Ubc9p is the sole E2-conjugating enzyme for SUMOylation, and its proper function is required for regulating key nuclear events such as transcription, DNA repair, and mitosis. In *Tetrahymena thermophila*, the genome is separated into a diploid germ line micronucleus (MIC) that divides by mitosis and a polyploid somatic macronucleus (MAC) that divides amitotically. This unusual nuclear organization provides novel opportunities for the study of SUMOylation and Ubc9p function. We identified the *Ubc9* gene and demonstrated that its complete deletion from both MIC and MAC genomes is lethal. Rescue of the lethal phenotype with a GFP-UBC9 fusion gene driven by a metallothionein promoter generated a cell line with CdCl₂-dependent expression of green fluorescent protein (GFP)-Ubc9p. Depletion of Ubc9p in vegetative cells resulted in the loss of MICs, but MACs continued to divide. In contrast, expression of catalytically inactive Ubc9p resulted in the accumulation of multiple MICs. Critical roles for Ubc9p were also identified during the sexual life cycle of *Tetrahymena*. Cell lines that were depleted for Ubc9p did not form mating pairs and therefore could not complete any of the subsequent stages of conjugation, including meiosis and macronuclear development. Mating between cells expressing catalytically inactive Ubc9p resulted in arrest during macronuclear development, consistent with our observation that Ubc9p accumulates in the developing macronucleus.

Posttranslational modification by small ubiquitin-related modifiers (SUMO) is a major regulator of protein function (reviewed in references 1–5). Unlike ubiquitin, which primarily targets proteins for proteasome-mediated degradation, SUMOylation alters the intracellular localization, protein-protein interactions, or posttranslational modifications of the target (6, 7). The importance of SUMOylation is evident from its roles in the regulation of transcription, mitosis, meiosis, and DNA damage repair (2, 8–10). The SUMO protein is expressed in known eukaryotes, and many proteins required for SUMOylation, including Ubc9p, are highly conserved from protozoa to multicellular species (8). Like ubiquitin, mature SUMO proteins are activated by a heterodimeric E1-activating enzyme (9) in an ATP-dependent reaction. Subsequently, SUMO is transferred from the E1 enzyme active-site Cys to a Cys residue-linked thioester bond in the E2 enzyme known as Ubc9p (10). In the last step, SUMO is attached to the target protein through a Lys-linked isopeptide bond. In vitro, conjugation of SUMO onto substrates can be done directly by Ubc9p; in vivo, E3 ligases increase the specificity and efficiency of the reaction (11, 12).

Ubc9p is the only known SUMO E2 enzyme and therefore is a key modulator of SUMOylation. Ubc9p was first described as an essential protein for mitosis in fission yeast (13). Studies of several eukaryotes highlight its importance in multiple aspects of mitosis, including the maintenance of chromosome integrity, proper chromosome segregation, cell cycle progression, kinetochore assembly, and cytokinesis (14–16). In *Xenopus* egg extracts, the dissociation of sister chromatids is blocked at the metaphase-anaphase transition when SUMOylation of topoisomerase II is inhibited by dominant negative Ubc9p (DN-Ubc9p) (17). Reduction of Ubc9p activity in zebrafish shows that Ubc9p is required for the G₂/M transition and progression through mitosis during vertebrate organogenesis (18).

Apart from its function in mitosis, Ubc9p is also involved in DNA damage repair. SUMOylation plays important roles in the repair of DNA double-strand breaks (DSBs) via homologous recombination (HR) and nonhomologous end joining (NHEJ). For example, both Rad51 and Rad52, key components of HR machinery, interact with both SUMO1 and Ubc9p (19–21).

Ciliated protozoa offer a unique platform for studies of nuclear functions of SUMOylation. Like other ciliates, *Tetrahymena thermophila* displays “nuclear dimorphism” where germ line and somatic genome functions are separated between two nuclei: the micronucleus (MIC) and macronucleus (MAC), respectively (22). The diploid micronuclei possess features of typical eukaryotic nuclei: they divide by mitosis during vegetative cell division and undergo meiosis during sexual reproduction, also known as conjugation. Unlike a typical eukaryotic nucleus, the macronucleus is transcriptionally inert. Gene transcription is limited to the macronucleus, which is composed of an amplified subset (~45 copies) of the sequences present in the MIC. Both nuclei replicate their genomes and divide during vegetative growth, but the MAC divides by an amitotic process. Previous studies demonstrated that RNA interference (RNAi) gene silencing of *UBA2* and SUMO in another ciliate, *Paramecium tetraurelia*, had little effect on vegetative cells but prevented the programmed excision of short regions of DNA called internal eliminated sequences (IESs) during formation of the somatic macronucleus (23). More recent studies of *Tetrahymena* demonstrated that a large increase in SUMOylation occurs during the sexual life cycle when SUMO protein and Uba2p accumulate in the developing macronucleus (24). Although we antic-
imated that depletion of SUMO or Uba2p would result in arrest during macronuclear development, these cells failed to pair, and therefore, later stages of development could not be evaluated (24).

In this study, we found that complete deletion of UBC9 was lethal, but reduced expression of Ubc9p resulted in different effects on MACs and MICs. The MICs were lost from cells during vegetative growth, but MACs continued to divide. In contrast, lethal, but reduced expression of Ubc9p resulted in different effects on MACs and MICs. These findings provide evidence for a prominent role of Ubc9p in micronuclear mitosis and strengthen our hypothesis that SUMOylation is critical during sexual reproduction for cell pairing and macronuclear development.

### MATERIALS AND METHODS

**Strains and cell culture.** *Tetrahymena thermophila* cell lines were obtained from the *Tetrahymena* Stock Center (Cornell University, Ithaca, NY). Cells were cultured in 1X SPP medium (2% proteose peptone, 0.1% yeast extract, 0.2% glucose, and 0.003% FeCl₃) at 30°C according to essential growth requirements (58).

**PCR to confirm knockout lines**

- **UBC9 knockout cassette**
  - UBC9's flank K = TGTTATCCATTTATGACCAAAATTTTCTA
  - UBC9's flank R = TTAAGCCCTATATGAGTTCGCTGCT
  - MTTpR = TTTCGTAACCATGCAAAATT

- **RT-PCR assay of conditional lines**
  - UBC9WTs' UTRF = TCAATTCAAGATCGGAAAA
  - UBC9WTs' UTRR = GCTGATTTCCAATCTTCTTCC
  - UBC9CodingF + start = ATGGGGACATGTGCAAACAAAATA
  - UBC9CodingR - stop = ACTGGAAATTCGTTTTTTTCATGGGT

- **GFP-UBC9 construct**
  - UBC9-startF = CACCTAGCAACAAAAATAAAGAAGTAAATGAATTAG
  - UBC9 +stopR = TCAGTCCTTTTTTTTTCATGTCAGAAA

- **UBC9-mCherry construct**
  - UBC9CodingF + start = ATGGGGATCATGTGCAAACAAAATA
  - UBC9CodingR - stop = ACTGGAAATTCGTTTTTTTCATGGGT
  - UBC9 FlankF = ATGGGGGCCGCGCAAAATTCAACAAATTTAAAA
  - UBC9 FlankR = TGAGCGCTCAGAAGGAGAACAA

### TABLE 1: Oligonucleotides used in this study

<table>
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<th>Purpose</th>
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<th>Sequence</th>
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<td>GTACACTGAGAGGAAACCTAATGCGGTATTAGTACA</td>
</tr>
<tr>
<td>UBC9 5' flank R</td>
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<tr>
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</tr>
<tr>
<td>UBC9 3' flank R</td>
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</tr>
<tr>
<td>PCR to confirm knockout lines</td>
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<td>TTGTATCCATTTATGACCAAAATTTTCTA</td>
</tr>
<tr>
<td>UBC9WTUpstreamR</td>
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<td></td>
</tr>
<tr>
<td>MTTpR</td>
<td>TTTCGTAACCATGCAAAATT</td>
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<td>RT-PCR assay of conditional lines</td>
<td>UBC9WTs' UTRF</td>
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<tr>
<td>UBC9WTs' UTRR</td>
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<td></td>
</tr>
<tr>
<td>UBC9CodingR - stop</td>
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<td>GFP-UBC9 construct</td>
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<td>UBC9-mCherry construct</td>
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</tr>
<tr>
<td>UBC9 FlankR</td>
<td>TGAGCGCTCAGAAGGAGAACAA</td>
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**Construction of plasmids.** A *Tetrahymena* UBC9 knockout construct (pUbC9KO) was generated by ligating UBC9 flanking sequences into pMNBL, which contains a paromomycin-selectable cassette expressed via the metallothionein 1 promoter that is induced by CdCl₃ from *T. thermophila* (30). UBC9 upstream sequences (1,202 bp; positions 354009 to 355211 of scaffold 8254664) were amplified from a genomic DNA template from wild-type strain B2086 by PCR using Phusion DNA polymerase (Thermo Fisher Scientific Inc., Waltham, MA). The knockout cassette primers are shown in Table 1. The PCR product was cloned into the unique Xhol and BglII restriction sites of pMNBL. The corresponding downstream flanking sequences (1,404 bp; positions 356442 to 357846 of scaffold 8254664) were amplified and cloned into the unique BamHI and NsiI restriction sites.

**A GFP-UBC9 fusion construct was made in a pENTR Gateway plasmid (Life Technologies, Carlsbad, CA).** The 997-bp coding region of the UBC9 gene (TThERM_00522720, NW_002476431.1 [GI:22955896]) from the second codon (eliminating the initiating methionine) to the TGA stop codon was PCR amplified and cloned into the pENTR-D entry vector. The gene cassette in the entry vector was then inserted into a PBS-MTT-GFP-gtw destination vector (obtained from Doug Chalker, Washington University, St. Louis, MO) by using the LR recombination in the Gateway cloning system (Life Technologies, Carlsbad, CA). Successful integration of the GFP-UBC9 fusion gene at the *Tetrahymena* RPL29 locus conferred cycloheximide resistance (12.5 μg/ml) (31).

**Ubc9p-mCherry C-terminal fusions were made in plasmid pmCherryLAP-Neo2 as described previously (32).** Approximately 1 kb of the 3' end of the UBC9 coding region was ligated into the Kpn1 and NotI sites adjacent to the mCherry-LAP (localization and affinity purification) tag, and 1 kb of the 3' downstream sequence was placed adjacent to the NEO2 selectable marker, which confers paromomycin resistance.

**Construction of UBC9 germ line knockout heterokaryons.** Plasmid pUBC9KO was purified by using a Qiagen Plasmid Maxi kit (Qiagen, Valencia, CA) and linearized with Xhol and NotI digestion. Gold particles (S550d DNSadel Gold carrier particles; Seashell Technology LLC) were coated with DNA and transfected into the macronuclear compartment of the parental strain. The resulting heterokaryons were selected on media containing paromomycin (12.5 μg/ml) and cycloheximide (12.5 μg/ml). The introduction of the UBC9 deletion was confirmed by PCR and Epi-fluorescence microscopy.
the DNA and introduced to mating B2086 and CU428 cells at 2.5 to 3.5 h postmixing (33) by biolistic bombardment, as described previously (34). Putative microcultural UBC9 knockouts (ΔUBC9) were selected by growth in the presence of paromomycin (100 μg/ml) followed by 6-methylpurine (6-MP) (7.5 μg/ml). Paromomycin selects for insertion of the knockout construct in the genome, and 6-MP selects for cells that have completed conjugation and formed a new macronucleus. The heterozygous (ubc9(neo3/ubc9 [pm-r; paromomycin resistant]) transformants were test crossed to wild-type strains to confirm the expected genetic segregation of 50% paromomycin resistance among progeny. After confirmation, the heterozygous cell line was mated to star strains B( VI) and B(VII) to generate two homoygous germ line knockout heterokaryon strains of different mating types, BV1 ΔUBC9 (ubc9(neo3/ubc9[VI; UBC9” pm-s]) and BVII ΔUBC9 (ubc9(neo3/ubc9[ VII; UBC9” pm-s; paromomycin sensitive]). Star strains contain defective micronuclei that do not contribute mitotic products in the cross and therefore result in endoreduplication of the haploid genome from the heterozygous microcultural knockout strain and generation of a homozygous knockout MIC genome. Star crosses do not complete conjugation; they maintain their parental MAC, and the progeny of the star parent contains a wild-type MAC with a homozygous knockout MIC. BV1 and BVII ΔUBC9 germ line knockout heterokaryons were crossed to obtain progeny that are complete ΔUBC9 homoygous homokaryon strains. These cells were used for phenotypic analyses with the initially generated complete micronuclear and macronuclear knockout lines described above. Elimination of the UBC9 gene was confirmed by genomic PCR (see Fig. 4C) and genetic crosses to wild-type strains (see Fig. 3).

Generation of a conditional UBC9 mutant line. The pGFP-UBC9 construct that was inserted adjacent to the RPL29 locus was biologically transformed (33) into mated heterokaryon homoygous ΔUBC9 cells at 8 h postmixing. Cells were selected with 12.5 μg/ml cycloheximide and induced with CdCl2 to initiate the expression of the GFP-UBC9 fusion gene. Background cycloheximide-resistant cell lines could be generated by transformation of the parental heterokaryon cell lines, but these cells would have paromomycin-sensitive MACs. The cycloheximide-resistant GFP-UBC9 progeny of the cross were tested for paromomycin resistance to demonstrate successful mating and generation of the new UBC9 knockout MAC. 

Isolation of DNA and RNA and RT-PCR. Genomic DNA was isolated by phenol-chloroform extraction followed by isopropanol precipitation, as described previously (34). Wild-type Tetrahymana thermophila strain B2086 and conditional UBC9 KO strains were used for isolation of total RNA by using a Qiagen RNeasy minikit (Qiagen, Valencia, CA) with a complete protocol. For reverse transcription-PCR (RT-PCR), total RNA was reverse transcribed-PCR (RT-PCR), using an M-MLV reverse transcriptase (Promega, Madison, WI). The cDNA reaction was amplified using specific UBC9 primers (UBC9 forward, 5′-CAGACATGTGAGCTGACAGA-3′; UBC9 reverse, 5′-CTGACATCTAAGAGCGGATG-3′) and fluorescently labeled M-MLV primers to amplify the 160-bp fragment of UBC9. The PCR products were visualized on an agarose gel and quantitated by densitometry.

RESULTS

UBC9 is an essential gene. We identified a single UBC9 homolog in the Tetrahymena genome. Searches of the Tetrahymena Genome Database (http://ciliate.org/index.php/home/welcome/) with UBC9 orthologs from Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Drosophila melanogaster revealed the same top BLAST hits (TTHERM_00522720). Reciprocal searches of the S. pombe and D. melanogaster genomes using the Tetrahymena ortholog identified the corresponding UBC9 genes as the top BLAST hits. The Tetrahymena Ubc9p deduced amino acid sequence has ~55% identity with UBC9 orthologs from yeast and Drosophila (Fig. 1). The amino acid sequence is 87% identical across the conserved catalytic domain (amino acids 92 to 100) and contains the conserved catalytic cysteine residue found in other Ubc9 proteins (35).

Previous studies showed that UBC9 orthologs are essential in budding yeast, nematodes, zebrafish, and mice (10, 15, 18, 36). An exception is S. pombe, where the deletion of hus5 (UBC9 ortholog) causes severe mitotic defects and slow growth but not death (14). To determine if UBC9 is essential in Tetrahymena, micronuclear (germ line) UBC9 knockout cell lines were constructed. The MIC knockout was constructed according to standard procedures by inserting the neo3 cassette (conferring paromomycin resistance) into one allele of the MIC UBC9 locus (Fig. 2A). This cell line was subsequently mated to star strains to generate cells with a homozygous knockout of UBC9 in the MIC and a wild-type MAC (Fig. 2B). Details of the construction of these homozygous MIC heterokaryons can be found in Materials and Methods (construction of UBC9 germ line knockout heterokaryons). Since the MIC is not transcribed, the knockout heterokaryon cell lines are phenotypically paromomycin sensitive (wild-type MAC), but PCR amplification generated the expected 480-bp product derived from the neo3 insertion in the UBC9 MIC gene (Fig. 2C). The homozygous ΔUBC9 heterokaryon cell lines were mated to wild-type strains and generated progeny that were 100% paromomycin resistant. This showed that the heterokaryon lines were fertile and homozygous for neo3 in the MIC (Fig. 3). The two heterokaryon...
lines were mated to each other to generate complete deletions of UBC9 in both the MIC and MAC of the progeny. Individual pairs were isolated and placed into drops with nutrient medium. The number of cells in each drop was counted when cell division was arrested (between 48 and 72 h). The average number of cells per drop for the 75% of cell lines that arrested and subsequently died was ~420. Assuming geometric growth and starting with 2 cells per drop, we estimate that progeny died roughly 7 to 8 cell divisions after mating (Fig. 3). The remaining drops contained live cells after 72 h, but these cells could result from a failure to complete conjugation rather than the survival of progeny with a complete UBC9 deletion. To distinguish between these possibilities, surviving cells were tested for resistance to paromomycin. True progeny that successfully completed conjugation by exchanging genetic material and forming a new MAC should be paromomycin resistant. All drops were sensitive to paromomycin, consistent with the low level of expression when cells were grown in the presence of CdCl2 (Fig. 4B, middle and right). In order to verify GFP-UBC9 CdCl2-dependent regulation, conditional mutants were grown in medium without CdCl2, and in the presence of different concentrations of CdCl2. Cells were fixed with 4% paraformaldehyde and examined by using fluorescence microscopy. As shown in Fig. 4B (left), no fluorescence signal was detected in the cells cultured without CdCl2, whereas fluorescence was observed only in wild-type cells and was not present in conditional mutants (Fig. 4B, middle and right). RT-PCR was carried out to confirm the presence of RNA transcripts containing UBC9. Two sets of primers were designed, as indicated in Fig. 4C. The first set was used to detect UBC9 transcripts from the endogenous locus. The forward primer was in the conditional mutants expressed only the transgene into the RPL29 locus of the new MAC conferred cycloheximide resistance (37). Transformants were selected with cycloheximide and induced with CdCl2, as soon as progeny were placed into growth medium to initiate the expression of the GFP-UBC9 fusion gene. The resulting progeny cell lines were green fluorescent protein (GFP) positive and paromomycin resistant, as expected. As predicted by the localization of Ubc9p orthologs in other species, we observed nuclear GFP localization when cells were grown in the presence of CdCl2 (Fig. 4B, middle and right). In order to verify GFP-UBC9 CdCl2-dependent regulation, conditional mutants were grown in medium without CdCl2, and in the presence of different concentrations of CdCl2. Cells were fixed with 4% paraformaldehyde and examined by using fluorescence microscopy. As shown in Fig. 4B (left), no fluorescence signal was detected in the cells cultured without CdCl2, whereas fluorescence was observed only in wild-type cells and was not present in conditional mutants (Fig. 4B, middle and right). RT-PCR was carried out to confirm the presence of RNA transcripts containing UBC9. Two sets of primers were designed, as indicated in Fig. 4C. The first set was used to detect UBC9 transcripts from the endogenous locus. The forward primer was in the 5’untranslated region (UTR) of endogenous UBC9, and the reverse primer was inside the UBC9 coding region (Fig. 4C, arrowheads). The second set of primers was inside the UBC9 coding region (Fig. 4C, arrows). The band corresponding to the endogenous UBC9 transcript was observed only in wild-type Tetrahymena cells and was not present in ΔUBC9 conditional mutants (Fig. 4D, top). On the other hand, bands corresponding to transcripts from the UBC9 coding region were detected in both the wild type and conditional mutants (Fig. 4D, middle), which demonstrated that ΔUBC9 conditional mutants express only the GFP-UBC9 form and not wild-type UBC9. We therefore conclude that a conditional CdCl2-dependent UBC9 expression strain was generated.

## Graphical Abstract

**Figure 1** Sequence alignment of Ubc9 proteins from different species. Dark shading represents residues with high levels of identity across all species. Light shading indicates less conserved residues. The arrow indicates the conserved active-site cysteine residue that was mutated as part of this study. The GenBank accession numbers for Ubc9 are as follows: NM_194261.2 for Homo sapiens, NM_001177761.1 for Mus musculus, NM_001087289.1 for Xenopus laevis, BC059506.1 for Danio rerio, AB017607.1 for D. melanogaster, Z74112.1 for S. cerevisiae, XM_004034933.1 for Ichthyophthirius multifilis, XM_001430839.1 for P. tetraurelia, and XP_001014450 for T. thermophila.

**Figure 2** Generation of a cell line with conditional Ubc9p expression. The lethal phenotype of strains with a complete deletion of UBC9 is essential for cell viability. Presumably, cells survive for several fissions after conjugation (with the formation of a new MAC) because Ubc9p from the parental cell is stable and is gradually diluted and/or degraded as the progeny cells divide. The results also demonstrate that there is no requirement for expression of the UBC9 gene from the developing MAC to complete conjugation.

**Figure 3** Insertion of the transgene into the RPL29 locus of the new MAC conferred cycloheximide resistance (37). Transformants were selected with cycloheximide and induced with CdCl2, as soon as progeny were placed into growth medium to initiate the expression of the GFP-UBC9 fusion gene. The resulting progeny cell lines were green fluorescent protein (GFP) positive and paromomycin resistant, as expected. As predicted by the localization of Ubc9p orthologs in other species, we observed nuclear GFP localization when cells were grown in the presence of CdCl2 (Fig. 4B, middle and right). In order to verify GFP-UBC9 CdCl2-dependent regulation, conditional mutants were grown in medium without CdCl2, and in the presence of different concentrations of CdCl2. Cells were fixed with 4% paraformaldehyde and examined by using fluorescence microscopy. As shown in Fig. 4B (left), no fluorescence signal was detected in the cells cultured without CdCl2, whereas fluorescence was observed only in wild-type cells and was not present in conditional mutants (Fig. 4B, middle and right). RT-PCR was carried out to confirm the presence of RNA transcripts containing UBC9. Two sets of primers were designed, as indicated in Fig. 4C. The first set was used to detect UBC9 transcripts from the endogenous locus. The forward primer was in the 5’untranslated region (UTR) of endogenous UBC9, and the reverse primer was inside the UBC9 coding region (Fig. 4C, arrowheads). The second set of primers was inside the UBC9 coding region (Fig. 4C, arrows). The band corresponding to the endogenous UBC9 transcript was observed only in wild-type Tetrahymena cells and was not present in ΔUBC9 conditional mutants (Fig. 4D, top). On the other hand, bands corresponding to transcripts from the UBC9 coding region were detected in both the wild type and conditional mutants (Fig. 4D, middle), which demonstrated that ΔUBC9 conditional mutants express only the GFP-UBC9 form and not wild-type UBC9. We therefore conclude that a conditional CdCl2-dependent UBC9 expression strain was generated.
Depletion of UBC9 leads to reduced cell growth and nuclear defects. To determine whether depletion of Ubc9p affects the Tetrahymena growth rate, equal numbers of cells (200 cells/ml) were inoculated into medium with or without 1.0 μg/ml CdCl2, and cells were counted at intervals of 4, 8, 18, 24, and 48 h. As shown in Fig. 5, ΔUBC9 conditional cells had a lower growth rate than did wild-type cells in the absence of CdCl2. However, the same cell lines cultured in CdCl2 exhibited growth rates similar to those of wild-type cells in CdCl2 during the first 24 h, and cell numbers then increased over the next 24 h to levels nearly as high as those of wild-type cells. In contrast, ΔUBC9 conditional cells were highly sensitive to the presence of CdCl2, as indicated by the rapid decrease in cell numbers upon treatment with the metal ion. These results suggest that Ubc9p plays an essential role in the survival of Tetrahymena in the presence of CdCl2, likely by facilitating the removal of damaged or misfolded proteins.
the wild type without CdCl₂ (Fig. 5). It should be noted that the conditional mutant grown in the absence of CdCl₂ also showed growth, most likely due to leaky expression from the MTT1 promoter, as we have seen in previous studies (24). In other species, Ubc9p has been shown to regulate cell cycle progression, including mitosis and chromosome segregation (14, 15, 38). Depletion of Ubc9p has been shown to cause chromosome missegregation in zebrafish (18). DAPI staining revealed that many Ubc9p-depleted cells lost detectable micronuclei (Fig. 6A, middle). Conditional mutants as well as wild-type cells, cultured with or without CdCl₂, were examined after 24 h. Micronuclei were counted, and a summary of the data is shown in Fig. 6B. Nearly half of the Ubc9p-depleted cells contained no DAPI-detectable micronuclei, compared with none of the wild-type cells, in the presence or absence of CdCl₂. We observed unequal partitioning of DNA to daughter micronuclei in 6 of 9 cells that were identified at late stages of mitosis, consistent with defects in the separation of sister chromatids (Fig. 6C). Unlike most eukaryotes, Tetrahymena cells do not require accurate chromosome segregation during mitosis because

FIG 4. Generation of conditional UBC9 knockout cell lines. (A) Schematic drawing of the approach used to generate a conditional GFP-UBC9-expressing strain. ΔUBC9 homozygous heterokaryons of different mating types were mixed to mate cells. The GFP-UBC9 construct was biolistically transformed into mated cells at 8 h postmixing. (B) Expression of GFP-Ubc9p is regulated by CdCl₂. The conditional GFP-Ubc9p strains were grown in SPP medium supplemented with different concentrations of CdCl₂. Cells were fixed, stained with DAPI, and viewed by fluorescence microscopy. Exposure times were identical for all pictures. FITC, fluorescein isothiocyanate. (C and D) RT-PCR was used to confirm that endogenous UBC9 transcript cannot be detected in conditional mutants. The location of the primers used to amplify regions from cDNA are shown. (C) One set of primers was used to assay the UBC9 transcript at its endogenous locus. The forward primer is located in the UBC9 5′ UTR, and the reverse primer is located inside UBC9 (arrowheads). A second set of primers was used to assay the UBC9 coding region (arrows). (D) Endogenous UBC9 transcript was detected only in the wild-type strain but not conditional mutants, while the UBC9 coding region was present in both the wild type and conditional mutants.

FIG 5. Growth curve of the UBC9 depletion strain. ΔUBC9 conditional mutant and wild-type cells were inoculated into 10 ml of SPP medium at a concentration of 200 cells/ml in either the presence or absence of CdCl₂. Cell populations were measured by direct cell counts at 0, 4, 8, 18, 24, and 48 h. Cond. + Cd, conditional mutant with CdCl₂; Cond. −Cd, conditional mutant without CdCl₂. Error bars indicate standard deviations from three independent experiments.
played a multiple-MIC phenotype similar to that of DN-UBC9 KO cells (66% versus 72%). To determine whether overexpression of wild-type UBC9 had the same effect on the number of MICs, we scored cells expressing dual-tagged 6×His-, 3×FLAG-Ubc9p (6H3F-UBC9) that was also driven by the MTT1 promoter and induced by CdCl2. Only 9% of cells had >2 MICs (Fig. 7A). This is similar to what we saw in wild-type cells in the presence of CdCl2 (5%), which is apparently due to the effect of CdCl2. This rather remarkable difference in phenotype between the depletion of Ubc9p and overexpression of defective Ubc9p cannot be readily explained. We examined the DN-UBC9 cell lines for defects in cytokinesis, but no obvious problems were identified. Individual cells were cultured in drops, and total cells were counted after 24 h, but no statistical differences between DN-UBC9 and wild-type cells could be found (data not shown). In addition, a time course experiment showed that >50% of induced DN-UBC9 cells contained >2 MICs within the first 9 h after the addition of CdCl2 (see Fig. S3 in the supplemental material). This short time interval suggests that we should have detected a cytokinesis defect in cell division if it was responsible. No cells showed evidence of incomplete MAC separation between cells at division, analogous to the cut phenotype of yeast (40) (data not shown). There are possible alternative explanations for the increased number of MICs presented in the Discussion.

Depletion of Ubc9p results in hypersensitivity to DNA-damaging agents. A role for SUMOylation in DNA damage repair, especially in response to double-strand breaks, has been established for mammalian species and yeast (41–45). To evaluate whether UBC9 is involved in DNA damage repair in Tetrahymena, we tested the effect of the DNA-damaging agents methyl methanesulfonate (MMS) and cisplatin on Ubc9p-depleted cells. ΔUBC9 conditional mutants and wild-type cells were cultured separately in SPP medium containing 1.0 μg/ml CdCl2 or no CdCl2. Cells were treated with 8 mM MMS or 2 mM cisplatin for 2 h and then washed twice with 10 mM Tris (pH 7.5). Single cells were placed into drops (~15 μl) of SPP medium containing 0.5 μg/ml CdCl2. CdCl2 in the posttreatment drops provides conditions that are optimal for growth of conditional cell lines that would have poor growth without CdCl2. After 48 h, the cells in each drop were counted and scored as viable if >500 cells were in a drop and nonviable if there were 0 to 10 cells. No drops had cell numbers between 10 and 500. As shown in Fig. 8, ΔUBC9 conditional cells cultured in the absence of CdCl2 showed only 12% survival in MMS, compared with 90% survival when cultured with CdCl2. The survival rate with cisplatin increased ~15-fold (5% versus 80%) in the presence of CdCl2. Wild-type cells showed little change in sensitivity regardless of the presence or absence of CdCl2 (Fig. 8). This finding is consistent with a role of Ubc9p and SUMOylation in DNA break repair.

Ubc9p localizes to developing macronuclei. We recently showed that a large increase in the amount of SUMOylated protein is observed during Tetrahymena sexual reproduction and that the increased signal is centered in the developing macronucleus (24). If this SUMOylation occurs within or around the developing MAC, then the signal for Ubc9p should also accumulate in the anlagen. To establish the location of Ubc9p during conjugation, we generated Tetrahymena cell lines in which mCherry is fused to the C terminus of UBC9. The transgene is created by homologous recombination in the MAC and is driven by the endogenous UBC9 promoter. Cells were starved and mixed to induce mating

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No changes in formatting or content are necessary for this task.
Ubc9p is required for pair formation in conjugating Tetrahymena cells. The localization of Ubc9p-mCherry to the developing macronucleus suggested a role in conjugation; consequently, we mated our UBC9 conditional cell lines to assay for a conjugation-defective phenotype. UBC9 conditional mutants of different mating types were cultured overnight in SPP medium with or without CdCl₂ to promote or reduce the expression of the GFP-UBC9 transgene. Cells were washed twice in 10 mM Tris (pH 7.5) and cultured in the same medium plus or minus CdCl₂. Wild-type cells served as controls. UBC9 conditional cells from non-CdCl₂-treated cultures (Ubc9p depleted) were mixed to initiate mating. Separate cultures of CdCl₂-treated Ubc9p conditional lines or wild-type cells were also mixed to initiate cell pairing. Cells were evaluated at 2, 4, 6, and 8 h postmixing for pair formation. As shown in Fig. 10, UBC9 conditional mutant cultures that were not exposed to CdCl₂ (Ubc9p depleted) were unable to form mating pairs at 2 h postmixing and formed only 10% pairs after 8 h, well past the expected pairing period. In contrast, the same cell line supplemented with CdCl₂ was able to generate 40 to 60% mating pairs. Wild-type cells exhibited mating efficiencies of >90%. We consistently observed a slight loss of mating efficiency when wild-type cells were exposed to CdCl₂ (Fig. 10), so a large increase upon exposure to CdCl₂ is particularly significant. In addition to Ubc9p effects on pairing, other experiments showed a pairing defect for Uba2p- and Smt3p (SUMO)-depleted cell lines (24). When UBC9 conditional cells cultured in the absence of CdCl₂ (UBC9 depleted) were mixed with wild-type cells, pairs formed and subsequently entered meiosis (see Fig. S2 in the supplemental mate-
Regardless of CdCl2 treatment (data not shown). Cells expressing dominant negative Ubc9p showed normal pairing re-
tection. As an alternative approach, we examined matings between
two different mating types were cultured in growth medium
in independent experiments.

Nonviable. Viability is expressed as the percentage of viable drops out of the
500 cells were scored as viable, and drops containing
10 cells were scored as
data not shown).

Dominant negative Ubc9p results in arrest at the macronu-
clear development stage. The inability of Ubc9p-depleted cells to
form pairs prevented the analysis of later stages during conjugation.
As an alternative approach, we examined matings between
cells expressing the above-described DN-Ubc9p. DN-Ubc9p cells of two different mating types were cultured in growth medium
and subsequently starved without CdCl2. Cells were then mixed to
start conjugation. Half the volume of mixed cells was placed into a
separate petri dish and supplemented with 0.1 μg/ml CdCl2 at 6 h
postmixing. At 24 h postmixing, samples were fixed, stained with
DAPI, and examined by fluorescence microscopy. DN-
UbC9p cells that were not treated with CdCl2 progressed through anlagen forma-
tion normally, as did wild-type cells and cells expressing an
affinity-tagged version of UBC9 (6H3F-UBC9) (Fig. 11, micro-
graphs). DN-Ubc9p cells that were treated with CdCl2 (inducing the expression of DN-Ubc9p) contained a large fraction of cells
that remained as mating pairs that retained their parental MAC
(42% of total cells scored) (Fig. 11). To test whether an earlier
addition of CdCl2 would result in an earlier arrest in conjugation,
a small volume of mixed DN-Ubc9p cells was placed into a sepa-
rate petri dish and induced with 0.1 μg/ml CdCl2 at the time of
mixing. A similar fraction of cells also arrested with the same phe-
notype at 24 h (data not shown). These crosses using cells with
excess catalytically defective Ubc9p demonstrate a role for Ubc9p
in completion of conjugation.

DISCUSSION

Ubc9p regulates micronuclear chromosomal segregation. Se-
veral results from our study are consistent with data from previous
investigations of Ubc9: (i) complete deletion of UBC9 was lethal, a
result consistent with findings for S. cerevisiae, Caenorhabditis el-
egans, and mammalian cells (10, 18, 36, 46); (ii) deletion of
Ubc9p resulted in greater sensitivity to MMS and cisplatin, con-
sistent with its role in DNA damage repair seen in other studies
(41, 47); and (iii) disruption of Ubc9p function resulted in defec-
tive mitosis of the micronucleus. MICs were undetectable in a
large fraction of UBC9-depleted cells during vegetative growth.
Nine cells were identified during cell division and six displayed
large differences in the intensity of DAPI-stained MICs such as
those shown in Fig. 6C. The results are consistent with a misseg-
regation of MIC chromosomes during mitosis. The requirement
for SUMOylation in chromosome segregation is well established
for other species (48–51). In budding yeast, deletion of UBC9
results in gross defects in chromosome structure and integrity as
well as aberrant segregation and polyploidy (15). There is also
evidence that SUMOylation plays critical roles in centromere
function. The Saccharomyces cerevisiae Sbx5/8 complex is the
founder member of a recently defined class of SUMO-targeted ubiq-
tin ligases (STUbLs) (52). The Sbx5/8 complex is preferen-
tially located near centromeres, and deletion of either gene shows
severe mitotic defects that include aneuplody and spindle misposi-
tioning (53). Defective chromosome segregation is also linked to
defective SUMOylation of topoisomerase II (54) and the mecha-
nism of kinetochore assembly and disassembly (reviewed in refer-
ence 51).

A defective mitotic phenotype was described previously for
Tetrahymena by Cui and Gorovsky (55) after deletion of a cen-
tromeric H3 protein called Cna1p. The CNA1 gene encodes a
centromere-specific histone H3 variant, Cna1p, also known as
CenH3 (56–58), that is associated with centromeric DNA in place
of the typical H3. CenH3 is required for functional centromeres
and recruitment of other centromeric and spindle checkpoint
proteins (59). A reduction in the MAC copy number of CNA1
results in MIs that are smaller than MIs in wild-type cells (55),
rather than the loss-of-MIC phenotype observed for our Ubc9p
deletion cells. These small MIs continue to undergo mitosis,
but DNA is unequally distributed to daughter nuclei (55), which
is similar to our observations for cells with reduced expression of
Ubc9p.

Surprisingly, the overexpression of catalytically inactive dom-
inant negative Ubc9p resulted in multiple MIs per cell. In
chicken cells, the depletion of Ubc9p generated a fraction of cells
with multiple nuclei (46), and the authors of that report suggested
that a cytokinesis defect could be responsible. In our Tetrahymena
DN-UBC9 cells, it is possible to generate multi-MIC cells without
any effects on the genetic phenotype because the MIC is transcrip-
tionally silent. However, we did not detect any defect in cytokine-
sis. Cell counts after 24 hours for wild-type, 6H3F-UBC9, and
DN-UBC9 single cells in the presence or absence of CdCl2 did not

![FIG 8 Depletion of Ubc9p results in hypersensitivity to DNA-damaging agents. UBC9 conditional mutant strains were grown in SPP medium in the presence or absence of 1.0 μg/ml CdCl2, for 24 h. Cells were treated with 8 mM MMS or 2 mM cisplatin for 2 h and then washed twice with 10 mM Tris-Cl (pH 7.5). Eighty single cells were isolated in drops containing growth medium in the presence of 0.5 μg/ml CdCl2 and incubated for 48 h. Drops containing >500 cells were scored as viable, and drops containing <10 cells were scored as
nonviable. Viability is expressed as the percentage of viable drops out of the
total number of drops. Error bars indicate standard deviations from three
independent experiments.](http://ec.asm.org)
result in a statistically significant difference between cell lines (data not shown). A time course experiment performed after CdCl₂ induction showed that 50% of DN-UBC9 cells had MICs within 9 h (see Fig. S3 in the supplemental material), suggesting that if a cytokinesis defect was responsible, it should have been detectable. We have considered alternatives to a defect in cytokinesis, and these include either a defective mitotic checkpoint that allows reentry to mitosis or the formation of MICs with partial genomes. While we are unaware of any example of the former, there is some precedence for the latter in organisms with open mitosis that involves nuclear envelope breakdown and reformation (reviewed in reference 60). Chromosomes that do not segregate with the majority of the genome can be enclosed in a nuclear envelope. This theory has the appeal of connecting segregation defects with both depletion and dominant negative UBC9 phenotypes, but there is no evidence for this phenomenon in organisms with closed mitosis such as *Tetrahymena*. Multiple MICs were previously reported for *Tetrahymena* cells, resulting from a MAC knockout of *TGP1* (61), encoding a G-quartet DNA-binding protein, yet these cells showed normal division rates. It is possible that a novel mechanism for multiple MICs could be operating in *Tetrahymena*. Interestingly, Tgp1p has appeared as a candidate SUMO substrate and a Ubc9p-interacting protein in our recent

**FIG 9** Localization of Ubc9p during conjugation. Ubc9p was tagged with mCherry on the C terminus at its endogenous locus. UBC9-mCherry-expressing cells of different mating types were mixed to initiate conjugation. Cells were fixed at regular intervals through conjugation with 4% paraformaldehyde, stained with 1 μg/μl DAPI, and viewed with a 40× objective under a fluorescence microscope. The following developmental stages were observed: prophase meiosis I (T = 3 h), zygotic nuclei (T = 4 h), postzygotic division (T = 6 h), and formation of developing macronuclei (T = 8 h). Asterisks indicate parental MACs, arrows indicate MICs, and arrowheads indicate anlagen.

**FIG 10** Depletion of Ubc9p results in reduced pairing. ΔUBC9 conditional mutants were grown and starved in the presence and absence of CdCl₂ and mixed. Cells were fixed at 2, 4, 6, and 8 h postmixing, and 100 cells were scored for mating efficiency. CdCl₂-depleted cells were unable to form mating pairs. Error bars indicate standard deviations from three independent experiments.

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**FIG 11** Depletion of Ubc9p results in reduced pairing. ΔUBC9 conditional mutants were grown and starved in the presence and absence of CdCl₂ and mixed. Cells were fixed at 2, 4, 6, and 8 h postmixing, and 100 cells were scored for mating efficiency. CdCl₂-depleted cells were unable to form mating pairs. Error bars indicate standard deviations from three independent experiments.
proteomics studies (Q. Yang and A. M. Nasir, unpublished data). Together, our results indicate that Ubc9p plays an indispensable role in Tetrahymena mitosis through regulating chromosome segregation. Having a separate germ line nucleus that does not contribute to gene expression makes Tetrahymena a unique system for studies of mitotic chromosome segregation because other organisms are not able to survive with aneuploid nuclei.

Role of Ubc9p in Tetrahymena sexual reproduction. Our initial interest in SUMOylation in Tetrahymena was stimulated by studies of the related ciliate Paramecium tetraurelia, where RNAi-induced silencing of UBA2 or SUMO prevented the excision of micronucleus-specific DNA elements during formation of the somatic macronucleus (23). For Tetrahymena, analysis of SUMOylation requirements during sexual reproduction has proven to be more complex. A large increase in the amount of SUMOylated substrates during MAC development (24) and the accumulation of Ubc9p (Fig. 9), SUMO, and Uba2p in MAC anlagen (24) are consistent with important roles during MAC development. However, depletion of the SUMO pathway protein Ubc9p (Fig. 10), Uba2p, or SUMO (24) prevents cell pairing, the first step in sexual reproduction. The observation that deficient SUMOylation inhibits cell pairing was surprising based on the expression profile of Tetrahymena SUMO pathway genes, yet in yeast, degradation of the mating-type factor α1 protein, important for the establishment of mating type, requires the STUbLs Shk5 and Shk8 (62). The mechanism required for SUMO-dependent pairing in Tetrahymena is not known, but the recent identification of the mating-type protein (63) provides an opportunity to examine whether it involves direct SUMOylation of the mating-type protein or an indirect signaling effect. This block in cell pairing at the first step of conjugation complicated efforts to investigate later stages, including MAC development.

To overcome this SUMOylation-dependent block, we expressed dominant negative Ubc9p after the start of conjugation. A large fraction of cells (36%) remained in pairs 24 h after the start of conjugation, and the parental MAC was not destroyed. Genetic analysis confirmed that crosses between cells expressing dominant negative Ubc9p resulted in fewer true progeny than for wild-type controls (data not shown). The results provide evidence for an additional SUMO-dependent step during conjugation. Failure to degrade the parental MAC is an uncommon mutant phenotype, but Tetrahymena cells treated with nicotinamide, an inhibitor of sirtuin histone deacetylase, arrest in this stage (64). These results suggest that increased acetylation of substrates results in retention of the parental MAC. Competition between SUMOylation and acetylation is known to alter the balance of sirtuin expression in human cells (65). Another example of parental MAC retention was observed after deletion of ATG8-2, a gene encoding a protein in the autophagy pathway (66). Although there is as yet no evidence for regulation of nuclear autophagy in Tetrahymena by SUMOylation, there is evidence that SUMOylation and acetylation regulate p53-mediated autophagy in mammalian cells (67).

Considering the importance of SUMOylation in the nuclear events of mitosis, meiosis, DNA repair, and transcriptional control, we are not surprised to find that multiple steps of Tetrahymena conjugation are dependent on SUMOylation. We expect that our current efforts to identify SUMOylated substrates during sexual reproduction will reveal key targets that are required for genome reorganization during macronuclear development as well as cell pairing and nuclear degradation.

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