Depletion of UBC9 Causes Nuclear Defects during the Vegetative and Sexual Life Cycles in *Tetrahymena thermophila*

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UBC9 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 modifier (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 *in vitro*, conjugation of SUMO onto substrates can be done directly by Ubc9p; in *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-
ipated 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 depletion of UBC9 was lethal, but reduced expression of Ubc9p resulted in different effects on MCIs and MACs. The MCIs were lost from cells during vegetative growth, but MACs continued to divide. In contrast, expression of catalytically inactive DN-Ubc9p resulted in the accumulation of multiple MCIs. Consistent with data from reports on other species, Ubc9p-depleted cells were hypersensitive to DNA-damaging agents that promote double-strand DNA breaks. During the sexual life cycle of *Tetrahymena*, tagged Ubc9p localized to the developing macronucleus. Cell lines that were depleted for Ubc9p, like SUMO and Uba2p, did not form mating pairs. To overcome the block in pair formation, we overexpressed dominant negative mutants of Ubc9p, like SUMO and Uba2p, did not form mating pairs. To overcome the block in pair formation, we overexpressed dominant negative mutants of Ubc9p, like SUMO and Uba2p, did not form mating pairs.

**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 1% SPP medium (2% proteose peptone, 0.1% yeast extract, 0.2% glucose, and 0.003% FeCl₃) at 30°C according to established procedures (25). Inbred wild-type strains B2086 (MPR1/MPR1 [MPR1; II]) and CU428 (mp-s VII) were used for generating conditional lines (26–29).

**Construction of plasmids.** A *Tetrahymena* UBC9 knockdown construct (pUBC9KO) was generated by ligating UBC9 flanking sequences into pMNBL, which contains a paromomycin-selectable cassette expressed via the metallothionein I (*MTT1*) promoter that is induced by CdCl₃ from *T. thermophila* (30). UBC9 upstream sequences (1,202 bp; positions 35,000 to 35,521 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 knockdown cassette primers are shown in Table 1. The PCR product was cloned into the unique XhoI and BglII restriction sites of pMNBL. The corresponding downstream flanking sequences (1,404 bp; positions 35,542 to 35,786 of scaffold 8254664) were amplified and cloned into the unique BamHI and XhoI restriction sites of *Tetrahymena* RPL29 locus (31).

**Construction of UBC9 germ line knockout heterokaryons.** Plasmid pUBC9KO was purified by using a Qiagen Plasmid Maxi kit (Qiagen, Valencia, CA) and linearized with XhoI and NotI digestion. Gold particles were coated with DNAdel Gold carrier particles (Seashell Technology LLC) were coated with unique sequences into *Tetrahymena* scaffold 8254664) were amplified and cloned into the unique BamHI and XhoI restriction sites of *Tetrahymena* RPL29 locus (31). Gold particles (S550d) were coated with unique sequences into *Tetrahymena* scaffold 8254664) were amplified and cloned into the unique BamHI and XhoI restriction sites of *Tetrahymena* RPL29 locus (31).

**TABLE 1 Oligonucleotides used in this study**

<table>
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<th>Purpose</th>
<th>Oligonucleotide</th>
<th>Sequence</th>
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<tr>
<td>Knockout cassette</td>
<td>UBC9 3’ flankF</td>
<td>GTCACTCAGAGGAAACCTATGCCTGATGATCACA</td>
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<td></td>
<td>UBC9 3’ flankR</td>
<td>GACTGATCTGGTTAAAATTAAAGTAAAGCGGTAAGTGCCTG</td>
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<tr>
<td></td>
<td>UBC9 3’ flankF</td>
<td>TTCGGATCCCTAAGAGAATTTCGTTAACACTAG</td>
</tr>
<tr>
<td></td>
<td>UBC9 3’ flankR</td>
<td>ATTACGCGCCGAGCTATTGTAGTATTT</td>
</tr>
<tr>
<td>PCR to confirm knockout lines</td>
<td>UBC9KO Upstream F</td>
<td>TTGTTATCCTATAGCCTACAAATTTC</td>
</tr>
<tr>
<td></td>
<td>UBC9KO Upstream R</td>
<td>TGGGAATATTGATGATCCTGCT</td>
</tr>
<tr>
<td></td>
<td>MTTP_r</td>
<td>TTTGCTAAACCATACGAAAA</td>
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<td>RT-PCR assay of conditional lines</td>
<td>UBC9WTS UTRF</td>
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<tr>
<td></td>
<td>UBC9WTS UTRR</td>
<td>GTCTATTCGAACTCTTCTCC</td>
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<tr>
<td></td>
<td>UBC9 CodingF + start</td>
<td>ATGGGATTACATGTAGACAAACAAAAATA</td>
</tr>
<tr>
<td></td>
<td>UBC9 CodingR - stop</td>
<td>ACTGGATATTGCTGTTTTTTTCTAGGT</td>
</tr>
<tr>
<td>GFP-UBC9 construct</td>
<td>UBC9- start F</td>
<td>CACCTAGCAACAAATTAAAGAAGTATAAGTAAATAG</td>
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<tr>
<td></td>
<td>UBC9+stop R</td>
<td>TCAGTCCTTTTTTTATGCTGTTACGAAAA</td>
</tr>
<tr>
<td>UBC9-mCherry construct</td>
<td>UBC9 CodingF + start</td>
<td>ATGCGGTACCTAGTACCAACAAAAATA</td>
</tr>
<tr>
<td></td>
<td>UBC9 CodingR - stop</td>
<td>ACTGGATATTGCTGTTTTTTTCTAGGT</td>
</tr>
<tr>
<td></td>
<td>UBC9 Flank F</td>
<td>ATCAGGGCGGCCAAAATTCCAAAATTTAA</td>
</tr>
<tr>
<td></td>
<td>UBC9 Flank R</td>
<td>TGACAGGTCTAAAAGAGAAGACCA</td>
</tr>
</tbody>
</table>

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the DNA and introduced into mating B2086 and CU428 cells at 2.5 to 3.5 h postmixing (33) by biologic bombardment, as described previously (34). Pu-

tative micronuclear UBC9 knockouts (\(\Delta UBC9\)) were selected by growth in

the presence of paromomycin (100 \(\mu\)g/ml) followed by 6-methylpurine (6-

MP) (7.5 \(\mu\)g/ml). Paromomycin selects for insertion of the knockout con-

struct in the genome, and 6-MP selects for cells that have completed conju-

gation and formed a new macronucleus. The heterozygous (\(ubc9^{+}\)), re-

combinants were then tested to cross to a wild-type strain 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 homozygous germ

line knockout heterokaryons of different mating types, BV1 \(\Delta UBC9\)

\((ubc9^{+}\)), BVII \(\Delta UBC9\) (\(ubc9^{+}\)), and BV1 \(\Delta UBC9\) (\(ubc9^{+}\)) and BVII \(\Delta UBC9\) (\(ubc9^{+}\)). Star strains contain defective microcnuclei that do not contribute mitotic products in the cross and therefore result in endoreduplication of the haploid genome from the heterozygous micronuclear 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 \(\Delta UBC9\) germ line knockout heterokaryons were crossed to obtain progeny that are complete \(\Delta UBC9\) homozygous 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 transfor-

med (33) into mated heterokaryon homozygous \(\Delta UBC9\) cells at 8 h postmixing. Cells were selected with 12.5 \(\mu\)g/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 \(Tetrahymena\) 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 concentration of 200 cells/ml in the presence or absence of 1.0 mM Tris-Cl (pH 7.5), and starved in the same buffer in the presence of 0.05 \(\mu\)g/ml or no CdCl2 for 16 to 24 h at 30°C. Conditional mutant or wild-type cells of different mating types were mixed (time zero), and samples were taken at later time

points, fixed with an equal volume of 4% paraformaldehyde, and then stained with DAPI (4',6-diamidino-2-phenylindole) = [\(\text{DAPI} = 4',6\text{-diamidino-2-phenylindole}\)] = UTTR) (Table 1).

The pGFP-\(UBC9\) expression plasmid \(\Delta UBC9\) was used for isolation of total RNA by using a Qiagen RNeasy minikit (Qiagen, Valencia, CA) with a concentration of 200 cells/ml in the presence or absence of 1.0 mM Tris-Cl (pH 7.5), and starved in the same buffer in the presence of 0.05 \(\mu\)g/ml or no CdCl2 for 16 to 24 h at 30°C. Conditional mutant or wild-type cells of different mating types were mixed (time zero), and samples were taken at later time

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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 MT1 promoter activity in the absence of heavy metal. On the other hand, an increasing fluorescence signal was observed in the nucleus when the cells were cultured without CdCl2, and in the presence of different concentrations of CdCl2 (Fig. 4B, left). No fluorescence signal was detected in the cells cultured with 0.1 μg/ml CdCl2 (Fig. 4B, middle and right). In order to verify GFP-UBC9CdCl2-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, consistent with the low level of MT1 promoter activity in the absence of heavy metal. On the other hand, an increasing fluorescence signal was observed in the nucleus when the cells were cultured in 0.1 μg/ml or 1.0 μg/ml CdCl2 (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 demonstrates 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.

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-UBC9CdCl2-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, consistent with the low level of MT1 promoter activity in the absence of heavy metal. On the other hand, an increasing fluorescence signal was observed in the nucleus when the cells were cultured in 0.1 μg/ml or 1.0 μg/ml CdCl2 (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 demonstrates 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.

Depletion of UBC9 is vegetative lethal. Strains with a complete deletion of UBC9 (MIC and MAC) were generated by mating two UBC9 homozygous germ line knockout heterokaryon strains. Individual mating pairs were isolated and placed into drops containing growth medium. All progeny of UBC9 deletion strains died ~7 to 8 cell divisions after conjugation. Progeny of UBC9 heterokaryon knockout and wild-type cells were viable. Successful conjugation was confirmed by replica plating of cells in paromomycin-containing medium.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of individual pairs examined</th>
<th>No. of drops with live cells (72h)</th>
<th>No. of drops with paromomycin resistant cells</th>
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<tbody>
<tr>
<td>ΔUBC9- neo3 x ΔUBC9-neo3</td>
<td>80</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>ΔUBC9- neo3 x wt</td>
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<td>ΔUBC9- neo3 x wt</td>
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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 MT1 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...
Depletion of Ubc9p causes loss of micronuclei. ΔUBC9 conditional mutants were cultured in SPP medium in the presence or absence of 1.0 μg/ml CdCl2 for 24 h. Wild-type cells were cultured similarly, in the presence or absence of CdCl2. (A) Cells sampled after 24 h of culturing under different conditions were fixed with 4% paraformaldehyde, stained with DAPI, and then viewed with a 40× objective under fluorescence. Representative images show that ΔUBC9 conditional cells cultured in the absence of CdCl2 lost micronuclei but that samples from the same cell lines in the presence of CdCl2 contained at least one micronucleus. (B) The total number of micronuclei was scored and is summarized in the pie chart. One hundred cells under each condition were scored and characterized into three subsets: cells with no MIC, cells with 1 or 2 MICs, and cells with >2 MICs. (C) Ubc9p is required for proper chromosome segregation. Wild-type or ΔUBC9 conditional mutant cells cultured in 1.0 μg/ml or no CdCl2 were fixed and stained with DAPI. Examples of dividing cells (a and b) were selected to illustrate nuclear division under each condition. Arrows indicate MICs in both panels A and C.

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.
(see Materials and Methods), and samples were then fixed at various time points, stained with DAPI, and observed by using a fluorescence microscope (Fig. 9). Wild-type cells without an mCherry tag were used as controls. A faint signal was observed in the macronucleus of starved cells, indicating low expression levels of Ubc9p during starvation (Fig. 9). During early conjugation, the signal was observed exclusively in parental macronuclei during the pronuclear exchange and postzygotic division stages (T/H11005, T/H11005-4, and T/H11005-6) (Fig. 9). At 8 h postmixing, the signal accumulated in the developing MAC (T/H11005-8) (Fig. 9). The mCherry signal was much stronger in the developing macronuclei than in the DNA-rich parental MAC, which stained brightly with DAPI (Fig. 9, asterisks). The efficiency of integration of this construct was too low to successfully rescue the knockout heterokaryons; therefore, we were not able to demonstrate that Ubc9p-mCherry was fully functional. As an alternative approach, we used the GFP-UBC9 conditional mutants described above. These cells were mated to wild-type cells in the presence of CdCl2. As shown in Fig. S1 in the supplemental material, GFP-Ubc9p localization was consistent with the Ubc9p-mCherry results. The protein was found exclusively in the parental MAC during meiotic prophase and postzygotic divisions. The signal then migrated to the developing MAC and eventually disappeared from the parental MAC. Although we cannot evaluate the relative signal strength in these experiments because the expression of GFP-Ubc9p is driven by CdCl2, the results provide confidence in the localization of Ubc9p to the developing MAC, the site of developmentally regulated genome reorganization.

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-
expressing dominant negative Ubc9p showed normal pairing reaction. As an alternative approach, we examined matings between form pairs prevented the analysis of later stages during conjugation clear development stage.

of 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 /H11022 500 cells were scored as viable, and drops containing /H11021 /H11020

agents.

(42% of total cells scored) (Fig. 11). To test whether an earlier that remained as mating pairs that retained their parental MAC -depleted cells progressed through meiosis. This phenotype indicates an additional role for UBC9 in meiosis. Cells expressing dominant negative Ubc9p showed normal pairing regardless of CdCl2 treatment (data not shown).

Dominant negative Ubc9p results in arrest at the macronuclear 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-UBC9 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-UBC9 cells that were not treated with CdCl2 progressed through anlagen formation normally, as did wild-type cells and cells expressing an affinity-tagged version of UBC9 (6H3F-UBC9) (Fig. 11, micrographs). DN-UBC9 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 separate 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 phenotype 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. Several 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 elegans, and mammalian cells (10, 18, 36, 46); (ii) depletion of Ubc9p resulted in greater sensitivity to MMS and cisplatin, consistent with its role in DNA damage repair seen in other studies (41, 47); and (iii) disruption of Ubc9p function resulted in defective 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 misregulation 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 Sb5/8 complex is the founding member of a recently defined class of SUMO-targeted ubiquitin ligases (STUbLs) (52). The Slx5/8 complex is preferentially located near centromeres, and deletion of either gene shows severe mitotic defects that include aneuploidy and spindle mispositioning (53). Defective chromosome segregation is also linked to defective SUMOylation of topoisomerase II (54) and the mechanism of kinetochore assembly and disassembly (reviewed in reference 51).

A defective mitotic phenotype was described previously for Tetrahymena by Cui and Gorovsky (55) after deletion of a centromeric 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 MICs that are smaller than MICs in wild-type cells (55), rather than the loss-of-MIC phenotype observed for our Ubc9p deletion cells. These small MICs 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 dominant negative Ubc9p resulted in multiple MICs 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 transcriptionally silent. However, we did not detect any defect in cytokinesis. Cell counts after 24 hours for wild-type, 6H3F-UBC9, and DN-UBC9 single cells in the presence or absence of CdCl2 did not
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 10 Depletion of Ubc9p results in reduced pairing. ∆UBC9 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 from mating pairs. Error bars indicate standard deviations from three independent experiments.

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 macronuclei (T = 8 h). Asterisks indicate parental MACs, arrows indicate MICs, and arrowheads indicate anlagen.
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 Slt5 and Slt8 (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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