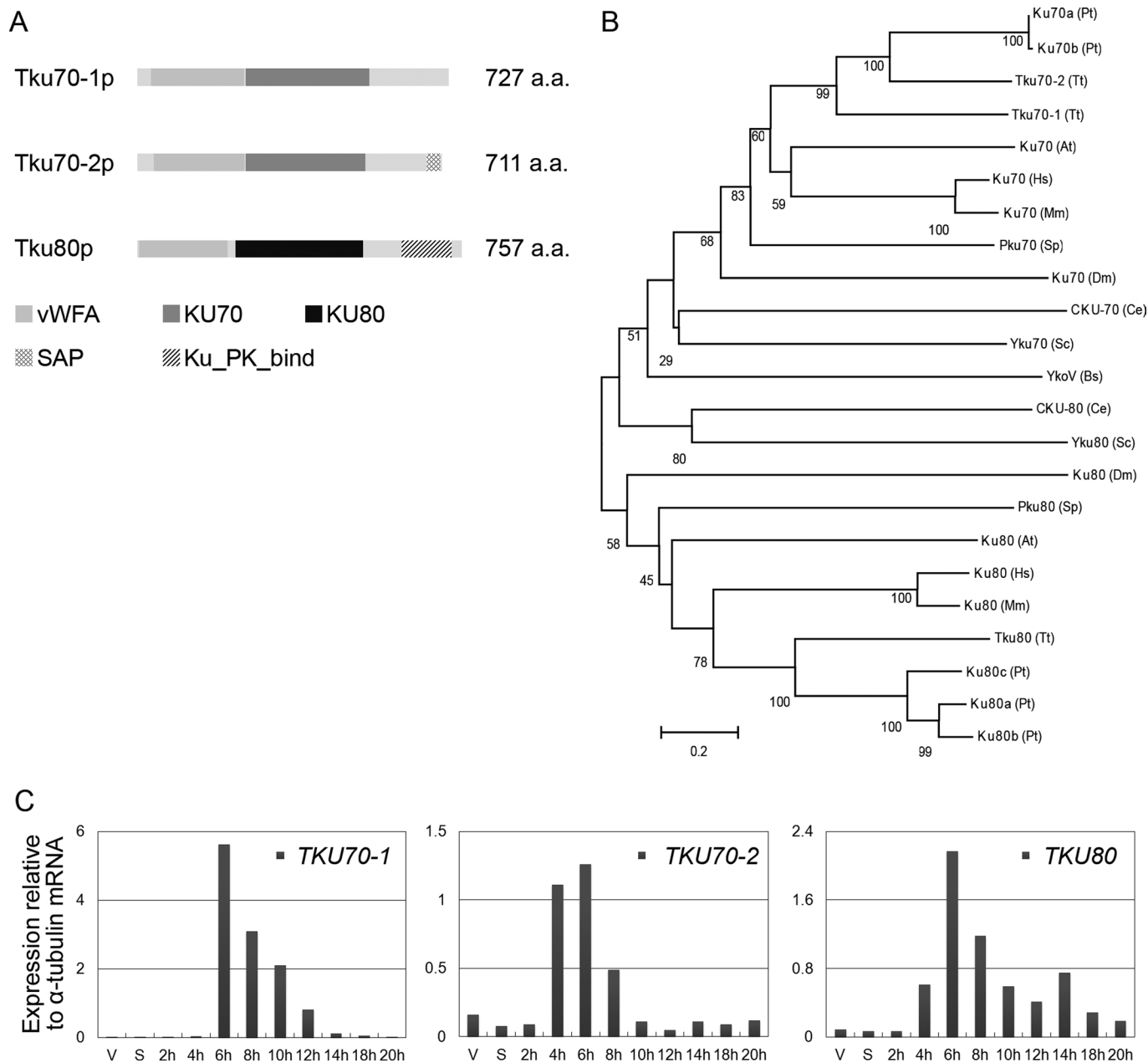
FIGURE 1. Three *Ku* orthologous proteins are expressed in *T. thermophila*. (A) Domain organization and predicted length of Ku proteins in *Tetrahymena*. These Ku proteins consist of a Ku70/Ku80 N-terminal α/β domain (vWFA), a central Ku-core domain, and a variant C-terminal region. The C-terminal region of Tku70-2p contains a SAP (SAF-A/B, Acinus, and PIAS) domain. Tku80p contains a Ku C terminal domain–like region (Ku_PK_bind). (B) A phylogenetic tree of Ku proteins using the neighbor-joining method. Numbers at each branch represent the percentage of 1000 bootstrap tests. The scale bar represents the branch length that corresponds to 0.2 amino acid substitution per site. At, *Arabidopsis thaliana*; Bs, *Bacillus subtilis*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Pt, *P. tetraurelia*; Sc, *S. cerevisiae*; Sp, *Schizosaccharomyces pombe*; Tt, *T. thermophila*. (C) Expression profiles of the three *Ku* orthologues in *Tetrahymena*. Total RNA from vegetative (V), starved (S), and conjugating cells (2, 4, 6, 8, 10, 12, 14, 18, and 20 h postmixing) were extracted and analyzed by using quantitative RT-PCR. Expression level of each gene was normalized to α-tubulin mRNA.

of *Tetrahymena Ku* orthologues were analyzed by quantitative real-time PCR (Figure 1C). *TKU70-1* was specifically expressed during conjugation. The expression level increased rapidly and reached the highest level at 6 h, around the time when new macronuclei began to develop, and then gradually decreased. Unlike *TKU70-1*, *TKU70-2* and *TKU80* were constitutively expressed in vegetative growth, starvation, and conjugation. Notably, expression of *TKU70-2* and

TKU80 were both highly up-regulated during conjugation, and their elevated patterns were only slightly different. The expression of *TKU70-2* reached a peak at 4–6 h after cell mixing, whereas *TKU80* expression reached the maximum at 6 h. These expression patterns are consistent with the microarray data in the *Tetrahymena* Gene Expression Database (Miao *et al.*, 2009). Taken together, the expression patterns suggest that the *Tetrahymena Ku* orthologues may

have important functions during conjugation. In the following studies, we focused on the analysis of the only Ku80 gene, *TKU80*, as a representative for understanding the role of Ku proteins and DNA double-strand break repair in programmed DNA rearrangements. Studies of the other two Ku proteins were complicated by difficulties in obtaining gene deletion mutants.

Green fluorescent protein–tagged Tku80p is localized in all nuclei during conjugation

To understand the role of Tku80p, we analyzed its subcellular localization by expressing Tku80p fused with green fluorescent protein (GFP) at the C-terminus under the control of its endogenous promoter. The intensities of Tku80p-GFP were consistent with the *TKU80* RNA expression profile. Tku80p-GFP was observed in both the micronucleus and macronucleus of vegetatively growing cells. During conjugation between the GFP-tagged strain and a normal strain (CU427), Tku80p-GFP appeared initially in only one of the paired cells but later in both cells (Figure 2). In the cells that did not express the fusion protein, Tku80p-GFP was localized in the new micronuclei and new macronuclei but not the parental macronucleus. This suggests that newly synthesized Tku80p may be targeted preferentially to sites actively involved in new DNA synthesis, which occurs in these newly emerging nuclei. At 10–12 h postmixing, Tku80p-GFP was detected predominantly in the new macronucleus. The Tku80p-GFP signal gradually decreased after the paired cells separated. The large accumulation of Tku80p-GFP in the new macronucleus indicates that Tku80p may have important functions in macronuclear development.

TKU80 is essential for completing conjugation

To investigate the role of *TKU80* during conjugation, we replaced *TKU80* by a paromomycin-resistant knockout construct using homologous recombination and generated three different types of *TKU80* mutant strains. The *TKU80* somatic knockout strains (lacking *TKU80* in the macronucleus) grew normally. Mating between two *TKU80* somatic knockout strains generated viable progeny, although the progeny production rate was partially affected (Supplemental Table S1). The *TKU80* germline knockout strains (lacking *TKU80* in the micronucleus) grew normally, and the progeny production rate was similar to that of control strains. The *TKU80* homozygous homokaryon knockout (Δ*TKU80*) strains (lacking *TKU80* in both the somatic and germline nucleus, verified by Southern hybridization, as shown in Figure 3A) also propagated well in vegetative growth. RNA analysis of these mutants revealed the relative contributions of these two nuclei. *TKU80* expression appeared at 8 h postmixing (after new macronucleus formation) and peaked at 10 h in somatic knockout cells, which represented the contributions from the new macronucleus (Figure 3B). On the other hand, the expression of *TKU80*, presumably from the old macronucleus, reached a maximum at 6 h and decreased rapidly at 8 h in germline knockout cells. These results suggest that expression of *TKU80* from parental and zygotic nuclei both contribute to the conjugation process, and expression from either nucleus alone is sufficient to generate viable progeny.

To determine whether *TKU80* was required at all for conjugation, mating between two homokaryon Δ*TKU80* strains was performed, and the developmental stages during conjugation were analyzed (Figure 3C). In late conjugation, the mutant cells were arrested at two–new micronuclei/two–new macronuclei stage, similar to other mutants defective in genes (e.g., Δ*TWI1*, Δ*DCL1*, Δ*PDD1*, and Δ*TPB2*) involved in DNA rearrangements (Coyne *et al.*, 1999; Mochizuki *et al.*, 2002; Mochizuki and Gorovsky, 2005; Malone *et al.*, 2005; Cheng *et al.*, 2010), and failed to produce viable prog-

eny (Figure 3D). This sterile phenotype could be rescued by mating to a control strain. These results indicate that *TKU80* is essential for completing the conjugation process.

IES elimination and chromosome breakage are both affected in the absence of *TKU80*

To investigate whether *TKU80* was required for DNA rearrangement, we analyzed DNA deletion by PCR. To distinguish the new rearranged products from the parental macronuclear DNA background, we analyzed deletion sites that can produce different-length products. IES elimination of M element generates two alternative products in the macronucleus, a ~0.9- or ~0.6-kb deletion. We selected parental strains that contained only one M element deletion product in the macronucleus. In this way, the presence of the other deletion form during conjugation would indicate successful M element rearrangements. We could not detect the other rearranged form in conjugation of Δ*TKU80* cells (Figure 4A). With the same strategy, two other IES elimination sites (rdn element and Tlr1 element) were analyzed, and no new rearranged macronuclear DNA could be detected (Supplemental Figure S2). These results indicate that Tku80p is required for IES deletion.

The failure to produce the final DNA deletion products could be due to failures to reach the DNA cleavage stage, as in most RNAi and heterochromatin mutants, or failures in rejoining the macronucleus-destined sequences after DNA cutting. To examine whether cleavage at IES elimination sites had occurred in the Δ*TKU80* strains, we looked for the existence of circular deletion products that were generated during deletion (Saveliev and Cox, 1994; Yao and Yao, 1994). In M element elimination, two alternative forms (0.6 and 0.9 kb) are excised, and both circular forms have been detected. To detect these circular deletion products, we used two inversely oriented primers located inside M element for PCR in DNA isolated from conjugating cells at different time points (Figure 4B). We detected both circular forms of the M element in late conjugation stages of Δ*TKU80* strains. The circular deletion products of R and mse2.9 elements were also observed in the mutant cells (Supplemental Figure S3). The strong signal of the circular deletion products in the mutant cells could be caused by delayed developmental progression and/or accumulation during conjugation. Given that the formation of these products requires DNA cleavage, the results suggest that Tku80p is not required for the DNA cleavage step of IES elimination and thus is likely required for rejoining the flanking regions after DNA cutting.

To analyze whether *TKU80* was required for chromosome breakage, we examined the existence of the 11-kb rDNA molecules, which can be distinguished from the parental macronuclear DNA background. In contrast to the 21-kb palindromic rDNA found in mature macronuclei, the 11-kb rDNA contains a single rRNA gene with a distinctive 5' end (Pan and Blackburn, 1981; Challoner and Blackburn, 1986). It is generated by chromosome breakage followed by de novo telomere addition during conjugation and is lost after several generations of growth. By detecting its unique telomerized 5' ends generated after breakage by PCR, we found that the 11-kb rDNA was not observed in conjugating Δ*TKU80* cells (Figure 4C), indicating that the DNA cleavage step and/or the de novo telomere addition step were affected in the absence of Tku80p. This result suggests that *TKU80* is essential for chromosome breakage during conjugation.

Loss of new macronuclear DNA in the absence of *TKU80*

Absence of *TKU80* during conjugation not only caused arrest of the cells, but also led to loss of bulk DNA in the new macronucleus. By
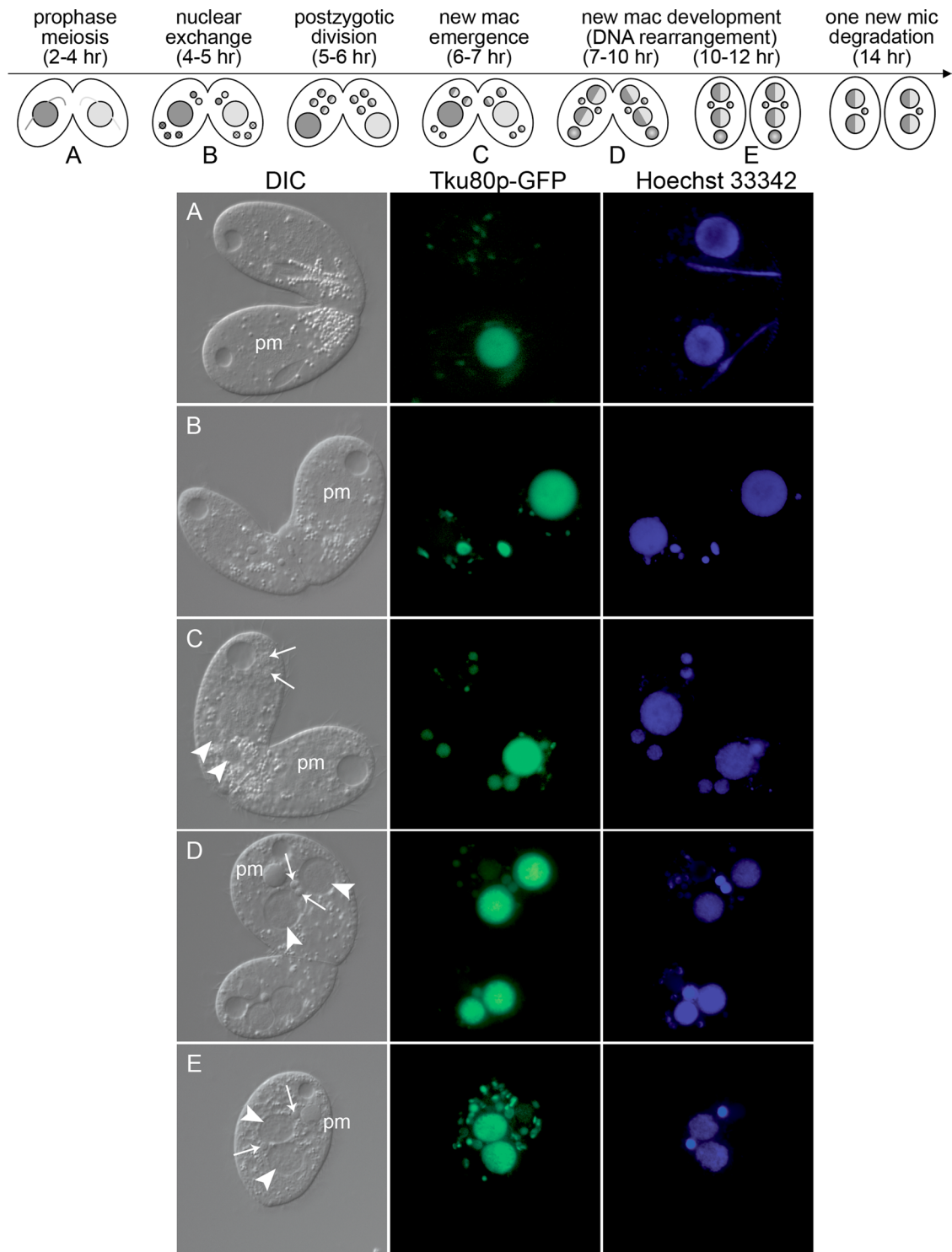
FIGURE 2: Localization of GFP-tagged Tku80p. Top, the nuclear developmental process of *Tetrahymena*. Bottom, the subcellular localization of Tku80p-GFP in living conjugating cells. Nuclear DNA was stained with Hoechst 33342. Pm, parental macronucleus; arrows and arrowheads denote new micronuclei and new macronuclei, respectively.

means of 4′,6-diamidino-2-phenylindole (DAPI) staining, abnormal morphology of the new macronucleus was first observed in the exconjugants of the Δ*TKU80* strains at 19–22 h postmixing. About 21–32% of the exconjugants contained macronuclei that had irregular shape, uneven DNA contents (some regions contained decreased DAPI staining), or complete loss of DNA (no DAPI staining). Meanwhile, their two micronuclei remained intact (Figure 5A). This pheno-

type of abnormal new macronuclear DNA degradation bears some similarity to that found in the Δ*DIE5* mutant cells, in which both IES elimination and chromosome breakage fail (Matsuda *et al.*, 2010).

To determine whether this DNA degradation was due to the presence of unrepaired chromosome ends, the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was performed. In previous studies, positive TUNEL labeling was
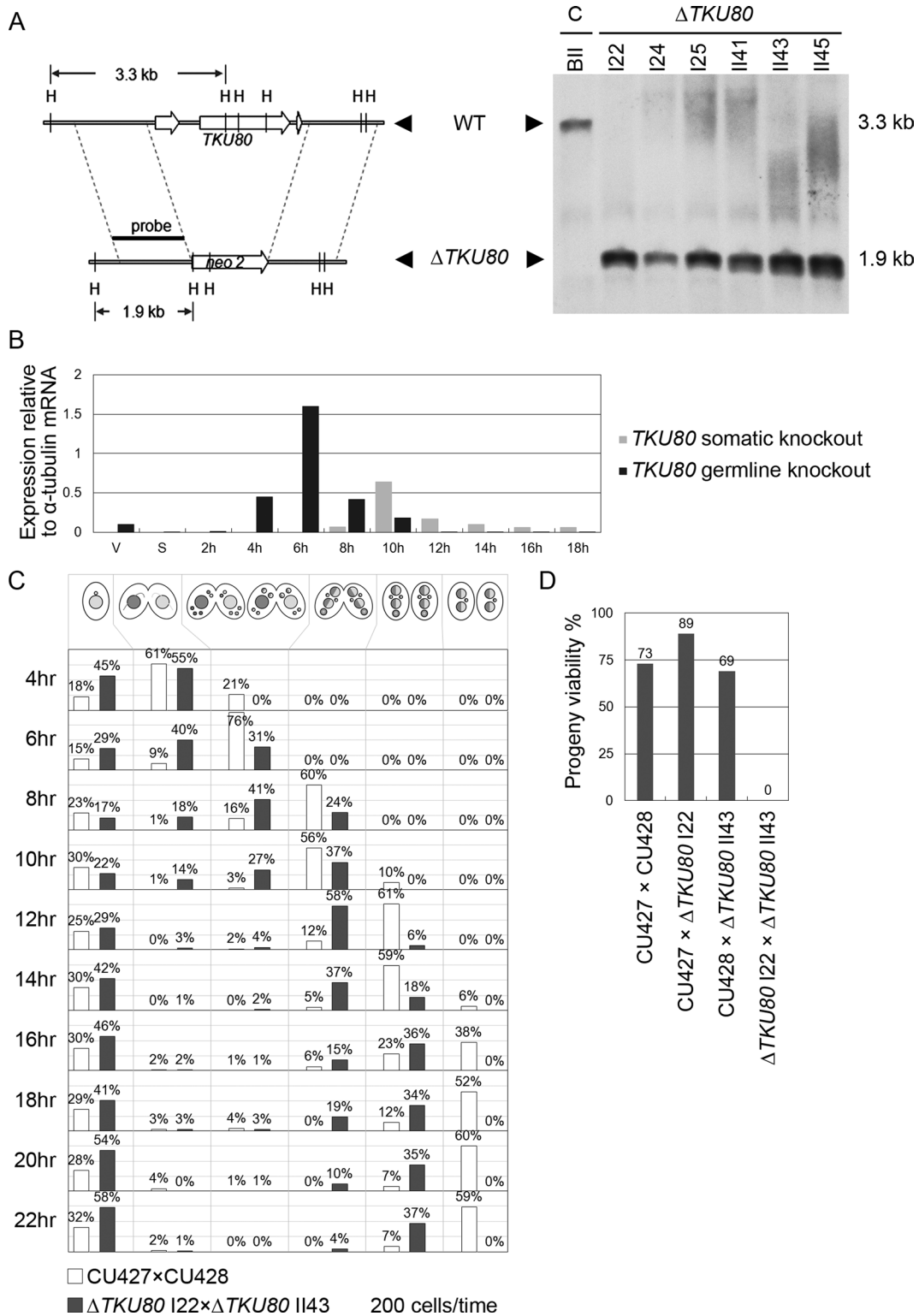
FIGURE 3: *TKU80* is essential for completing conjugation. (A) *TKU80* homozygous homokaryon knockout (Δ*TKU80*) strains were verified by Southern hybridization. Genomic DNA was extracted from control cells and Δ*TKU80* cells and analyzed. Probe used in the assay is indicated. (B) The *TKU80* expression profiles of the *TKU80* mutant strains. Total RNA from vegetative (V), starved (S), and conjugating cells (2, 4, 6, 8, 10, 12, 14, 16, and 18 h postmixing) were extracted and analyzed by quantitative RT-PCR. Expression level in each mutant was measured separately and normalized to α-tubulin mRNA. The *TKU80* expression of *TKU80* somatic knockout strains (light gray) were observed after 8 h postmixing. On the other hand, the expression of *TKU80* germline knockout strains (dark gray) reached the maximum at 6 h but decreased rapidly at 8 h. (C) Top, the developmental stages during conjugation. In late conjugation, development of the *TKU80*-mutant cells was arrested at two–new micronuclei/two–new macronuclei stage. (D) Percentage of viable progeny. Paired cells from different mating sets were transferred into medium after 8 h postmixing, and the progeny production rate was assessed by drug resistance.
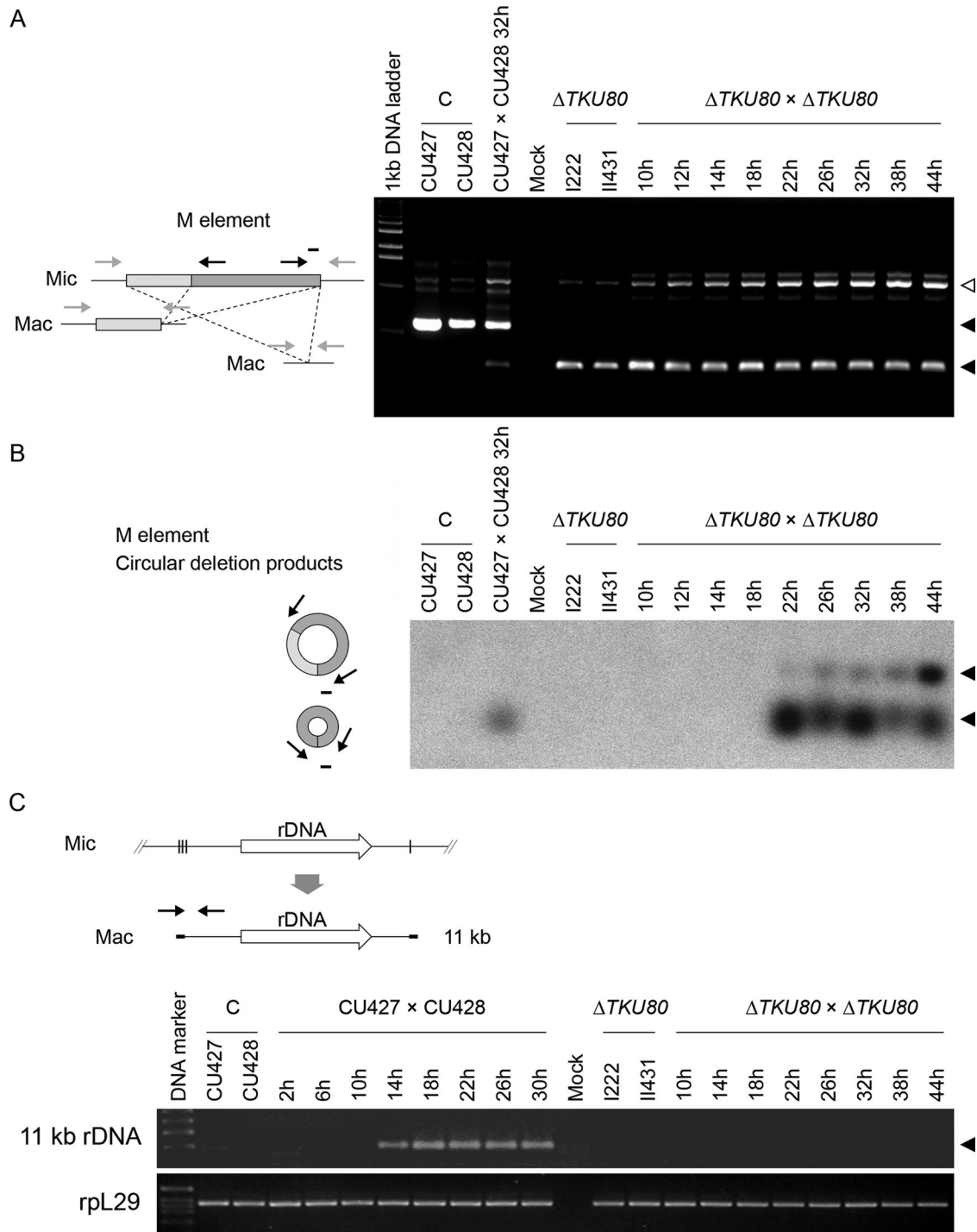
FIGURE 4: Loss of *TKU80* affected IES elimination and chromosome breakage. (A) Schematic representations of PCR-based M element elimination assay. Elimination of M element (light and dark gray box) generates two alternative products in the macronucleus, a ~0.9-kb deletion (light and dark gray box) or a ~0.6-kb deletion (dark gray box). The arrows represent the PCR primers used in the deletion assay (gray arrows) and in the circular deletion product detection (black arrows). The genomic DNA of vegetative cells and conjugating cells (10, 12, 14, 18, 22, 26, 32, 38, and 44 h postmixing) was analyzed. The open and black arrowheads indicate the micronuclear and macronuclear form products, respectively. The new, rearranged macronuclear DNA was not detected during conjugation of Δ*TKU80* cells. (B) Circular deletion products of M element were analyzed by using PCR coupled with Southern hybridization. The PCR primers (black arrows) and the probe (black rectangle) are illustrated. The circular products of M element (indicated by black arrowheads) were observed in both control cells and Δ*TKU80* cells. (C) Chromosome breakage detection. The 11-kb rDNA molecule is produced by chromosome breakage and followed by de novo telomere (black rectangle) addition. Chromosome breakage sites are indicated by the vertical black lines. The 11-kb rDNA can be detected by PCR using telomere sequence as one of the PCR primers (black arrows). The genomic DNA of vegetative cells and conjugating cells collected at different stages was analyzed. The rpL29 was detected as a template control. The 11-kb rDNA (indicated by the black arrowhead) was not detected during conjugation of Δ*TKU80* cells.
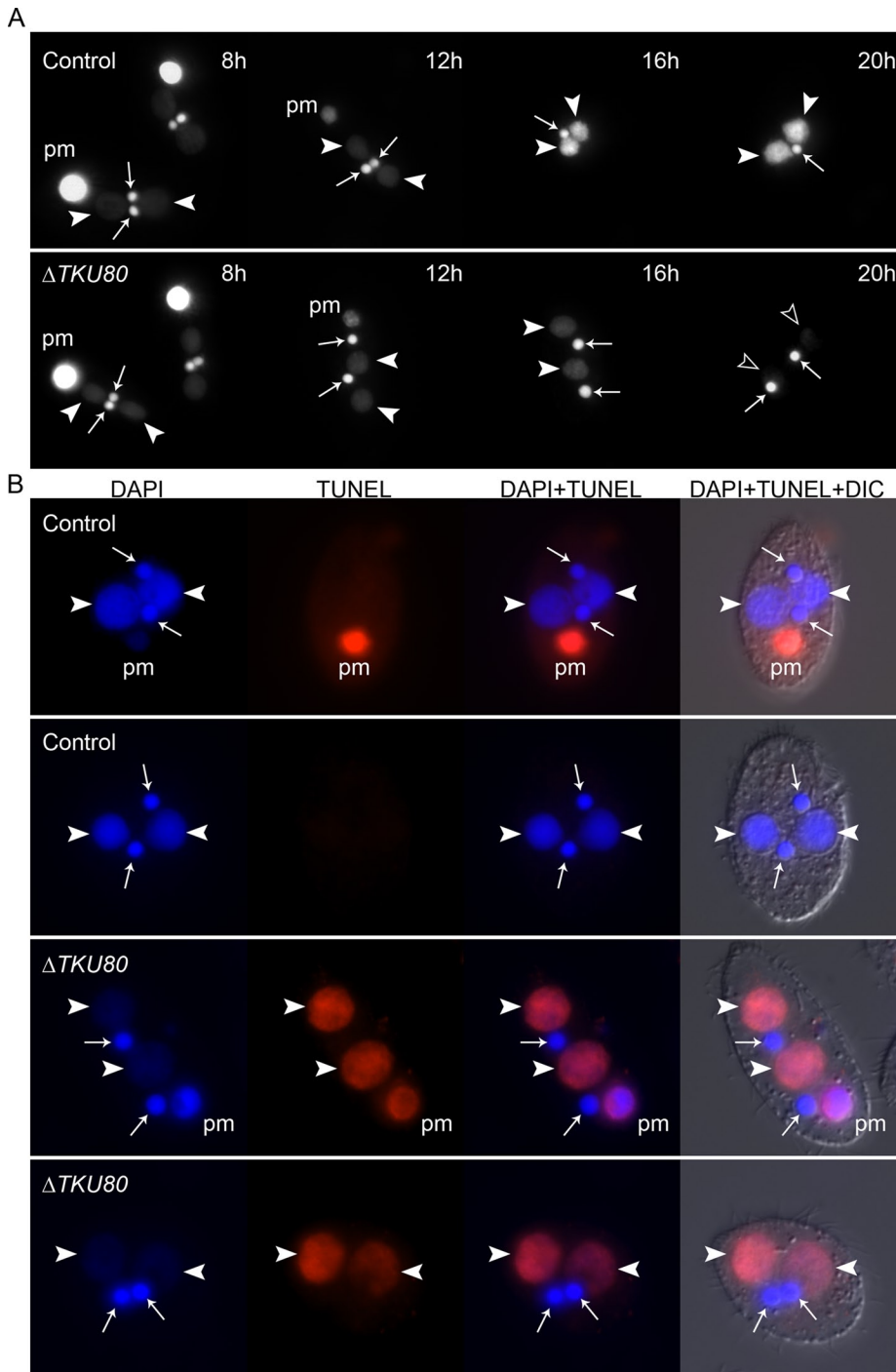
FIGURE 5: Loss of new macronuclear DNA in Δ*TKU80* cells. (A) Fluorescent images of late conjugation of control and Δ*TKU80* cells. Weak DAPI signals appeared in the new macronuclei in late conjugation of Δ*TKU80* cells (open arrowheads, 20 h postmixing). (B) TUNEL assay. TUNEL-positive signals were observed in the parental macronucleus of control cells and the developing macronucleus and parental macronucleus of Δ*TKU80* cells. Pm, parental macronucleus; arrows and arrowheads indicate new micronuclei and new macronuclei, respectively.

Δ*DIE5* mutant cells, in which TUNEL signals were not observed in the new macronucleus (Matsuda *et al.*, 2010). The results suggest that loss of *TKU80* may lead to accumulation of unrepaired and/or unprotected ends during nuclear development.

TKU80 is important for the formation of large, Pdd1p-containing structures in new macronucleus

Chromatin modifications, including H3K9-me2 and H3K27me3, have been shown to be associated with the IES elimination sites during conjugation (Taverna *et al.*, 2002; Liu *et al.*, 2007). Like the chromodomain-containing protein Pdd1p, the modified histones are homogeneously distributed in new, developing macronucleus at early stages. Subsequently, they form large, distinct spherical structures in the nuclear periphery (Taverna *et al.*, 2002; Liu *et al.*, 2007). The appearance of the Pdd1p-containing structures coincides with IES elimination (Austerberry *et al.*, 1984; Madireddi *et al.*, 1994, 1996; Chalker, 2008), although their precise roles remain unclear. Besides Pdd1p, several proteins (e.g., Pdd2p, Lia1p, and Tpb2p) essential for DNA rearrangements are required for the formation of the subnuclear structures in late conjugation (Nikiforov *et al.*, 1999; Rexer and Chalker, 2007; Cheng *et al.*, 2010). By means of immunofluorescence staining, we found that two histone modification marks (H3K9me3 and H3K27me3) and Pdd1p were also observed in developing macronuclei of Δ*TKU80* cells. However, the large subnuclear structure failed to form in these mutant cells (Figure 6). Occasionally, large Pdd1p aggregates were observed in the cytoplasm of mutant cells (unpublished data). The result indicates that Tku80p is important for the formation of the Pdd1p-containing structures, even though it is not required for IES cleavage.

DISCUSSION

Programmed DNA rearrangements excise thousands of DNA elements from the *Tetrahymena* genome and break the chromosomes into hundreds of smaller units. It is critical for the organism to precisely and efficiently repair the broken ends generated by this large number of excision events. In this study, we found that the NHEJ core component Ku80 protein plays an essential role.

found in the degenerating meiotic nuclei and the condensed parental macronuclei but not in the new, developing macronuclei of normal cells (Madireddi *et al.*, 1996; Mpoke and Wolfe, 1996). In the absent of *TKU80*, we found that at late conjugation stages, TUNEL-positive signals were observed in the new, developing macronucleus of ~86% of exconjugants (Figure 5B). This was different from the

The localization of GFP-tagged Tku80p suggests that Tku80p function is related to the development of the new macronucleus. Perhaps the most direct support comes from the gene knockout studies. In the absence of *TKU80*, nuclear development of the exconjugants was arrested at the two–new micronuclei/two–new macronuclei stage, a phenotype typical of mutant strains (Δ*TWI1*, Δ*DCL1*,
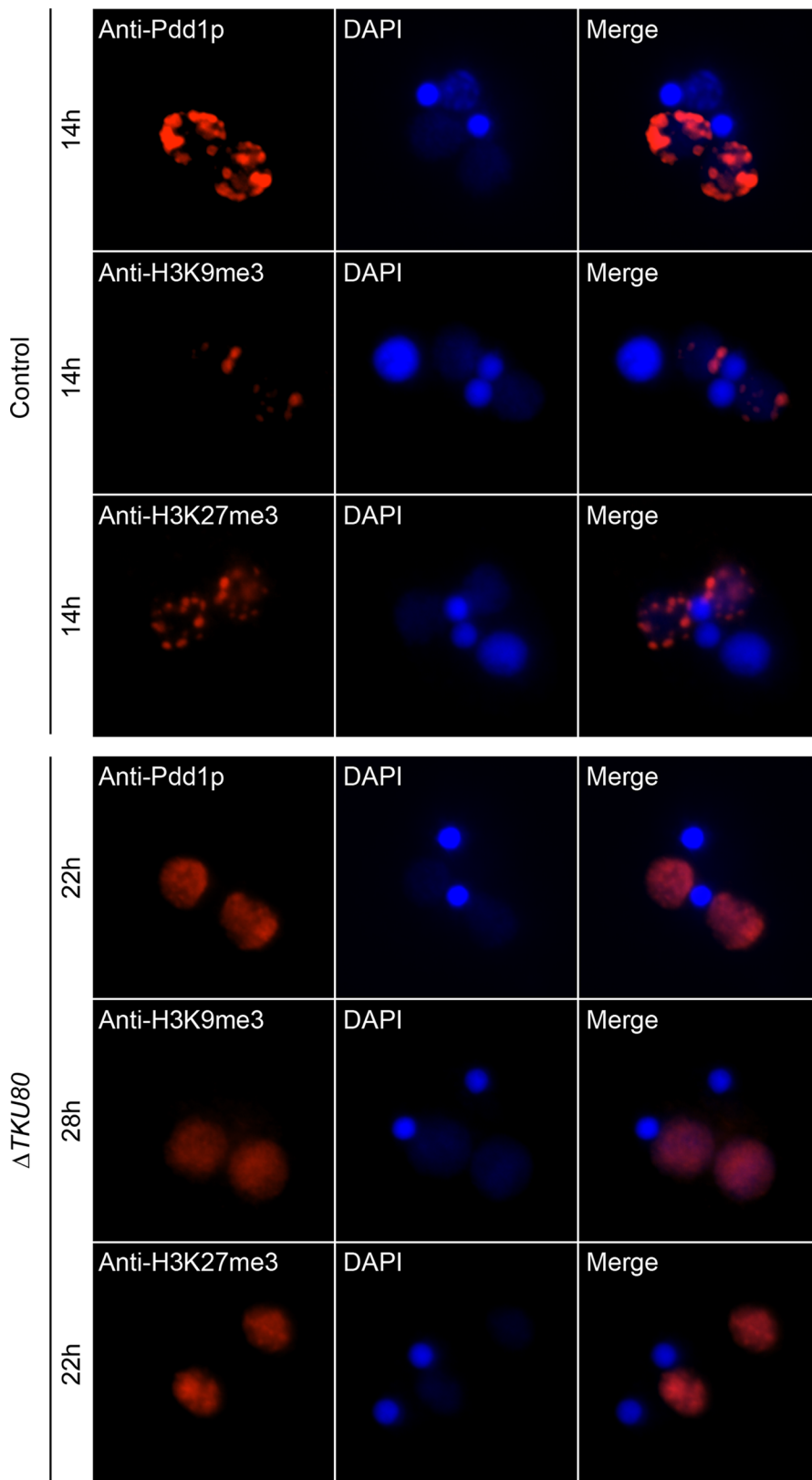
FIGURE 6: Nuclear localization of histone H3K27 methylation, H3K9 methylation, and Pdd1p. The subcellular localization of H3K27me3, H3K9me3, and Pdd1p was analyzed by immunofluorescence staining. Pdd1p-containing heterochromatin structures were observed in the new macronucleus of control cells (14 h postmixing). The Δ*TKU80* cells shown here were examined at 22 h (Pdd1p and H3K27me3) or 28 h (H3K9me3) postmixing. Although Pdd1p, H3K27me3, and H3K9me3 were observed in the new macronucleus of Δ*TKU80* cells, these large subnuclear structures failed to be detected in Δ*TKU80* cells.

Δ*EMA1*, Δ*EZL1*, Δ*PDD1*, Δ*PDD2*, Δ*LIA1*, Δ*TPB2*, and Δ*DIE5*) defective in DNA rearrangements (Coyne *et al.*, 1999; Nikiforov *et al.*, 1999; Mochizuki *et al.*, 2002; Malone *et al.*, 2005; Liu *et al.*, 2007; Rexer and Chalker, 2007; Aronica *et al.*, 2008; Cheng *et al.*, 2010; Matsuda *et al.*, 2010). Direct DNA analysis indeed revealed the failure of this mutant to generate final deletion products and to finish de novo telomere addition. Thus *TKU80* is involved in the DNA-deletion process and is essential for chromosome breakage in *Tetrahymena*.

The mutant analysis revealed additional details that illuminated the role of *TKU80*. Most mutant strains studied previously are defective in various steps leading to DNA deletion, and all of them showed the retention of IESs in the new macronucleus. In Δ*TKU80* cells, however, the defect appeared to be in the rejoining of the IES flanking regions that are to be retained in the macronucleus. Thus the mutant was not able to generate the final, properly rearranged DNA product. Although we have not been able to directly detect the nascent free ends generated from DNA cutting, we have detected the circular form of the excised IES, which indicated that cuttings at the IES boundaries occurred to produce these molecules. Without proper protection by proteins, including Ku80, these free ends are expected to be unstable and difficult to detect directly by PCR. Nonetheless, their presence was strongly suggested by our TUNEL assay results. Even though there are thousands of DNA breaking and rejoining (or telomere addition) events occurring, the normal new macronucleus is TUNEL negative. This suggests that the free DNA ends generated in this process are very short-lived and/or shielded from detection by TUNEL. In mutant cells, however, TUNEL signals were readily detected, indicating the presence of unprotected free ends, a phenotype not previously seen in any *Tetrahymena* mutant. Furthermore, DNA in these mutant nuclei appeared to be degraded rapidly, leading to the loss of bulk DNA (as indicated by the lack of DAPI staining) from developing macronuclei, agreeing with the presence of unprotected ends. This phenotype of DNA degradation in the new macronucleus has seldom been observed, with the possible exception of Δ*DIE5*, in which chromosome breakage and DNA deletion failed to occur, but the aggregation of Pdd1p-containing structures occurred normally (Matsuda *et al.*, 2010). *DIE5* does not share significant sequence homology with known genes, and it is unclear whether Δ*DIE5* cells carry out DNA cleavage and generate free ends. If they do, the degradation of DNA in Δ*DIE5* cells could conceivably be slower due to the presence of Tku80p and might be less detectable by TUNEL assay at the time points analyzed.

In earlier studies, deletion products of two IES elements (M and R) were examined, and both the circular and linear deletion forms were detected during conjugation (Saveliev and Cox, 1994, 1996, 2001; Yao and Yao, 1994). Saveliev and Cox found free double-stranded ends at both boundaries that shared the same structure of 4-base 5′ overhang. Because of the 4–base pair heterologous extensions at different ends, two types of chromosome junctions would be produced by a cleavage–ligation mechanism, but only one type was detected in some deletion events. They proposed that DNA deletion is initiated by a double-strand cleavage at one boundary of an IES element, followed by transesterification to generate the macronuclear junction on one DNA strand and liberate a linear deleted product after a single-strand cleavage (Saveliev and Cox, 1996, 2001). Their results could not exclude that a DNA double-strand break was also produced at the other boundaries, followed by ligation to rejoin the two macronuclear chromosome ends (Saveliev and Cox, 1996, 2001). In our study, we found that DNA cleavages occurred, but the final rejoined macronuclear DNA was not observed in the absence of *TKU80*. This favors the cleavage and religation mechanism of DNA deletion but does not exclude the transesterification mechanism, since the first cleavage products might also require Ku for their protection. Our results suggest that *TKU80* is not essential for the events leading to DNA cutting, which is probably carried out by the domesticated transposase Tpb2p (Cheng *et al.*, 2010), but is required for the subsequent ligation of the ends adjacent to the retained macronuclear DNA. Of interest, the appearance of the circular deletion intermediates in the Δ*TKU80* exconjugants demonstrated that ligation of the IES element ends could occur in a Ku-independent pathway and perhaps even by a different ligase. Because they were the two ends of the same DNA molecule, Tku80p may be dispensable in holding them together for ligation. This phenomenon is different from V(D)J recombination, in which both the coding-end and the signal-end joinings are severely defective in Ku80-deficient mammalian cells (Pergola *et al.*, 1993; Taccioli *et al.*, 1993; Zhu *et al.*, 1996).

In normal *Tetrahymena* cells, Pdd1p-containing structures occur in the developing new macronucleus, first as widely distributed punctuations and then gradually concentrated into a few large, spherical structures preferentially located in the nuclear periphery at late conjugation stages. These structures contain most IESs as determined by in situ hybridizations and are lost later, consistent with the timing of DNA rearrangements (Madireddi *et al.*, 1996; Taverna *et al.*, 2002; Liu *et al.*, 2007; Yao *et al.*, 2007; Cheng *et al.*, 2010). However, the exact function of these subnuclear structures remains unknown. It was believed that these Pdd1p-containing structures might serve to concentrate the activity of the DNA rearrangement machinery and provide specific compartments in the new macronucleus for DNA deletion. In this study, we found that the aggregation of the Pdd1p-containing structures failed to form in the absence of Tku80p, and yet DNA cutting at IES elimination sites appeared to have occurred. Thus, all events leading to and including DNA cutting are likely independent of the assembly of Pdd1p-containing structures. This finding helps to focus the role of these structures, and it could be in the subsequent ligation step. With the participation of Ku proteins, the free ends generated may form aggregates that are important for the rejoining reaction, in a way similar to the formation of DNA double-strand breakage repair foci observed in other organisms (Lisby *et al.*, 2004; Lukas *et al.*, 2005; Bekker-Jensen *et al.*, 2006; Misteli and Soutoglou, 2009). In addition, these structures may form specialized compartments to sequester the deleted IESs for subsequent degradation, thus preventing them from further improper rearrangements. In the absence of Ku proteins, unpro-

tected ends might activate a DNA-damage checkpoint that arrests cell differentiation and prevents all subsequent steps from occurring, including the formation of Pdd1p-containing structures.

Three Ku proteins were found in the *Tetrahymena* genome database, and all of them were highly expressed at the time when the new macronucleus starts to develop. In addition, we searched for the expression profiles of evolutionarily conserved genes known to participate in the NHEJ pathway in the *Tetrahymena* Gene Expression Database (Miao *et al.*, 2009) and found that the transcription of *DNA-PK$_{CS}$* and *Ligase-IV* (TTHERM_00203010 and TTHERM_00387050) also increased during development of the new macronucleus. These gene expression patterns supported the idea that major components of NHEJ pathway might be involved in DNA rearrangements during conjugation. In a recently study, the NHEJ component DNA-Ligase-IV-XRCC4 was shown to be required for DNA rearrangements in *Paramecium*. In the *LIG4*- or *XRCC4*-silenced cells, reduced DNA content was observed in the new macronucleus, and the IES flanking DNA failed to be rejoined (Kapusta *et al.*, 2011). These observations are similar to the phenotype of *TKU80* mutants in *Tetrahymena*, suggesting that NHEJ is a common step in ciliate genome rearrangements. Although it shares important features with *Tetrahymena*, *Paramecium* has the unusual ability to repair free ends of injected linear DNA through new telomere addition during growth (Gilley *et al.*, 1988). It can also generate telomerized ends at some IES sites during conjugation, presumably as an alternative to DNA ligation after cutting (Caron, 1992; Le Mouel *et al.*, 2003). It would be interesting to see whether deficiency in Ku proteins affects these special abilities in *Paramecium*.

Tetrahymena and *Paramecium* are distantly related ciliates, yet both appear to use a domesticated piggyBac transposase for DNA cutting and the NHEJ pathway for end joining during DNA rearrangements. In another distantly related ciliate, *Oxytricha*, transposases are required for the programmed excision of transposons and the deletion of IESs (Nowacki *et al.*, 2009). Remarkably, this strategy of programmed DNA cutting and joining also appears to be shared by mammals, in which the domesticated transposase RAG1 and NHEJ proteins are involved in V(D)J recombination (Agrawal *et al.*, 1998; Kapitonov and Jurka, 2005). Perhaps this strategy reflects the prevalence of transposons, which provides opportunities to evolve programmed DNA rearrangements in diverse groups. Transposons appear to play a major role in shaping the ciliate genome (Chalker and Yao, 2011). Both *Tetrahymena* and *Paramecium* use the RNAi machinery to mark DNA for deletion, possibly for the defense against invading genetic elements, including transposons and other foreign genes, similar to what RNAi does in other eukaryotes. In ciliates the process does not lead simply to gene silencing as in other eukaryotes. Instead, it ends with the deletion of the targeted DNA, through the recruitment of transposases possibly from transposons that are being defended against. In this study we found that the free ends generated by this process are linked back together through the conserved cellular NHEJ repair pathway. We speculate that Tpb2p generates a DNA double-strand break at one IES boundary, leaving a chromosome free end and an IES end. Cutting at the other IES boundary could occur by Tpb2p or through a nucleophilic attack using the IES 3′ hydroxyl terminus. The chromosome ends are protected by Ku and are rejoined through the Ku-dependent DNA repair pathway (Figure 7). The excised IES (linear form or circular form) form large foci with associated proteins and subsequently are degraded. Thus, the intricate interplays between transposon invasion, host defense, and chromosome repair may have driven the evolution of massive DNA
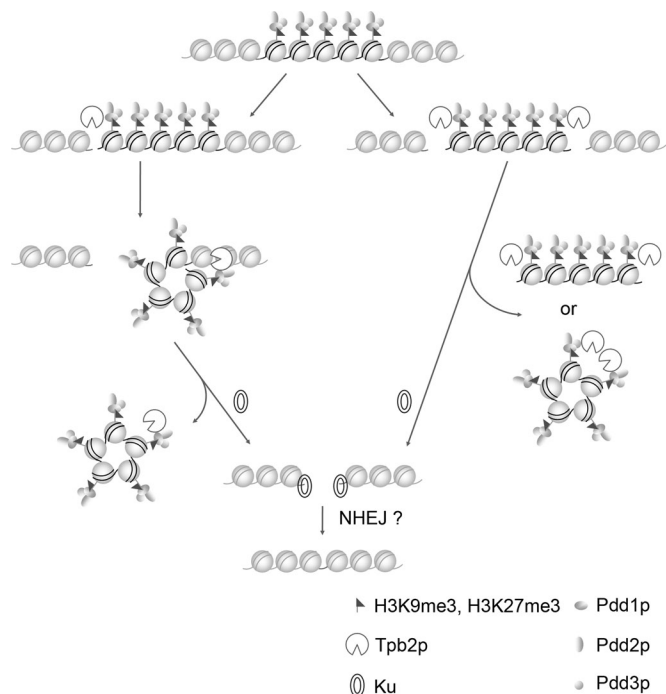
FIGURE 7: A possible scheme for IES elimination in *T. thermophila*. After the establishment of heterochromatin marks H3K9me3 and H3K27me3 on IESs, the domesticated transposase Tpb2p is recruited to cut the DNA at one IES boundary. DNA cutting at the other IES boundary could be generated by either the IES 3′ hydroxyl terminus (left) or by Tpb2p (right), leaving the circular form or linear form of excised IES. Ku then binds the DNA free ends and mediates rejoining of the IES flanking sequences.

rearrangements in ciliates, which provides a special window for looking into the details of chromosome dynamics.

MATERIALS AND METHODS

Strains and cell culture

Tetrahymena thermophila obtained from P. Bruns (Cornell University, Ithaca, NY) were cultured in axenic media as described before (Orias et al., 2000). Inbred strains B2086, CU427, and CU428 were used for gene expression, cell transformation, and all other analysis. Star strains B*VI and B*VII that possess defective micronuclei were obtained from P. Bruns and used for generating *TKU80* homozygous germline knockout strains. Conjugation was initiated by mixing cells starved overnight in 10 mM Tris-HCl solution (pH 7.4).

Sequence analysis

Three *Tetrahymena Ku* orthologues—*TKU70-1*, *TKU70-2*, and *TKU80*—were identified by BLAST search in the *Tetrahymena* genome database (Stover et al., 2006) using *Saccharomyces cerevisiae* Yku70p (NCBI reference sequence: NP_014011) and Yku80p (NCBI reference sequence: NP_013824) and also by gene annotations in *Tetrahymena* genome database. Sequences of Ku proteins were obtained from the National Center for Biotechnology Information Protein Database. The phylogenetic tree using the neighbor-joining method was produced by using the software MEGA4 (Tamura et al., 2007). The evolutionary distances were computed using the Poisson correction method. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). Multiple sequence alignments were performed by using the program

CLUSTAL W with the default parameters in the software BioEdit Sequence Alignment Editor.

Quantitative real-time PCR

Total RNA was extracted from inbred normal cells by using an RNA isolation kit (Roche, Indianapolis, IN). First-strand cDNA synthesis was performed using Transcriptor reverse transcriptase and oligo $(dT)_{18}$ as a primer. The quantitative real-time PCR (qRT-PCR) analysis was carried out by using the LightCycler Carousel-Based PCR system and LightCycler FastStart DNA Master^{PLUS} SYBR Green I. The relative amounts of the three *Ku* orthologues were normalized by using α-tubulin as an internal control. The primers used in qRT-PCR are listed in Supplemental Table S2.

Generation of *TKU80-GFP*–tagged strains and *TKU80*-knockout strains

To generate *TKU80-GFP*–tagged constructs, we PCR amplified the *TKU80* C-terminal 0.95-kbp fragment (nucleotides 70454–71394 of *Tetrahymena thermophila* SB210 genomic scaffold scf_8254446), which lacked a stop codon, and inserted it between the *Acc65*I and *Sal*I restriction sites of the *neo2* cassette (Gaertig et al., 1994), which conferred paromomycin resistance. The GFPrpL29 fragment amplified from pIGF-1 (Malone et al., 2005) by PCR was inserted in frame and downstream of the *TKU80* C-terminal fragment into the *Sal*I and *Cla*I sites of the vector. The 1.3-kbp downstream fragment of *TKU80* (nucleotides 68994–70299 of scf_8254446) was PCR amplified and cloned into the *Sma*I and *Sac*I sites of the vector. The sequences of the primers are listed in Supplemental Table S1. The *TKU80-GFP*–tagged construct was digested with *Acc65*I and *Sac*I and introduced into conjugating cells by biolistic transformation. Transformants of *TKU80-GFP* were selected in media containing paromomycin (Bruns and Cassidy-Hanley, 2000).

To create a *TKU80*-knockout construct, we PCR amplified the *TKU80* upstream 1.3-kbp fragment (nucleotides 73405–74767 of scf_8254446) and inserted it into the *Apa*I and *Sal*I sites of the *neo2* cassette. The downstream 1.3-kbp fragment (nucleotides 68994–70299 of scf_8254446) of *TKU80* was PCR amplified and cloned into the *Sma*I and *Sac*I sites of the plasmid. The sequences of the primers are listed in Supplemental Table S2. The construct was linearized by digestion with *Xho*I and *Sac*I and transformed into mating cells by biolistic transformation. The knockout mutant strains were selected with paromomycin. The germline transformants were confirmed by the existence of the drug resistance phenotype in the next generation. The homozygous germline knockout strains were created through genomic exclusion, by crossing the heterozygous germline knockout strains to a star strain. The *TKU80* homozygous homokaryon knockout (Δ*TKU80*) strains were produced by mating two homozygous germline knockout strains. Genomic DNA of the Δ*TKU80* strains was examined by Southern blot analysis. Total DNA extracted from the mutant strain was digested with *Hind*III and subjected to electrophoresis in a 0.8% agarose gel. DNA was transferred to an IMMOBILON-NY+ nylon membrane (Millipore, Bedford, MA) and hybridized with digoxigenin-labeled probes at 42°C overnight. The membranes were washed in 2× saline–sodium citrate (SSC) with 0.1% SDS and 0.5× SSC with 0.1% SDS at 65°C several times before detection of the luminescence using x-ray films.

IES elimination and chromosome breakage analysis

Genomic DNA was purified from conjugating cells collected at different time points. The elimination of M, Tlr-1, and rdn elements was examined by PCR analysis (Coyne et al., 1999; Aronica et al., 2008). The circular elimination products of M, R, and mse2.9 elements

were detected by PCR and Southern blot as described in previous studies (Yao and Yao, 1994). The 11-kb rDNA was detected by PCR. The primers used in these experiments are listed in Supplemental Table S2.

Immunofluorescence analysis

To elucidate Tku80p-GFP localization, conjugants of CU427 and *TKU80-GFP*–tagged cells were collected at different time points without fixation and stained with the vital fluorescent dye Hoechst 33342 (2 μg/ml) for the analysis.

To analyze nuclear development during conjugation, conjugating cells were collected and fixed in PBS containing 2% paraformaldehyde, followed by DAPI staining (100 ng/ml).

The presence of unrepaired or damaged DNA in nuclei was characterized by the TUNEL assay. Conjugating cells were fixed in phosphate-buffered saline (PBS) containing 2% paraformaldehyde for 15 min, followed by washing in PBS several times. The cells were air dried on slides before permeabilization in PBT (PBS containing 0.3% Triton X-100) for 1 h at room temperature. TUNEL reaction mixture from the *In Situ* Cell Death Detection Kit, TMR red (Roche) was applied, and samples were incubated in a moisture box at 37°C for 1 h. The cells were then washed with PBS and counterstained with DAPI.

For immunofluorescence analysis, conjugating cells were fixed, air dried on slides, and permeabilized as described. Anti-Pdd1p antibody (ab5338, 1:1000; Abcam, Cambridge, MA), anti-H3K9me3 (07-442, 1:300; Millipore), or anti-H3K27me3 (07-449, 1:500; Millipore) was added and incubated at 4°C overnight. After washing with PBT, cells were incubated with Alexa Fluor 488 F(ab') fragment of goat anti–rabbit immunoglobulin G (1:500; Invitrogen) for 1 h at room temperature, followed by washing with PBT and counterstaining with DAPI.

All images were captured using a fluorescent microscope (Zeiss Axio Imager Z1; Carl Zeiss, Jena, Germany) coupled to a CoolSNAP HQ Photometrics camera (RoperScientific, Tucson, AZ).

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