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

Qianyi Yang,^a Amjad M. Nasir,^a Robert S. Coyne,^b James D. Forney^a

Department of Biochemistry, Purdue University, West Lafayette, Indiana, USA;^a J. Craig Venter Institute, Microbial and Environmental Genomics, Rockville, Maryland, USA^b

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.

![Image](http://ec.asm.org/)

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

Qianyi Yang,^a Amjad M. Nasir,^a Robert S. Coyne,^b James D. Forney^a

Department of Biochemistry, Purdue University, West Lafayette, Indiana, USA;^a J. Craig Venter Institute, Microbial and Environmental Genomics, Rockville, Maryland, USA^b

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 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 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-
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 deletion of UBC9 was lethal, but reduced expression of Ubc9p resulted in different effects on MICS and MACs. The MICS were lost from cells during vegetative growth, but MACs continued to divide. In contrast, effects on MICS and MACs. The MICS were lost from cells during the formation of the macronucleus. 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 Ubc9p localizing conjugation and found that a substantial fraction of cells arrested during the formation of the macronucleus. 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 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 (mpr1-1/mpr1-1 [MPR1; mp-s VII]) were used for cell transformation and all other analyses. Star strains B*(VI) and CU428 ([MPR1; II]) and CU428 (mpr1-1/mpr1-1 [MPR1; mp-s VII]) were used for generating germ line knockout heterokaryons. UBC9

TABLE 1 Oligonucleotides used in this study

| Purpose            | Oligonucleotide | Sequence |
|--------------------|----------------|----------|
| Knockout cassette  | UBC9 5′ flankF | GTCACTCGAGGAGGAACCTATGGCGTATTTGATACA |
|                    | UBC9 5′ flankR | GACTAGATCTGTTTTAAAATAAAGTACGAGTTGCTG |
| PCR to confirm knock out lines | UBC9KUpstreamF | TGTGTATCTTATGAGCAAAATTTTCA |
|                    | UBC9WTupstreamR| TGGAGCTAATTTGATGTCCTG |
|                    | MTTPr         | TTTGCTAACCATTGACCAAAAT |
| RT-PCR assay of conditional lines | UBC9WT5′ UTRF | TCAATTCAAGATCTAGGAAAA |
|                    | UBC9WT5′ UTRR | GCCTGATTTCAACCTCTTTC |
|                    | UBC9CodingF+start | ATCGGATACCATGTGCAAAACAAAAT |
|                    | UBC9CodingR-stop | ACGCAGATTGCCTTTTTTTCATGG |
| GFP-UBC9 construct | UBC9-startF | ATCGGATACCATGTGCAAAACAAAAT |
|                    | UBC9+stopR    | TCACTTTTTTTCGTGTCTACCAA |
| UBC9-mCherry construct | UBC9CodingF+start | ATCGGATACCATGTGCAAAACAAAAT |
|                    | UBC9CodingR-stop | ACGCAGATTGCCTTTTTTTCATGG |
|                    | UBC9′ FlankF  | ATCGGATACCATGTGCAAAACAAAAT |
|                    | UBC9′ FlankR  | TGGAGCTAATTTGATGTCCTG |

Plasmid pUBC9KO was purified by using a Qiagen Plasmid Maxi kit (Qiagen, Valencia, CA) and linearized with XhoI and NotI digestion. Gold particles (S550d DNA delivery particles; Seashell Technology LLC) were coated with 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 XhoI 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 NotI 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:229558596]) 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-green fluorescence protein vector (obtained from Doug Chalker, Washington University, St. Louis, MO) by using the LR recombinase in the Gateway cloning system (Life Technologies, Carlsbad, CA). Successful integration of the GFP-UBC9 fusion gene at the Tetrahymena UBC9 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 KpnI 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.

The dominant negative UBC9 (pDN-UBC9) construct was generated by inserting an N-terminal His6-FLAG-tagged version of UBC9 containing a mutation of the conserved cysteine in the 100th position to a serine (C100S). The gene was chemically synthesized by IDT (Coralville, IA) with BsiWI and Apal restriction sites adjacent to the 5′ and 3′ coding regions, respectively. The coding region was then inserted into plasmid pBS-MTT-GFP-green fluorescence protein (obtained from Doug Chalker, Washington University, St. Louis, MO) after digestion with BsiWI and Apal. The construct was linearized by HindIII digestion and transformed into Tetrahymena. This construct integrates adjacent to the RPL29 locus by homologous recombination and confers resistance to cycloheximide (12.5 μg/ml).

Construction of UBC9 germ line knockout heterokaryons. Plasmid pUBC9KO was used for generating germ line knockout heterokaryons. UBC9

December 2015 Volume 14 Number 12 Eukaryotic Cell ec.asm.org 1241
the DNA and introduced into mating B2086 and CU428 cells at 2.5 to 3.5 h postmixing (33) by biolistic bombardment, as described previously (34). Putative micronuclear \textit{UBC9} knockouts (\textit{\textDelta}UBC9) were selected by growth in the presence of paromomycin (100 \textmu g/ml) followed by 6-methylpurine (6-MP) (7.5 \textmu g/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 (\textit{ubc9}\textsuperscript{neo3/ubc9} [pm-r; paromomycin resistant]) transformants were test 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\textsuperscript{VII} and B\textsuperscript{VII} to generate two homozygous germ line knock out heterokaryon strains of different mating types, BV1 (\textit{UBC9-}\textit{ubc9}\textsuperscript{neo3} [\textsuperscript{VII}; \textit{UBC9}\textsuperscript{*} pm-s];) and BVII (\textit{UBC9-}\textit{ubc9}\textsuperscript{neo3} [\textsuperscript{VII}; \textit{UBC9}\textsuperscript{*} 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 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 (\textit{UBC9}\textsuperscript{*} pm-s) germ line knock out heterokaryons were crossed to obtain progeny that are complete \textit{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 \textit{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 biolistically transformed (33) into mated heterokaryon homozygous \textit{\textDelta}UBC9 cells at 8 h postmixing. Cells were selected with 12.5 \textmu g/ml cycloheximide and induced with CdCl\textsubscript{2} to initiate the expression of the \textit{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 \textit{GFP-UBC9} progeny of the cross were tested for paromomycin resistance to demonstrate successful mating and generation of the new \textit{UBC9} knock out 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 \textit{Tetrahymena thermophila} strain B2086 and conditional \textit{UBC9} KO strains were used for isolation of total RNA by using a Qiagen RNeasy minikit (Qiagen, Valencia, CA) with a tentative or conjugating cells was concentrated to 10\textsuperscript{6} cells/ml by low-speed concentration of 200 cells/ml in the presence or absence of 1.0 mM Tris-Cl (pH 7.5), and then transferred to 10 ml SPP medium at a concentration of 200 cells/ml. Cells were then examined under a fluorescence microscope (Olympus BX51 TF microscope).

**Growth rate.** \textit{UBC9} conditional mutant strains were first cultured overnight in SPP medium with 0.1 \textmu g/ml CdCl\textsubscript{2}, washed twice with 10 mM Tris-Cl (pH 7.5), and then transferred to 10 ml SPP medium at a concentration of 200 cells/ml in the presence or absence of 1.0 \textmu g/ml CdCl\textsubscript{2}. Cell populations were measured by direct cell counts at 0, 4, 8, 18, 24, and 48 h by using a hemocytometer.

**Drug sensitivity.** \textit{UBC9} conditional mutant cells and wild-type B2086 cells were cultured in SPP medium in the presence of 1.0 \textmu g/ml CdCl\textsubscript{2} or no CdCl\textsubscript{2} for 24 h, treated with 8 mM methyl methanesulfonate (MMS) or 2 mM cisplatin for 2 h, and then washed twice with 10 mM Tris-Cl (pH 7.5). Single cells were isolated in SPP drops containing 0.5 \mu g/ml CdCl\textsubscript{2}. After 48 h, the number of cells in each drop was counted. Drops containing <10 cells were scored as nonviable, and drops with >500 cells were scored as viable.

**Pairing efficiency.** Two different mating types of \textit{UBC9} conditional mutants and wild-type cells were cultured separately in SPP medium for 16 h, harvested at 1,100 × g, washed twice with 10 mM Tris-Cl (pH 7.5), and starved in the same buffer in the presence of 0.05 \textmu g/ml or no CdCl\textsubscript{2} 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 counted for pairing efficiency. One hundred cells were scored at 2, 4, 6, and 8 h postmixing. Pairing efficiency was calculated by counting the number of mating pairs, multiplying by 2 (2 cells per pair), and then dividing by the total number of cells.

**Analysis of DN-UBC9 during conjugation.** \textit{Tetrahymena} strains of different mating types expressing DN-\textit{UBC9} were grown in SPP medium for ~24 h to a concentration of 2 × 10\textsuperscript{6} cells/ml. Cells were washed twice in 10 mM Tris (pH 7.5), suspended at 2 × 10\textsuperscript{6} cells/ml in the same solution for 24 h, and then mixed to initiate mating. At 6 hours postmixing, the culture was split into two equal portions and one half was induced with 0.1 \textmu g/ml CdCl\textsubscript{2}. At 24 h postmixing, 200 \mu l of cells was fixed with an equal volume of 4% paraformaldehyde and stained with DAPI for microscopy.

**RESULTS**

\textit{UBC9} is an essential gene. We identified a single \textit{UBC9} homolog in the \textit{Tetrahymena} genome. Searches of the \textit{Tetrahymena} Genome Database (http://ciliate.org/index.php/home/welcome/) with \textit{UBC9} orthologs from \textit{Saccharomyces cerevisiae}, \textit{Schizosaccharomyces pombe}, and \textit{Drosophila melanogaster} revealed the same top BLAST hits (TTHERM_00522720). Reciprocal searches of the \textit{S. pombe} and \textit{D. melanogaster} genomes using the \textit{Tetrahymena} ortholog identified the corresponding \textit{UBC9} genes as the top BLAST hits. The \textit{Tetrahymena} Ubc9 deduced amino acid sequence has ~55% identity with \textit{UBC9} orthologs from yeast and \textit{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 \textit{UBC9} orthologs are essential in budding yeast, nematodes, zebrafish, and mice (10, 15, 18, 36). An exception is \textit{S. pombe}, where the deletion of \textit{hus5} (\textit{UBC9} ortholog) causes severe mitotic defects and slow growth but not death (14). To determine if \textit{UBC9} is essential in \textit{Tetrahymena}, micronuclear (germ line) \textit{UBC9} knockout cell lines were constructed. The MIC knockout was constructed according to standard procedures by inserting the \textit{neo3} cassette (conferring paromomycin resistance) into one allele of the MIC \textit{UBC9} locus (Fig. 2A). This cell line was subsequently mated to star strains to generate cells with a homozygous knockout of \textit{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 \textit{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 \textit{neo3} insertion in the \textit{UBC9} MIC gene (Fig. 2C). The homozygous \textit{UBC9} 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 \textit{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 were paromomycin resistant, as expected. As predicted by the localization of Ubc9 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 expression, 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 MTTI 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 Ubc9 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, ∆UBC9 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

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

| Genotype                  | No. of individual pairs examined | No. of drops with live cells (72h) | No. of drops with paromomycin resistant cells |
|---------------------------|---------------------------------|-----------------------------------|----------------------------------------------|
| ∆UBC9- neo3 × ∆UBC9-neo3  | 80                              | 19                                | 0                                            |
| wt × wt                   | 80                              | 76                                | 72                                           |
| ∆UBC9- neo3 × wt          | 80                              | 78                                | 78                                           |
| ∆UBC9- neo3 × wt          | 80                              | 75                                | 75                                           |

FIG 2 Generation of a UBC9 knockout strain by homologous recombination in Tetrahymena. (A) Sequences flanking UBC9 were PCR amplified and cloned into plasmid pMNBL (30). The plasmid was digested with NotI and Xhol to yield a fragment capable of homologous recombination with the complementary sequence on the chromosome. The shaded portion of the knockout construct represents the neomycin 3 cassette (30) that includes the neomycin resistance gene (neo3) and the metallothionein promoter (MTT1). PCR primers used to assay the integration of the neo3 cassette, one set specific for the KO allele and the other specific for the wild-type allele, are shown. (B) Schematic of the genetic cross to generate UBC9 knockout heterokaryon strains containing homozygous UBC9 deletions in the micronucleus and a wild-type macronucleus. The heterozygous ∆UBC9 cell line was crossed to a star strain, resulting in a homozygous micronuclear genome (see the text for additional details). (C) ∆UBC9 heterokaryon strains were confirmed by using the primers shown in panel A, followed by separation of the PCR-amplified products on a 2% agarose gel. The homozygous heterokaryon ∆UBC9 strains generated a KO product of 480 bp (lanes 5 to 8) despite a neomycin-sensitive (wild-type MAC) phenotype. The wild-type strains yielded only the 291-bp wild-type (WT) product, as expected (lanes 1 and 2).
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.
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. ∆UBC9 conditional mutants and wild-type cells were cultured separately in SPP medium containing 1.0 μg/ml CdCl$_2$ or no CdCl$_2$. 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 CdCl$_2$. CdCl$_2$ in the posttreatment drops provides conditions that are optimal for growth of conditional cell lines that would have poor growth without CdCl$_2$. 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, ∆UBC9 conditional cells cultured in the absence of CdCl$_2$ showed only 12% survival in MMS, compared with 90% survival when cultured with CdCl$_2$. The survival rate with cisplatin increased ~15-fold (5% versus 80%) in the presence of CdCl$_2$. Wild-type cells showed little change in sensitivity regardless of the presence or absence of CdCl$_2$ (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...
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. *ΔUBC9* 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. *ΔUBC9* 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, *ΔUBC9* 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 *ΔUBC9* 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-
Despite the CdCl2 treatment (data not shown), expressing dominant negative Ubc9p showed normal pairing phenotype indicates an additional role for Ubc9p in meiosis. Cells expressing dominant negative Ubc9p showed normal pairing regardless of CdCl2 treatment (data not shown).

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-Ubc9p 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) deletion 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 missegregation 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/S8 complex is the founding member of a recently defined class of SUMO-targeted ubiquitin ligases (STUbLs) (52). The Sbx5/S8 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
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 a1 protein, important for the establishment of mating type, requires the STUbLs Slx5 and Slx8 (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.

**ACKNOWLEDGMENTS**

This work was supported by NIH grant GM089662 to R.S.C. Cell lines were obtained from the Tetrahymena Stock Center at Cornell University, which is supported by National Institutes of Health grant 2 P40 RR019688-05. We thank Douglas Chalker (Washington University, St. Louis, MO) for the pBS-MTT-GFP-gtw plasmid. We thank Mark Winiey (University of Colorado, Boulder, CO) for the pmCherryLAP-NEO2 plasmid. We acknowledge the Tetrahymena Genome Database for publicly available genome sequences and annotation.

**REFERENCES**

1. Ulrich HD. 2009. The SUMO system: an overview. Methods Mol Biol 497:3–16. http://dx.doi.org/10.1007/978-1-59745-566-4_1

2. Wilkinson KA, Henley JM. 2010. Mechanisms, regulation and consequences of protein SUMOylation. Biochem J 428:133–145. http://dx.doi.org/10.1042/Bj20100158

3. Hannoun Z, Greenhough S, Jaffray E, Hay RT, Hay DC. 2010. Post-translational modification by SUMO. Toxicology 278:288–293. http://dx.doi.org/10.1016/j.tox.2010.07.013.
...
Bressan D, Dotiwalla F, Papusha A, Zhao X, Myung K, Haber JE, Aguilara A, Aragon L. 2006. Smc5-Smc6 mediate DNA double-strand-break repair by promoting sister-chromatid recombination. Nat Cell Biol 8:1032–1034. http://dx.doi.org/10.1038/ncb1466.

46. Hayashi T, Seki M, Maeda D, Wang W, Kawabe Y, Seki T, Saitoh H, Fukagawa T, Yagi H, Enomoto T. 2002. Ubc9 is essential for viability of higher eukaryotic cells. Exp Cell Res 280:210–221. http://dx.doi.org/10.1006/excr.2002.5634.

47. Prudden J, Perry JJ, Nie M, Vashisht AA, Arvai AS, Hitomi CH, Boddy MN. 2014. SUMO-dependent ubiquitin ligases. PLoS One 9:e1003837. http://dx.doi.org/10.1371/journal.pone.0065628.

48. Yuan YF, Zhai R, Liu XM, Khan HA, Zhen YH, Huo LJ. 2015. SUMOylation regulates p53-induced autophagy. Cell Cycle 14:2717–2728. http://dx.doi.org/10.4161/cc.121091.

49. Voelkel-Meiman K, Taylor LF, Mukherjee P, Humphreys N, Tsubouchi H, Macqueen AJ. 2013. SUMO localizes to the central element of synaptonemal complex and is required for the full synopsis of meiotic chromosomes in budding yeast. PLoS Genet 9:e1003837. http://dx.doi.org/10.1371/journal.pgen.1003837.

50. Klug H, Xaver M, Chaugule VK, Koidl S, Mittler G, Klein F, Pichler A. 2013. Ubc9 sumoylation controls SUMO chain formation and meiotic synopsis in Saccharomyces cerevisiae. Mol Cell 58:625–636. http://dx.doi.org/10.1016/j.molcel.2013.07.027.

51. Wan J, Subramonian D, Zhang XD. 2012. SUMOylation in control of accurate chromosome segregation during mitosis. Curr Protein Pept Sci 13:467–481. http://dx.doi.org/10.2174/138920312802430563.

52. Prudden J, Pebernard S, Raffa G, Slade KM, Freggioro S, Cottrell KA, Smith JJ, Wiley EA. 2011. Centromeric histone H3 is essential for vegetative cell division and for DNA elimination during conjugation in Tetrahymena thermophila. Mol Cell Biol 26:4499–4510. http://dx.doi.org/10.1128/MCB.00079-06.

53. Henikoff S, Dalal Y. 2005. Centromeric chromatin: what makes it unique? Curr Opin Genet Dev 15:177–184. http://dx.doi.org/10.1016/j.gde.2005.01.004.

54. Sharp JA, Kaufman PD. 2003. Chromatin proteins are determinants of centromere function. Curr Top Microbiol Immunol 274:23–52.

55. Mellone BG, Allshire RC. 2003. Stretching it: putting the CEN(P-A) in centromere. Curr Opin Genet Dev 13:191–198. http://dx.doi.org/10.1016/S0959-437X(03)00019-4.

56. Wieland G, Orthaus S, Ohndorf S, Diekmann S, Hemmerich P. 2004. Functional complementation of human centromere protein A (CENP-A) by Cse1p from Saccharomyces cerevisiae. Mol Cell Biol 24:6620–6630. http://dx.doi.org/10.1128/MCB.24.15.6620-6630.2004.

57. Webster M, Witkin KL, Cohen-Fix O. 2009. Sizing up the nucleus: nuclear shape, size and nuclear-envelope assembly. J Cell Sci 122:1477–1486. http://dx.doi.org/10.1242/jcs.073333.

58. Lu Q, Henderson E. 2000. Two Tetrahymena G-DNA-binding proteins, TGPI and TGPIII, share novel motifs and may play a role in micronuclear division. Nucleic Acids Res 28:2993–3001. http://dx.doi.org/10.1093/nar/28.15.2993.

59. Nixon CE, Wilcox AJ, Laney JD. 2010. Degradation of the Saccharomyces cerevisiae mating-type regulator alpha1: genetic dissection of cis-determinants and trans-acting pathways. Genetics 185:497–511. http://dx.doi.org/10.1534/genetics.110.115907.

60. Robinson R. 2013. Mating type determination in tetrahymena: last man standing. PLoS Biol 11:e1001522. http://dx.doi.org/10.1371/journal.pbio.1001522.

61. Slade KM, Freggiaro S, Gottrell KA, Smith JJ, Wiley EA. 2011. Sir2uin-mediated nuclear differentiation and programmed degradation in Tetrahymena. BMC Cell Biol 12:40. http://dx.doi.org/10.1186/1471-2121-12-40.

62. Dehennaut V, Loison I, Dubuissez M, Nassour J, Abbadie C, Leprince A, Tsubouchi H, Macqueen AJ. 2002. Ubc9 is essential for viability of budding yeast. Mol Cell Biol 22:523–533. http://dx.doi.org/10.1128/MB.00079-06.

63. Liu ML, Yao MC. 2005. Centromeric chromatin: what makes it unique? Curr Opin Genet Dev 15:177–184. http://dx.doi.org/10.1016/j.gde.2005.01.004.

64. Henikoff S, Dalal Y. 2005. Centromeric chromatin: what makes it unique? Curr Opin Genet Dev 15:177–184. http://dx.doi.org/10.1016/j.gde.2005.01.004.

65. Sharp JA, Kaufman PD. 2003. Chromatin proteins are determinants of centromere function. Curr Top Microbiol Immunol 274:23–52.

66. Mellone BG, Allshire RC. 2003. Stretching it: putting the CEN(P-A) in centromere. Curr Opin Genet Dev 13:191–198. http://dx.doi.org/10.1016/S0959-437X(03)00019-4.