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SUMOylation Is Developmentally Regulated and Required for Cell Pairing during Conjugation in *Tetrahymena thermophila*

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The covalent attachment of *s*mall *u*biquitin-like *mo*difier (SUMO) to target proteins regulates numerous nuclear events in eukaryotes, including transcription, mitosis and meiosis, and DNA repair. Despite extensive interest in nuclear pathways within the field of ciliate molecular biology, there have been no investigations of the SUMO pathway in *Tetrahymena*. The developmental program of sexual reproduction of this organism includes cell pairing, micronuclear meiosis, and the formation of a new somatic macronucleus. We identified the *Tetrahymena thermophila SMT3* (SUMO) and *UBA2* (SUMO-activating enzyme) genes and demonstrated that the corresponding green fluorescent protein (GFP) tagged gene products are found predominantly in the somatic macronucleus during vegetative growth. Use of an anti-Smt3p antibody to perform immunoblot assays with whole-cell lysates during conjugation revealed a large increase in SUMOylation that peaked during formation of the new macronucleus. Immunofluorescence using the same antibody showed that the increase was localized primarily within the new macronucleus. To initiate functional analysis of the SUMO pathway, we created germ line knockout cell lines for both the *SMT3* and *UBA2* genes and found both are essential for cell viability. Conditional Smt3p and Uba2p cell lines were constructed by incorporation of the cadmium-inducible metallothionein promoter. Withdrawal of cadmium resulted in reduced cell growth and increased sensitivity to DNA-damaging agents. Interestingly, Smt3p and Uba2p conditional cell lines were unable to pair during sexual reproduction in the absence of cadmium, consistent with a function early in conjugation. Our studies are consistent with multiple roles for SUMOylation in *Tetrahymena*, including a dynamic regulation associated with the sexual life cycle.

Protein posttranslational modifications are critical regulatory events in eukaryotic cells, adding another layer of complexity to protein function. Modification by ubiquitin (Ub) is known to regulate numerous proteins, most commonly by tagging them for subsequent degradation (reviewed in reference 1). SUMOylation involves the covalent attachment of a small protein called SUMO (*s*mall *u*biquitin-like *mo*difier) to lysine residues on target proteins. SUMO shares ~18% sequence similarity with ubiquitin, and both proteins are relatively small, with similar tertiary structures (reviewed in reference 2). In addition, SUMOylation employs a similar 3-step enzymatic pathway in its conjugation onto target proteins (3–5). SUMO protein, also called Smt3p (suppressor of Mif two 3) in *Saccharomyces cerevisiae* (6), is first activated by the E1-activating enzyme, a heterodimer consisting of Aos1p and Uba2p, which binds Smt3p via a high-energy thioester linkage in an ATP-dependent step. Activated Smt3p is then transferred to the E2-conjugating enzyme Ubc9p. Several E3 ligases then interact with Ubc9p and direct Smt3p conjugation onto substrates. SUMO-specific proteases (Ulps/SENPs) cleave Smt3p from substrate proteins, making SUMOylation a reversible and cyclical process. This interplay of proteins of the SUMOylation cascade makes it a dynamic and tightly regulated process.

The functions associated with SUMOylation include altering protein-protein interactions, causing changes in the subcellular localization of target proteins, and competing with ubiquitin sites to mask sites from ubiquitin attachment and subsequent degradation (reviewed in reference 4). The importance of SUMOylation in regulating critical processes in eukaryotes is reflected in its modification of proteins in processes such as maintenance of chromosome structure and segregation (7), cell cycle progression (8), and DNA repair mechanisms (9, 10). Depletion of Smt3p in budding yeast results in a phenotype where they are unable to segregate their chromosomes and suffer from short spindles (11, 12).

The ciliate *Tetrahymena thermophila* separates its somatic and germ line functions between two morphologically and functionally different nuclei. The polyploid macronucleus (MAC) is actively transcribed and responsible for gene expression that determines phenotype. The diploid micronucleus (mic) is transcriptionally silent and is responsible for germ line functions (reviewed in reference 13). Conjugation in *Tetrahymena* is a complex and dynamic process that starts with cell pairing, followed by meiosis, during which genetic exchange occurs, and terminates with MAC differentiation, during which a new MAC and mic are derived from the mitotic products of the zygotic nucleus in progeny cells. This process involves a series of highly synchronized events, including extensive programmed DNA rearrangements in the form of site-specific DNA deletion and chromosome breakage (reviewed in references 14 and 15), telomere addition (reviewed in reference 16), histone acetylation (17), and amplification of the MAC genome (18, 19).

TABLE 1 *SMT3* and *UBA2* are essential genes

	Types of mating cells[a]	No. individual pairs examined	No. wells with more than 2 cells/drop	No. of clones completed conjugation[b]
Δ/Δ × Δ/Δ	Δ*SMT3-neo3-1* × Δ*SMT3-neo3-2*	110	34	0
	Δ*UBA2-neo3-1* × Δ*UBA2-neo3-2*	124	32	0
Δ/Δ × +/+	Δ*SMT3-neo3-1* × B2086	110	91	81
	Δ*UBA2-neo3-1* × B2086	110	96	88
	Δ*SMT3-neo3-2* × CU428	110	89	84
	Δ*UBA2-neo3-2* × CU428	110	92	83
+/+ × +/+	B2086 × CU428	88	82	82

[a] Each row represents data obtained in the course of three independent experiments.
[b] To distinguish between cells that completed conjugation (progeny cells) and cells that had aborted conjugation, wild-type B2086 and CU428 strains were tested for resistance with 6-methylpurine (to which only progeny cells should be resistant), and cells from the Δ*SMT3-neo3* × Δ*SMT3-neo3* and Δ*UBA2-neo3* × Δ*UBA2-neo3* matings were checked for paromomycin resistance (progeny should be paromomycin resistant).

Our previous studies in the ciliate *Paramecium tetraurelia* showed that RNA transcripts encoding *SMT3* and *UBA2* are up-regulated during macronuclear development, which occurs during sexual reproduction. RNA interference (RNAi)-induced silencing of these two genes during conjugation resulted in inhibition of programmed DNA rearrangements and failure to form a functional macronucleus (20).

In the current study, we report that modification of substrates by Smt3p is differentially regulated between vegetative and mating *Tetrahymena*. During conjugation SUMOylation increases, with the highest Smt3p adduct formation observed during the MAC differentiation stage. This is consistent with a role for SUMOylation in regulating the nuclear events of conjugation, specifically, the formation of a somatic MAC during conjugation in *Tetrahymena*. Vegetative cells depleted of Uba2p or Smt3p show increased sensitivity to DNA damage, as expected based on their roles in maintaining genome integrity. Interestingly, depletion of Uba2p or Smt3p prior to conjugation prevented pair formation, demonstrating a requirement prior to MAC development. These studies lay the foundation for an exceptional system to investigate the dynamics of SUMOylation.
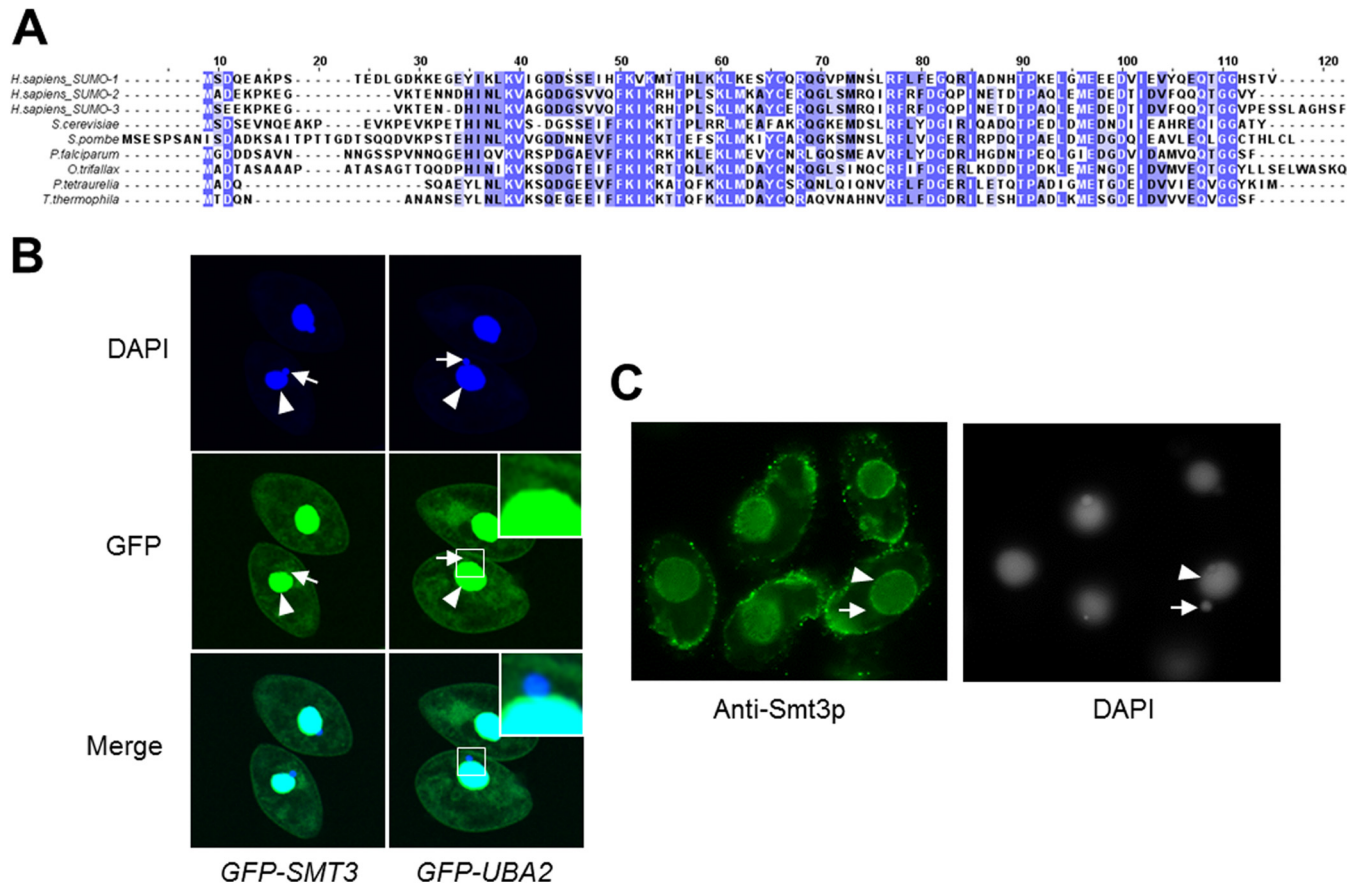
MATERIALS AND METHODS

Strains and culture conditions. Wild-type B2086 and CU428 strains of *Tetrahymena thermophila* were obtained from the *Tetrahymena* Stock Center, Cornell University, Ithaca, NY, USA. Strain B2086 contains 6-methylpurine-sensitive (*MPR1*) wild-type MACs that express mating type II. Strain CU428 contains 6-methylpurine-sensitive (*MPR1*) wild-type MACs that express mating type VII but contain micronuclei that have homozygous 6-methylpurine resistance (*mpr1-1/mpr1-1*). Cells were cultured in 1× SPP medium (2% proteose peptone, 0.1% yeast extract, 0.2% glucose, and 0.003% FeCl$_3$) at 30°C with shaking at 110 rpm.

Construction of SMT3 and UBA2 germ line knockouts. The targeting construct consisted of a *neo3* cassette conferring paromomycin (pm) resistance placed under the control of the metallothionein (*MTT1*) promoter (21). The drug resistance gene was flanked by sequences upstream and downstream of the *SMT3* coding sequence (the 5′ flank sequence was 1,141 bp [bp 187801 to 188942 of scaffold 8254555], and the 3′ flank sequence was 1,292 bp [bp 190002 to 191294 bp of scaffold 8254555]; GenBank accession number NW_002476326) or *UBA2* coding sequence (5′ flank sequence was 1,004 bp [bp 472623 to 473627 of scaffold 8254719] and 3′ flank sequence was 1,148 bp [bp 476286 to 477434 of scaffold 8254719]; GenBank accession number NW_002476484) (see Table S1 in the supplemental material for a list of the primers). Wild-type B2086 and CU428 strains were mated, and the targeting construct was

introduced 2.5 to 3.5 h postmixing, as described by Bruns and Cassidy-Hanley (22). Potential *SMT3* and *UBA2* micronuclear knockout (KO) strains were identified by selection with paromomycin (confirming insertion of the *neo3* cassette) and 6-methylpurine (confirming successful conjugation and formation of new macronuclei). The heterozygous mic KO strains were further analyzed by PCR and genetic crosses to test strains (e.g., CU427) to confirm germ line segregation of paromomycin resistance. These heterozygous germ line transformants were crossed with "star" strains B*VI and B*VII, which are deficient in donating a functional micronucleus. The subsequent uniparental transfer that occurs resulted in the generation of homozygous germ line knockout heterokaryon strains (23). The micronuclei (germ line) in these cells have homozygous deletions of the targeted gene, but the MAC genome is wild type because the two partners of "star" crosses do not proceed through conjugation to form new MACs. PCR analysis as well as genetic crosses confirmed the genotype of each KO heterokaryon (see Table 1 and Fig. 4, below).

Viability test. To test the viability of the progeny of *SMT3* or *UBA2* homozygous heterokaryons strains, starved *SMT3* or *UBA2* KO heterokaryons strains (~2 × 10^5 cells/ml) were mixed to initiate mating, and mating efficiency was assessed at 2 h. Mating pairs were then isolated at 8 to 10 h postmixing in drops containing 1× SPP medium and placed at 30°C. Cells in drops were examined at multiple times between 24 and 72 h postmixing. The number of cell divisions that occurred before death of homozygous *SMT3* and *UBA2* KO progeny was calculated by counting the total number of cells at the time of death, dividing by two (two exconjugants per pair), and calculating the number of cell divisions based on exponential growth. To determine whether cells successfully completed conjugation in *SMT3* or *UBA2* heterokaryon KO crosses, cells were cultured in 1× SPP containing 80 μg/ml paromomycin. Progeny of the control cell lines wild-type B2086.2 × CU428.1 were tested for 6-methylpurine (7.4 μg/ml) resistance. Additional matings between *SMT3* and *UBA2* heterokaryons to wild-type partners were also performed to ensure generation of viable progeny (Table 1). To check for progression through conjugation, cells were fixed and stained with the DNA-specific dye diamidinophenolindole (DAPI), as described below (see "Fluorescent and confocal microscopy").

Creation of GFP-SMT3 and GFP-UBA2 constructs. To examine Smt3p and Uba2p localization in *Tetrahymena*, coding regions for Smt3p and Uba2p were amplified and cloned into the pENTR/D-TOPO plasmid (Invitrogen, Grand Island, NY), which is used for recombination with the Gateway cloning system, to create pENTR-SMT3 and pENTR-UBA2, respectively. LR Clonase II (Invitrogen, Grand Island, NY) was used for directional cloning of *SMT3* or *UBA2* into the destination vector pBSmt-tGFPgtw, which contained an N-terminal *MTT1*-inducible green fluorescent protein (GFP) expression cassette cloned upstream of a cycloheximide-resistant *Tetrahymena* rpl29 locus (described in reference 24). For biolistic transformation, constructs were digested with HindIII or BlpI to

Mating *Tetrahymena* and assessment of pair formation. For conjugation, *T. thermophila* strains were cultured at 30°C in 1× SPP to logarithmic phase and harvested at an optical density at 540 nm (OD_{540}) of 0.3, corresponding to 2×10^5 cells/ml. Cells were then washed with starvation buffer (10 mM Tris, pH 7.5) and then subsequently starved (16 to 24 h at 30°C) in 10 mM Tris (pH 7.5). Equal numbers of cells were then mixed together to initiate mating. To assess pair formation, conditional mutants of *SMT3* or *UBA2* were cultured under three different conditions. The +Cd cultures were grown in SPP medium supplemented with 0.1 μg/ml $CdCl_2$ for 24 h at 30°C. These cells were then starved in starvation buffer containing 0.1 μg/ml $CdCl_2$ for 24 h at 30°C, after which they were mixed to initiate mating pair formation. The −Cd cultures were grown and starved in the absence of $CdCl_2$ and then mated to assess pair formation. The +Cd addition cultures were treated as described for the −Cd set except that at 2 h prior to mixing, cells were supplemented with 0.1 μg/ml $CdCl_2$ and then mated. The percentage of cells forming pairs was calculated as the number of cells in pairs divided by the total number of cells in the sample (paired plus single cells).

Preparation of whole-cell extracts. Cultures (100 ml) of vegetative wild-type strains B2086.2 and CU428.1 at log phase (2×10^5 cells/ml) and wild-type mated cell cultures (at 0, 2, 5, 7, 10, 24, and 30 h postmixing) were harvested by centrifugation and washed twice with 10 mM Tris-Cl (pH 7.5). The cell pellet was resuspended in 2 ml of lysis buffer (8 M urea, 50 mM Tris [pH 8.0]) containing 50 mM *N*-ethylmaleimide and lysed by sonication. The resulting lysate was centrifuged at 4°C for 30 min at 77,000 × *g* in an SW41 rotor (Beckman Coulter). The clarified supernatant was then prepared for Western blotting as described below.

Western blotting and Coomassie staining analyses. Clarified lysates from wild-type strains were prepared by the addition of SDS loading buffer to a final concentration of 1× (50 mM Tris [pH 6.8], 10% glycerol, 2% SDS, 100 mM dithiothreitol, and 0.05% bromophenol blue). Samples were separated on 8% Bis-Tris gels by using Tris-glycine-SDS running buffer and then transferred to a polyvinylidene difluoride membrane (Pall Corporation, Port Washington, NY) at 147 V for 1 h. Subsequent Western blot analysis was then performed with anti-Smt3p and horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Smt3p antibodies were custom generated by ProteinTech Group Inc. (Chicago, IL). Rabbits were immunized with a peptide corresponding to amino acids 26 to 43 of *Tetrahymena* Smt3p (FFKIKKTTQFKKLMDAYC), and antibodies were affinity purified from the resulting serum by using the same peptide. Results were visualized with the ECL Prime chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). Coomassie staining was performed by first treating the SDS gel with fixing solution (10% glacial acetic acid and 25% isopropanol) for 3 h and then incubating it in Coomassie R250 staining solution (0.05% Coomassie R250, 10% acetic acid, and 25% methanol) overnight at room temperature. Destaining was done in 10% glacial acetic acid for 3 h.

RESULTS

***Tetrahymena* Smt3p and Uba2p primarily accumulate in the macronucleus.** Previous studies on *Paramecium tetraurelia* revealed that RNAi-induced silencing of *UBA2* or *SUMO* resulted in the failure of programmed DNA rearrangements (20), but the underlying mechanism responsible for this effect was not established. *Tetrahymena* was selected for additional studies because of its technical advantages (gene knockouts, higher mating efficiency) and the opportunity for comparative analysis with *Paramecium*. To initiate our analysis of SUMOylation, we searched the *Tetrahymena thermophila* genome for homologs and identified a single gene encoding *SUMO* (26) with reciprocal top BLAST hits to SUMO proteins in *S. cerevisiae*, *Drosophila melanogaster*, and humans. We named the *Tetrahymena* gene *SMT3* (TTHERM_00410130) (20), consistent with *S. cerevisiae* nomenclature (sup-

pressor of Mif two 3). The alignment of *Tetrahymena* Smt3p with human SUMO isoforms, *S. cerevisiae*, *Schizosaccharomyces pombe*, and other protozoa is shown in Fig. 1A. *Tetrahymena* Smt3p shares 50% identity with *S. cerevisiae*. The N-terminal regions show substantial divergence, but most of the protein is highly conserved, including the diglycine motif found on the mature C terminus of ubiquitin-like proteins (Ubls). A *Tetrahymena* homolog for *UBA2* was previously reported based on its identity with *Paramecium tetraurelia* (TTHERM_00391590) (20). That study evaluated the developmental expression of transcripts from the *Tetrahymena UBA2* and *SMT3* (SUMO) genes with Northern hybridizations. Both showed substantial increases in expression during sexual reproduction, consistent with subsequent data from whole-genome microarrays (27). The developmental pattern of expression and sequence identity between these genes compared with the previously examined *Paramecium UBA2* and *SUMO* genes led us to perform additional studies.

To evaluate whether the cellular location of *Tetrahymena* SUMOylation pathway proteins is consistent with that in *Paramecium tetraurelia* and other eukaryotes, we examined the localization of Smt3p and Uba2p. Localization of Smt3p and Uba2p during vegetative growth was examined by expressing *GFP-SMT3* or *GFP-UBA2* transgenes from a metallothionein promoter regulated by cadmium. Both Smt3p and Uba2p were observed in the somatic MAC but not the germ line mic in vegetative *Tetrahymena* (Fig. 1B). Based on the coding sequence of *SMT3*, we generated rabbit polyclonal anti-Smt3p antibodies by immunizing animals with residues 21 to 43 of the full-length *Tetrahymena* Smt3p protein and used them to examine localization of Smt3p in vegetative cells (described in Materials and Methods). As seen in Fig. 1C, the predominant signal was in the macronucleus and no signal could be detected in the micronucleus. The signal at the cell cortex was detected with secondary antibody alone (no primary antibody) and is therefore unlikely to be significant. Additional experiments with the GFP transgenes showed that increasing or decreasing cadmium concentrations changed the strength of the GFP signal but did not alter its nuclear localization (data not shown). Although a small fraction of Uba2p and Smt3p are likely present in the cytoplasm, we are confident that the signal is predominantly macronuclear in vegetative cells. Studies of other developmental model systems, such as mice (28, 29), *Drosophila* (30, 31), and yeast (32), have reported that both Smt3p and Uba2p localize predominantly to the nucleus. This is expected, as many of the proteins modified by SUMO are nuclear, including promoter-specific transcription factors, DNA repair proteins, and chromatin-associated proteins. Our results in *Tetrahymena* are consistent with the role of SUMOylation in somatic nuclear processes such as the regulation of transcription, which is limited to the MAC in vegetative cells.

SUMOylation increases during conjugation in *T. thermophila*. Anti-Smt3p antibodies (Fig. 1C) were also utilized to determine SUMO expression throughout conjugation by Western blot analysis. Figure 2A shows schematic representations of the stages when protein was prepared for analysis. Figure 2B shows a typical SUMOylation pattern with several reactive bands consistent with a range of proteins conjugated to Smt3p. The image is from a short exposure to emphasize the difference in signal between vegetative cells and conjugating cells, but a longer exposure showed a large number of bands in the vegetative protein samples. The arrowhead labeled Smt3p indicates the expected migration of free
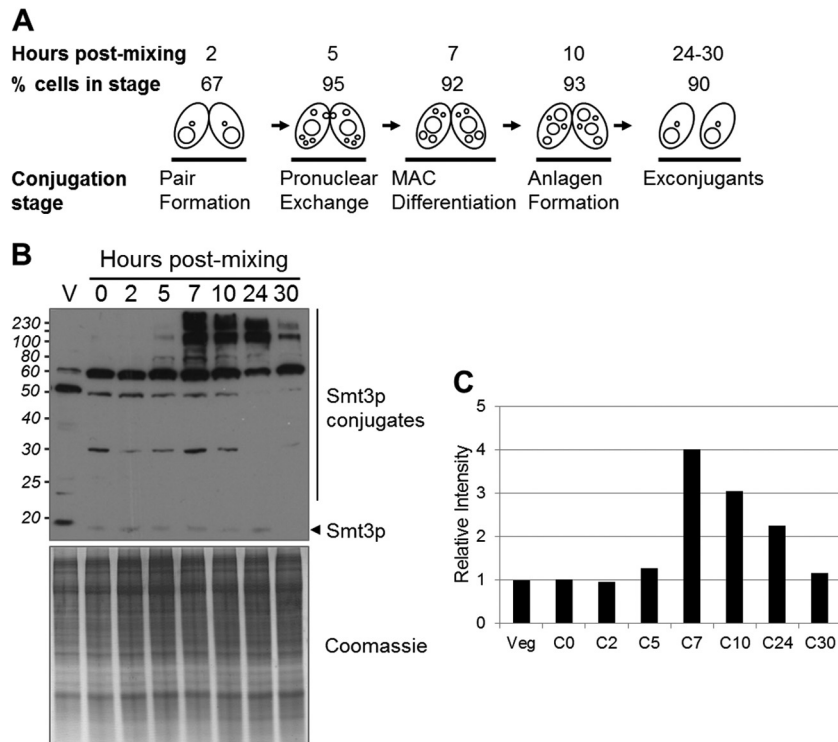
FIG 2 Differential modification of proteins by Smt3p during conjugation versus vegetative growth. (A) Schematic representation of the stages of conjugation at which cells were lysed. The following stages are shown: pair formation; pronuclear exchange; MAC differentiation; anlagen formation; exconjugants. (B) Whole-cell extracts (WCEs) were prepared from vegetative and mating B2086 and CU428 wild-type cells at 0, 2, 5, 7, 10, 24, and 30 h postmixing. WCEs were analyzed by 10% SDS-polyacrylamide gel electrophoresis followed by Coomassie blue staining (for loading control) (bottom panel) and Western blotting using polyclonal anti-Smt3p antibody (top panel). The arrowhead indicates the expected position of free Smt3p (~13 kDa) and high-molecular-mass proteins that are likely to be Smt3p conjugates. A Coomassie-stained gel was included as a control to evaluate equivalent sample loading. (C) The bar graph provides a quantitative assessment of the immunoblotting signal across different time points after normalizing for sample loading (Coomassie-stained gel).

Smt3p. Experiments with high-percentage gels (20%) showed that Smt3p migrates with a mass of approximately 13.5 kDa, but on the 10% gel shown in Fig. 2B proteins below 20 kDa were not well resolved. A slightly higher apparent molecular mass than the theoretical molecular mass (11.4 kDa) of Smt3p is not surprising, as SUMO proteins have been reported to exhibit anomalously slow migration on SDS-PAGE gels (33). The high-molecular-mass bands observed at the 40- to 200-kDa range were Smt3p substrates that represented a diverse set of target proteins that are modified by Smt3p in vegetative and mating cells. In mating cells, we observed that Smt3p adduct formation increased as cells progressed through conjugation, with the highest signal observed between 7 and 10 h postmixing, which corresponded to anlagen formation, implicating SUMOylation in its role in MAC differentiation (Fig. 2A and B). The bar graph in Fig. 2C provides a quantitative assessment of the increased signal above 40 kDa relative to total protein as measured by Coomassie staining. This increase in Smt3p signal is consistent with microarray expression data, which show an increase in transcript levels during conjugation, including anlagen formation (27). These results demonstrate that SUMOylation occurs differentially between vegetative and mating *Tetrahymena*, with a peak in SUMO conjugates observed during MAC differentiation.

To determine the cellular location of the increased Smt3p, we performed immunofluorescence with the same anti-Smt3p antibody used in Fig. 2. Mating cells were fixed at various times during conjugation leading up to the beginning of MAC development (9

h). A low signal relative to background fluorescence masked specific localization in early stages of conjugation. As conjugation progressed, Smt3p was detected in parental MACs by the end of meiosis, and the antibody revealed a strong accumulation in the developing anlagen (Fig. 3). We also used the GFP-Smt3p-expressing strains (Fig. 1) and found that Smt3p first localized to parental MACs early in conjugation and later accumulated in the developing anlagen, which mimicked our results with the anti-Smt3p immunofluorescence analysis (Fig. 3). Both techniques showed that the signal in the parental MAC disappeared as the anlagen developed and before the MAC was degraded. We did not detect Smt3p in the micronuclei or meiotic products during conjugation using anti-Smt3p or GFP-Smt3p. Although the GFP-Smt3p strains have the disadvantage of expression from the inducible *MTT1* promoter, the consistent results using antibodies and GFP fusions give us confidence in our observations.

In *Paramecium*, GFP-Uba2p localized to the developing MAC (20). Antibodies against *Tetrahymena* Uba2p are not available for immunofluorescence, but GFP-Uba2p was expressed from the inducible *MTT1* promoter in the absence of wild-type Uba2p (described in Materials and Methods). As shown in Fig. 3, GFP-Uba2p is located in the old MAC during the meiotic "crescent" phase (prophase I) of conjugation. The signal remains in the old MAC at later stages of meiosis and, unlike Smt3p, Uba2p is clearly visible in the meiotic haploid products as shown in Fig. 3. The signal is also detectable in haploid products during the period of
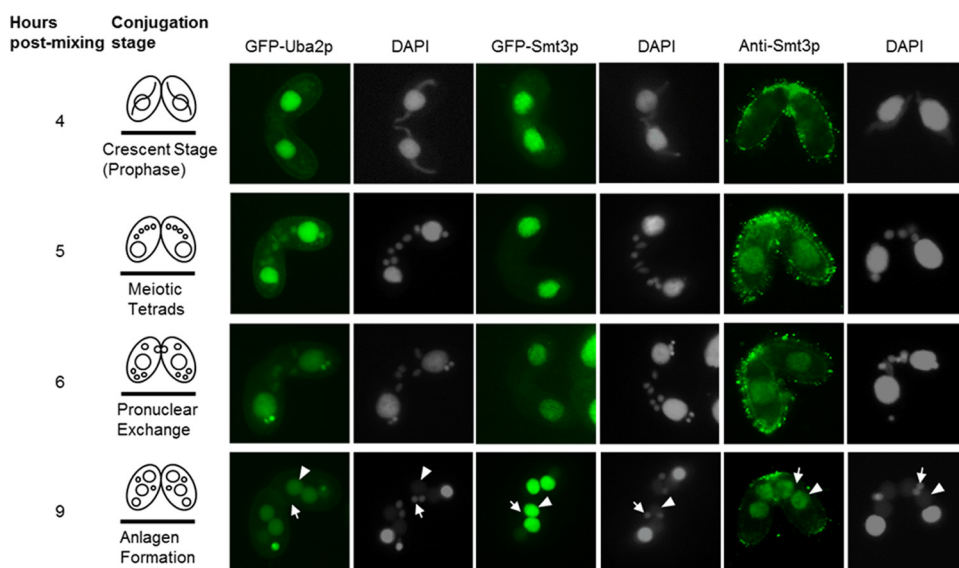
FIG 3 Localization of Uba2p and Smt3p in conjugation. *UBA2*-deleted cells were rescued with the *MTT1*-driven *GFP-UBA2* construct to generate N-terminally tagged GFP-Uba2p-expressing cells. Cells were mated and then fixed and stained with DAPI at various times throughout conjugation. GFP images were taken to visualize the distribution of *UBA2* in mating pairs. *GFP-SMT3* was transformed into B2086 and CU428 wild-type strains to produce N-terminal GFP-Smt3p-tagged strains. The GFP-Smt3p-expressing strains were mated and fixed at the indicated time points. Smt3p localization was also visualized in mated wild-type cells by using anti-Smt3p antibodies. A schematic of nuclear morphologies observed in wild-type mating is drawn for reference. The following developmental stages were observed: "crescent" micronuclei (prophase meiosis I) (4 h); meiotic haploid products generated after completion of meiosis II (5 h); pronuclear differentiation and exchange (6 h); second postzygotic mitosis (7 h); macronuclear anlagen formation and nuclear processing (9 h). Arrowheads indicate selected anlagen; arrows indicate selected mic's.

pronuclear exchange (Fig. 3). As the developing MACs (anlagen) appear, they contain increasing Uba2p signal, and the signal is simultaneously reduced in the parental (old) MAC. Consistent with our observations in vegetative cells, the signal is not detected in micronuclei at the two-mic two-MAC stage of development. Interestingly, *Paramecium* GFP-Uba2 expressed from its endogenous promoter showed little signal in the parental MAC, and the primary signal appeared in the anlagen (20). The *Tetrahymena* Uba2p and Smt3p localization results (Fig. 3), along with the immunoblot assay results (Fig. 2), are consistent with a major increase in SUMOylation of nuclear proteins during conjugation, particularly in the developing MAC. The absence of GFP-Smt3p localization in the meiotic products, in contrast to the GFP-Uba2p localization to those structures, is not readily explained but it is clearly not an issue of protein abundance (the result was independent of the level of expression) or stability (since the signal in the MAC was robust).

Complete deletions of *SMT3* and *UBA2* result in cell lethality. In budding yeast and invertebrates such as the nematode *Caenorhabditis elegans*, there is a single *SMT3* gene which is essential for viability (34, 35). Deletions in the SUMO-activating enzyme *UBA2* or the SUMO-conjugating enzyme *UBC9* are lethal in the budding yeast (11, 32, 35), *C. elegans* (34), *A. thaliana* (36), and in mice (37). In *Paramecium tetraurelia*, RNAi silencing of *UBA2* or *SUMO* had no detectable effect on vegetative cells, but it arrested conjugating cells. To test whether *SMT3* and *UBA2* are essential genes in *Tetrahymena*, we generated micronuclear (germ line) deletion strains that were subsequently mated to produce complete (mic and MAC) deletions. The initial heterozygous mic knockout strains were selected with paromomycin after transformation with KO constructs containing the neomycin resistance cassette (*neo3*)

flanked by sequences upstream and downstream of each coding region (Fig. 4A). The resulting heterozygous knockout cells were cultured without paromomycin selection to allow phenotypic assortment of the drug-resistant (KO) alleles and identification of paromomycin-sensitive cells with fully wild-type MACs. These heterozygous knockout cells were mated with "star" strains containing defective micronuclei and wild-type MACs. These crosses result in abortive conjugation, in which paired cells complete meiosis and exchange genetic material but separate without making new macronuclei. These postconjugation cells emerge with homozygous micronuclei but retain their original MACs, genetically wild type for *SMT3* and *UBA2* (Fig. 4A and B) (23). Phenotypically, these cell lines are paromomycin sensitive, but we used PCR amplification to identify the strains homozygous for the *neo3* cassette in place of the corresponding coding region in the micronucleus (Fig. 4C). These homozygous heterokaryon knockout strains of *SMT3* or *UBA2* were then mated, and individual pairs were isolated to nutrient medium. Analysis of >100 pairs from each cross revealed that the majority of pairs failed to survive (Table 1). Pairs that did survive were tested for paromomycin resistance to determine whether they were true exconjugant progeny or pairs that aborted conjugation. This test was used because the parental strains were phenotypically paromomycin sensitive (MAC genotype) but their germ line micronuclei were homozygous for paromomycin resistance. The formation of a new MAC would result in paromomycin resistance. As indicated in Table 1, none of the surviving lines was paromomycin resistant, and most likely they were abortive mating pairs containing wild-type MACs. Control crosses of the same knockout heterokaryon strains with wild-type cells (B2086 or CU428) resulted in high survival (~93%) of true exconjugant progeny that were paromomycin
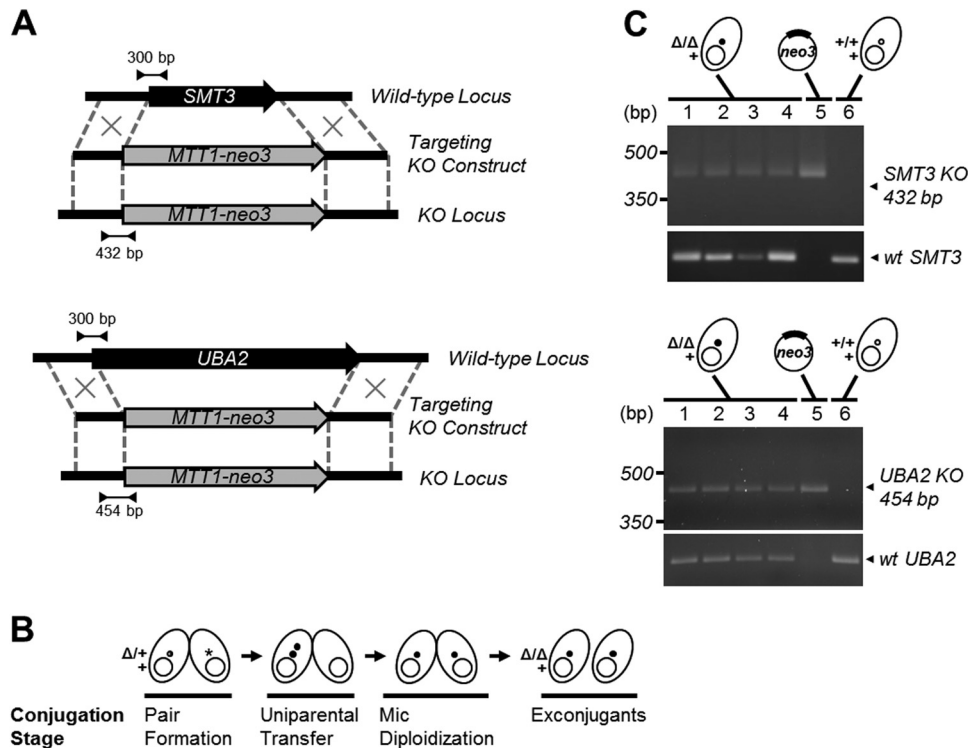
FIG 4 Generation of *SMT3* and *UBA2* knockout heterokaryons. (A) Schematic drawings of *SMT3* and *UBA2*, their targeted loci, and the knockout construct, as well as the resulting gene knockout formed by homologous recombination. The entire *SMT3* and *UBA2* coding sequences were replaced by insertion of the *neo3* knockout cassette, which confers paromomycin resistance in *Tetrahymena*. Arrows indicate the locations of primers used for genotyping PCR from total DNA. (B) To generate *Tetrahymena* with homozygous deletions in the mic, a special type of abortive mating called round I genomic exclusion was utilized, wherein wild-type cells were crossed with "star" strains that have defective micronuclei. Star strains form conjugal pairs with wild-type cells but fail to contribute a migratory gametic micronucleus to the wild-type partner at the fertilization stage of conjugation. As a result, a single haploid micronucleus is contributed by the wild-type partner to the star partner, where it is endoreplicated, leading to a homozygous, diploid micronucleus in each conjugant. At this point, conjugation is aborted prematurely, and there are no postzygotic nuclear divisions. (C) Total DNA isolated from wild-type CU428 (lane 6), *SMT3* (lanes 1 to 4; top panel), and *UBA2* (lanes 1 to 4; bottom panel) knockout homozygous heterokaryons was PCR amplified with the primers shown in panel A. Positions of the bands for the disrupted (KO) loci as well as wild-type (wt) genes are indicated. Genotypes of *SMT3* and *UBA2* homozygous germ line knockout heterokaryon strains and wild-type strains are illustrated at the top. Lane 5 in both gels contained the corresponding KO construct as the template.

resistant. This demonstrated that the knockout heterokaryons contain fertile micronuclei. The data are consistent with a lethal phenotype for complete deletions of *SMT3* or *UBA2*. This has been reported in other organisms, and we conclude that both are essential genes in *Tetrahymena*.

Interestingly, there was a substantial difference in the timing of postconjugation death for Δ*SMT3* and Δ*UBA2* strains. Δ*SMT3* progeny died before the first postzygotic cell division, but Δ*UBA2* progeny died much later, 6 to 8 cell divisions after mating (~72 h postisolation). Clearly, *UBA2* expression is not required from the zygotic macronucleus (anlagen) to complete conjugation. The *SMT3* and *UBA2* knockout heterokaryon crosses were monitored by DAPI staining to detect any grossly aberrant nuclear events (i.e., meiosis, pronuclear fusion, mitotic divisions, and anlagen development), but none was detected. The Δ*UBA2* vegetative progeny have the normal distribution of 1 MAC and 1 mic prior to death. The Δ*SMT3* progeny arrest in conjugation at the 2-MAC, 2-mic stage, but the events prior to that appear normal (data not shown). We also examined the Δ*SMT3* progeny and Δ*UBA2* progeny for defective programmed DNA elimination. Using a PCR strategy developed previously (38), we examined DNA from cells at the time of mixing (time zero) and 24 h later from several hundred conjugating pairs collected after mixing the same cul-

ture. A total of five eliminated DNA elements were examined for each cross. The results for the M and R elements in Δ*SMT3* progeny are shown in Fig. S1 in the supplemental material. For each eliminated element, the product expected for accurate DNA processing was detected and there was no evidence for the inhibition of DNA elimination. The explanation for the shorter life span of Δ*SMT3* cells compared with Δ*UBA2* is not clear but could result from faster depletion of parentally expressed Smt3p.

Gene rescue with the *MTT1* promoter converts *SMT3* or *UBA2* deletion lines into conditional mutants. As *SMT3* and *UBA2* are essential genes, complete deletions resulted in cell death, making it difficult to obtain information on the null phenotype of these genes. This problem was circumvented by the creation of conditional mutants of *SMT3* and *UBA2* in which the expression of these genes could be regulated by addition of CdCl$_2$ to the culture medium. As Δ*UBA2* progeny survive for up to 72 h postmixing, the parental *UBA2* heterokaryons were mated *en masse*, and at 8 h postmixing (corresponding to formation of new anlagen), mating cells were biolistically transformed with the *GFP-UBA2* construct (placed under *MTT1* control) to rescue the lethal phenotype (Fig. 5A). The only expressed functional copy of the *UBA2* gene in these cells was the *GFP-UBA2* transgene under
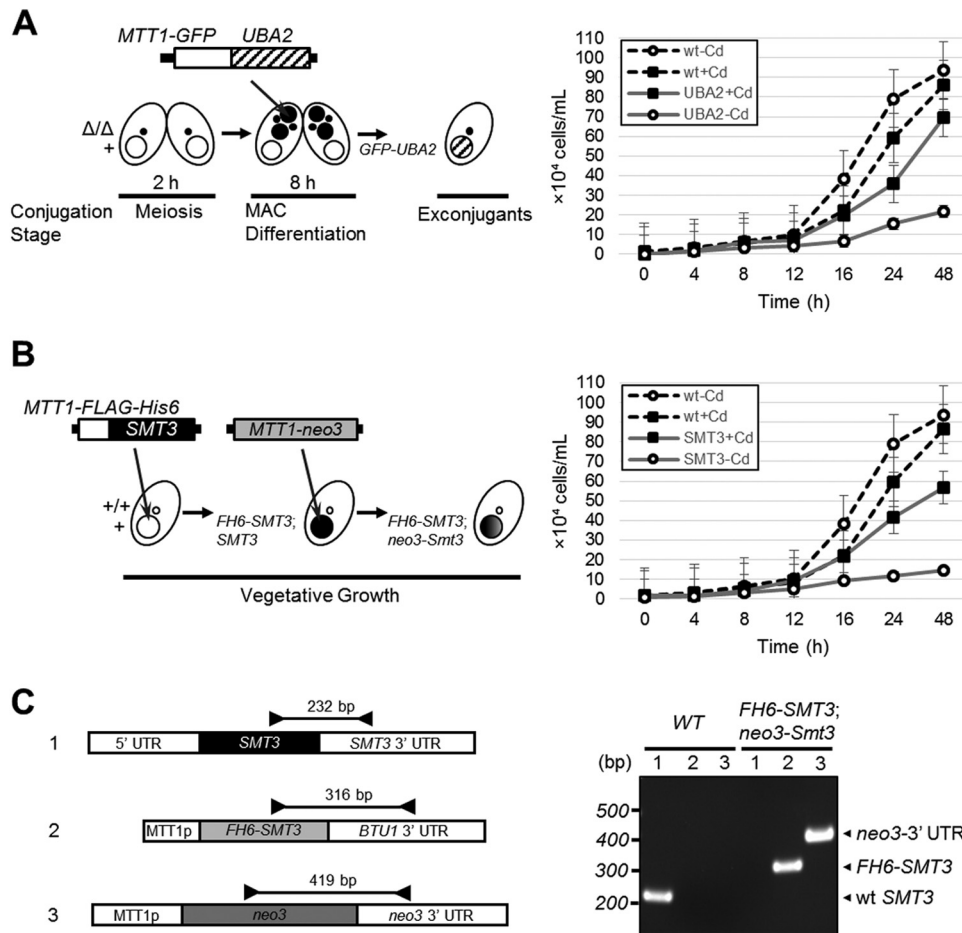
FIG 5 *Tetrahymena* expressing the *GFP-UBA2* or *FLAG-His6-SMT3* transgene regulated by the *MTT1* promoter behave as conditional mutants. (A) Schematic drawing of Δ*UBA2* homozygous heterokaryon strains that were mated and transformed with the *GFP-UBA2* transgene regulated by the *MTT1* promoter and inserted at the rpl29 locus. The resulting *UBA2* conditional cell lines and wild-type CU428 strains were grown vegetatively and transferred into 10 ml of SPP medium at an initial concentration of 200 cells/ml with either 0.1 μg/ml cadmium or no cadmium. At 0, 4, 8, 12, 20, 24, and 48 h after placement in growth medium, cells were fixed and scored using a hemocytometer. (B) Δ*SMT3* homozygous homokaryon progeny were lethal within 24 h, and therefore a different approach was used to generate a conditional mutant expressing *SMT3*. The schematic drawing shows the transformation of strain CU522 with *MTT1*-driven *FLAG-His6-SMT3* at the *BTU1* locus. The same strain was then transformed with the *neo3* knockout construct (again under the control of the *MTT1* promoter), disrupting the endogenous *SMT3* gene. These conditional *SMT3* cells were scored for growth as described for panel A. (C) RT-PCRs to detect wild-type (wt) *SMT3* transcripts in conditional mutants. The locations of the primers (arrows) used to amplify regions from the cDNA are shown on the left. Lanes 1, 2, and 3 on the gel correspond to PCR products with the primer sets (1, 2, and 3) indicated on the diagrams to the left. No wt *SMT3* transcript was detected in the *SMT3* conditional mutant cell line.

MTT1 control. For Δ*SMT3* mated cells, we were unable to rescue the lethal progeny with the *GFP-SMT3* transgene, possibly due to insufficient expression of the introduced copies at the end of conjugation. To bypass this issue, we generated *Tetrahymena* cell lines that were somatic transformants expressing introduced FLAG-His6 epitope-tagged *SMT3* (also driven by the *MTT1* promoter) at the *BTU1* locus in CU522 and CU725 strains. Incorporation at the *BTU1* locus in these strains confers resistance against the drug paclitaxel; the strains are otherwise paclitaxel sensitive. Next, we transformed these *FLAG-His6-SMT3*-expressing strains with the *neo3* knockout construct, which replaced the endogenous *SMT3* gene, and then we cultured cells in increasing concentrations of paromomycin and paclitaxel to select for cells in which wild-type copies of *SMT3* had been sorted out and only *FLAG-His6-SMT3* remained as the functional copy (Fig. 5B and C). Reverse transcription-PCR was used to confirm the absence of the wild-type

SMT3 transcript (Fig. 5C). To determine whether placing *SMT3* and *UBA2* under the control of the *MTT1* promoter led to a conditional, Cd-regulated mutant, we examined the growth rates of these cell lines compared to those of wild-type strains. The growth rates of the conditional strains were similar to that of wild-type cells when the culture medium was supplemented with cadmium but significantly slower when these strains were cultured in cadmium-free medium (Fig. 5A and B). The differences between the lethal phenotype of the complete gene deletion strains and the slow growth phenotype of the conditional cell lines in the absence of cadmium can be explained by "leaky" expression of the *MTT1* promoter even in the absence of cadmium. This has been reported previously (21), and we have seen this in our own analysis of *MTT1* promoter constructs. These results demonstrate a conditional phenotype in the absence of CdCl₂ that is consistent with Smt3p and Uba2p depletion.
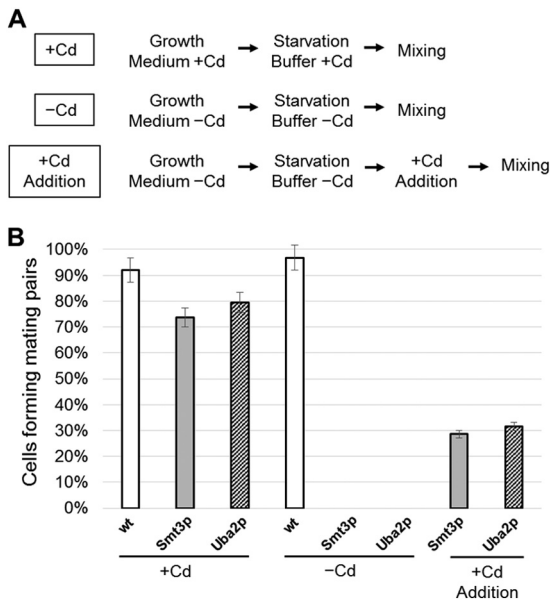
FIG 6 Depletion of Smt3p and Uba2p results in inability to form mating pairs. (A) Work flow for assaying formation of mating pairs in *SMT3* and *UBA2* conditional mutants. Strains of different mating types for each genotype were cultured with (+Cd) or without (–Cd) cadmium to early log phase and then starved in starvation buffer under the corresponding plus or minus cadmium condition (see Materials and Methods for details). A portion of each –Cd culture was supplemented with 0.1 μg/ml cadmium 2 h prior to mating; these cultures are referred to as +Cd addition. Cells were then mixed to initiate mating. (B) At 8 h postmixing, cells were fixed and scored using a hemocytometer to calculate mating efficiency. *SMT3* and *UBA2* conditional mutants cultured under cadmium-deficient conditions (–Cd) were unable to form mating pairs and did not progress through conjugation. Cells from +Cd cultures or –Cd cultures that were supplemented with cadmium 2 h prior to mixing (+Cd addition) formed mating pairs. Smt3p, *SMT3* conditional mutant; Uba2p, *UBA2* conditional mutant. Error bars (standard errors) are shown.

SMT3 and *UBA2* requirement in mating pair formation in conjugating cells.

We know from previous studies in *Paramecium tetraurelia* that silencing *UBA2* results in conjugation arrest during MAC development (20). Using our conditional Smt3p and Uba2p cell lines, we tested the effect of Smt3p or Uba2p depletion on conjugation in *Tetrahymena*. Conditional mutants of different mating types were cultured overnight in nutrient medium with or without cadmium, to either promote or reduce expression of *SMT3* or *UBA2* transgenes, respectively. Each culture was washed and cultured in starvation buffer for 24 h under the same +Cd or –Cd conditions. Two hours before mating, 0.1 μg/ml of cadmium was added to a flask containing a portion of the cadmium-free culture ("+Cd addition" cells). Wild-type cells were carried through the same procedure under the same conditions. Cells from cadmium-treated and non-cadmium-treated cultures were mated separately. Cells were evaluated at 2 h and 8 h postmixing for pair formation, and cell samples were DAPI stained to examine the nuclear events during conjugation. Smt3p and Uba2p conditional mutant cultures that had not been exposed to cadmium were unable to form mating pairs (Fig. 6). In contrast, the conditional mutants that had been exposed to cadmium had greater than 70% pair formation and progressed normally through the nuclear events of conjugation (data not shown). Smt3p and Uba2p conditional cells that were cultured without cadmium but treated with cadmium 2 h prior to mating were able to form pairs

and progress through the normal nuclear events of conjugation, as assayed by DAPI staining (data not shown). The finding that conditional cells can recover their ability to pair clearly demonstrates a dependence on Smt3p and Uba2p expression immediately prior to conjugation and eliminates the possibility that cells do not pair as a result of defective nuclei or other damage that could occur during vegetative growth. Wild-type control cells, both cadmium-treated and non-cadmium-treated cells, showed greater than 80% pairing and normal progress through conjugation, as expected (Fig. 6). The nonpairing phenotype of Uba2p- and Smt3p-depleted cells was unexpected and prevents the use of these cell lines to examine later stages of conjugation. Nevertheless, the elevated expression of SUMO pathway proteins and increased SUMOylation during MAC development make additional critical functions likely.

Smt3p- and Uba2p-deficient strains are sensitive to DNA-damaging agents.

Generation of conditionally mutant strains further allowed us to examine possible roles of this modification. Studies in budding yeast have shown that increased DNA damage sensitivity correlates with reduced SUMOylation (39–41). Multiple studies in mammals have shown that Smt3p modification of DNA repair proteins enhances the repair response (42, 43). To test whether *Tetrahymena* relies on SUMOylation-mediated DNA repair, we treated Smt3p and Uba2p conditional cell lines with the DNA-damaging agents methyl-methanesulfonate (MMS), which causes cell cycle arrest, and cisplatin, which cross-links DNA, causing cell cycle arrest, and induces apoptosis in yeast and mammalian cells (44–46). Wild-type, Smt3p conditional lines and Uba2p conditional lines were cultured in the presence or absence of cadmium. Cells were treated with either MMS or cisplatin and then placed in drops with cadmium (0.1 μg/ml) for 24 h to evaluate cell survival. In the absence of cadmium, Smt3p and Uba2p conditional mutants showed lower survival against MMS and cisplatin than did the same cells cultured with cadmium (Fig. 7). This increased survival after cadmium exposure occurred despite the negative effect of cadmium on wild-type cell survival (Fig. 7). This finding suggests that SUMOylation enhances the DNA damage repair response in *Tetrahymena* as it does in other species. The role of increased SUMOylation in conferring resilience to DNA damage in conditional mutants is consistent with a role for SUMOylation during the programmed DNA repair events of MAC development.

DISCUSSION

Developmentally regulated SUMOylation during conjugation in *Tetrahymena*. Global increases in SUMOylation are known to result from exposure to environmental conditions or specific reagents, for example, hydroxyurea, heat shock, or ethanol (43, 47, 48). In contrast, few examples of large developmentally regulated increases in SUMOylation have been reported. Our study revealed that the sexual cycle of *Tetrahymena* is one such example. Immunoblot analysis of whole-cell extracts revealed differential modification of substrates by Smt3p between vegetative and mating *Tetrahymena* cultures (Fig. 2). Smt3p increased as conjugation progressed, with the highest Smt3p signal observed during the MAC differentiation stage. This increase is consistent with elevated *SMT3* and *UBA2* transcript levels in conjugating cells compared with vegetative or starved cells (20). Our immunofluorescence studies also showed that Smt3p is predominantly a nuclear protein during the MAC development stage (Fig. 3). Together, the
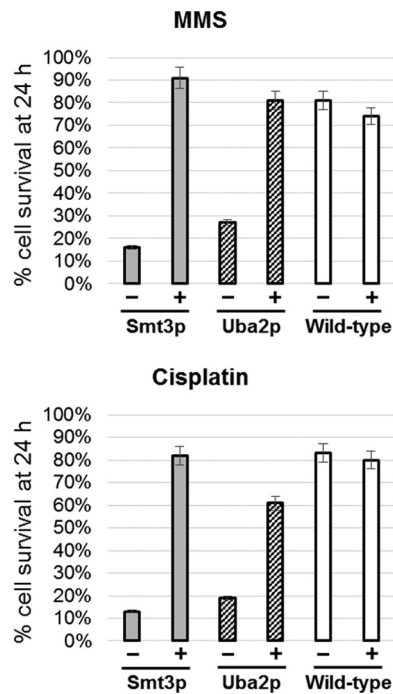
FIG 7 Smt3p- and Uba2p-depleted cell lines are sensitive to DNA-damaging agents. *SMT3* and *UBA2* conditional mutants grown in the absence or presence of cadmium (1 μg/ml) were treated with the DNA-damaging agents MMS (4.5 mM) and cisplatin (2 mM). Under each condition, 216 single lines were isolated into drops with medium supplemented with 0.1 μg/ml cadmium and incubated at 30°C for 48 h. Drops containing ≥500 cells were scored as viable, and drops containing ≤10 cells were counted as unviable. Viability is expressed as the percentage of viable drops out of the total number of drops. B2086 was used as the wild-type control. Error bars (standard errors) are shown.

immunoblotting and immunofluorescence data suggest a requirement for protein SUMOylation inside the developing MAC. During this period, extensive genome remodeling occurs, including heterochromatin formation, transcriptional regulation, DNA replication, and DNA repair (19, 49), processes that have known connections to SUMOylation in other species. In addition, our previous study of *UBA2* and *SUMO* genes in *Paramecium* revealed elevated transcript levels for both during conjugation. RNAi-generated knockdowns arrested conjugating cells and inhibited programmed DNA elimination (20). While the nuclear events in *Tetrahymena* and *Paramecium* are dramatic, other reports of global changes in SUMOylation are known. During mouse spermatogenesis, two distinct expression profiles of SUMO isoforms are detected, one during meiosis and the other in postmeiotic spermatids, suggesting an important role for SUMOylation in spermiogenesis (29). A recent study in human keratinocyte differentiation also highlighted SUMOylation as a regulator of cell differentiation. In these cells, SUMO expression was upregulated by calcium signaling at both the RNA and protein levels, while degradation of SUMO-activating and -conjugating enzymes resulted in abnormal differentiation of these cells, demonstrating key roles for SUMOylation in the keratinocyte differentiation process (50).

The SUMO pathway is conserved in *Tetrahymena*. Despite the dramatic changes in SUMOylation in *Tetrahymena*, we found that most features of the pathway are conserved with other eukaryotes. As expected based on other species, the localization of

Smt3p and Uba2p is predominantly nuclear (reviewed in reference 33). In vegetative *Tetrahymena* cells, both Smt3p and Uba2p localized to the somatic MAC (Fig. 1). Interestingly, we did not observe a GFP-Uba2p or GFP-Smt3p signal in the mic, nor did we detect the loss of mics in our conditional cell lines. We examined more than 50 cells during various stages of mitosis and could not detect a GFP-Smt3p signal from the micronucleus (data not shown). We cannot claim that Smt3p is completely absent from the mic, but our data support a much lower concentration in mics than in MACs during vegetative growth.

As is true for other eukaryotes, *UBA2* and *SMT3* are essential genes. We generated germ line deletions of the *SMT3* and *UBA2* genes by using homologous recombination. The deletions were carried in the mics of heterokaryon cell lines that express wild-type *SMT3* and *UBA2* from the somatic MAC. The progeny of these heterokaryon cells had complete deletions of *SMT3* or *UBA2* and died following conjugation (Table 1). This lethal phenotype is consistent with the deletion of *SUMO* and *UBA2* genes in yeast (32, 51) and mammalian model systems (37). Interestingly, our earlier work with the ciliate *Paramecium* showed that RNAi knockdown of *SMT3* and *UBA2* had no effect on vegetative growth (20). In retrospect, this lack of vegetative phenotype is likely due to incomplete knockdown providing a low level of *SMT3* and *UBA2* expression. As observed for other eukaryotes, *Tetrahymena* Smt3p- and Uba2p-depleted cell lines were hypersensitive to DNA-damaging agents. When we treated *Tetrahymena* with MMS or cisplatin, cells depleted for Smt3p and Uba2p showed increased sensitivity to both DNA-damaging agents (Fig. 7). Several proteins involved in DNA repair are SUMO substrates or proteins that interact with components of the SUMO pathway (42, 43, 52). Examples include DNA repair proteins, such as PCNA (40), 53BP1 and MDC1 (42), and XRCC4 (52). 53BP1 and MDC1 localize along with SUMO proteins to sites of double-strand breaks that occur as a result of DNA damage (42). XRCC4, an important protein in the mammalian nonhomologous end-joining (NHEJ) pathway, depends upon transient SUMOylation for localization to the nucleus (52). The NHEJ pathway is believed to be the key mechanism for repair of double-stranded DNA (dsDNA) breaks generated during programmed DNA rearrangements that occur naturally during conjugation in ciliates (15). In *Paramecium tetraurelia*, the RNAi-induced silencing of ligase IV (a partner of XRCC4 in NHEJ) during conjugation results in the persistence of free broken ends during genome reorganization (53). The increasing links between NHEJ pathway proteins and genome reorganization during ciliate conjugation suggest potential targets for SUMOylation. The increased sensitivity of conditional Smt3p mutants to DNA-damaging agents along with the accumulation of Smt3p and Uba2p in the MAC anlagen are consistent with a role for SUMOylation during MAC differentiation in ciliates.

The SUMOylation pathway is required for cell pairing. When we reduced Uba2p expression levels in conditional mutant strains by withdrawing cadmium, the cells were unable to form mating pairs. The same conditional strains growing in the presence of cadmium proceeded through conjugation normally, as did wild-type cells. Although this result was unexpected, there is precedence for SUMOylation-dependent effects on mating. In the budding yeast, SUMO modification of transcription factor Ste12 is stimulated by mating pheromone, thus increasing its half-life and committing the cell to the mating differentiation program (54). In

addition, turnover of the yeast mating type factor α1 protein is dependent on SUMO-targeted ubiquitin ligases SLX5 and SLX8, which is consistent with the involvement of SUMO-mediated pathways (55). In our system, the inability of Smt3p- and Uba2p-deficient *Tetrahymena* cells to form pairs could result from altered gene transcription, modified signaling pathways, or direct SUMOylation of the mating-type protein. The experiments presented here do not allow us to distinguish between direct and indirect SUMO-mediated effects, but the recent identification of the mating-type protein from *Tetrahymena* provides an opportunity to test some of these possibilities in the future (56). The dramatic upregulation of SUMOylation in *Tetrahymena*, coupled with the defined events of genome alteration (heterochromatin formation, DNA elimination, DNA replication) make this a rich system for analysis of SUMOylation function and dynamics. Although the cell-pairing defect prevented the use of these lines to analyze defects at later stages of conjugation, we expect strong phenotypes during macronuclear development. Efforts are currently focused on developing alternative approaches, such as inducible RNAi knockdowns of SUMO pathway genes and identification of SUMOylated proteins specific to conjugation for further analysis.

ACKNOWLEDGMENTS

REFERENCES

1. **Komander D, Rape M.** 2012. The ubiquitin code. Annu Rev Biochem **81:** 203–229. http://dx.doi.org/10.1146/annurev-biochem-060310-170328.
2. **Dohmen RJ.** 2004. SUMO protein modification. Biochim Biophys Acta **1695:**113–131. http://dx.doi.org/10.1016/j.bbamcr.2004.09.021.
3. **Gareau JR, Lima CD.** 2010. The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. Nature Rev Mol Cell Biol **11:**861–871. http://dx.doi.org/10.1038/nrm3011.
4. **Wilkinson KA, Henley JM.** 2010. Mechanisms, regulation and consequences of protein SUMOylation. Biochem J **428:**133–145. http://dx.doi.org/10.1042/BJ20100158.
5. **Flotho A, Melchior F.** 2013. Sumoylation: a regulatory protein modification in health and disease. Annu Rev Biochem **82:**357–385. http://dx.doi.org/10.1146/annurev-biochem-061909-093311.
6. **Meluh PB, Koshland D.** 1995. Evidence that the MIF2 gene of Saccharomyces cerevisiae encodes a centromere protein with homology to the mammalian centromere protein CENP-C. Mol Biol Cell **6:**793–807. http://dx.doi.org/10.1091/mbc.6.7.793.
7. **Dasso M.** 2008. Emerging roles of the SUMO pathway in mitosis. Cell Div **3:**5. http://dx.doi.org/10.1186/1747-1028-3-5.
8. **Lomeli H, Vazquez M.** 2011. Emerging roles of the SUMO pathway in development. Cell Mol Life Sci **68:**4045–4064. http://dx.doi.org/10.1007/s00018-011-0792-5.
9. **Bekker-Jensen S, Mailand N.** 2011. The ubiquitin- and SUMO-dependent signaling response to DNA double-strand breaks. FEBS Lett **585:**2914–2919. http://dx.doi.org/10.1016/j.febslet.2011.05.056.
10. **Bologna S, Ferrari S.** 2013. It takes two to tango: ubiquitin and SUMO in the DNA damage response. Front Genet **4:**106. http://dx.doi.org/10.3389/fgene.2013.00106.
11. **Dieckhoff P, Bolte M, Sancak Y, Braus GH, Irniger S.** 2004. Smt3/SUMO and Ubc9 are required for efficient APC/C-mediated proteolysis in budding yeast. Mol Microbiol **51:**1375–1387. http://dx.doi.org/10.1046/j.1365-2958.2003.03910.x.
12. **Biggins S, Bhalla N, Chang A, Smith DL, Murray AW.** 2001. Genes involved in sister chromatid separation and segregation in the budding yeast Saccharomyces cerevisiae. Genetics **159:**453–470.
13. **Karrer KM.** 2000. Tetrahymena genetics: two nuclei are better than one. Methods Cell Biol **62:**127–186. http://dx.doi.org/10.1016/S0091-679X(08)61529-0.
14. **Schoeberl UE, Mochizuki K.** 2011. Keeping the soma free of transposons: programmed DNA elimination in ciliates. J Biol Chem **286:**37045–37052. http://dx.doi.org/10.1074/jbc.R111.276964.
15. **Jahn CL, Klobutcher LA.** 2002. Genome remodeling in ciliated protozoa. Annu Rev Microbiol **56:**489–520. http://dx.doi.org/10.1146/annurev.micro.56.012302.160916.
16. **Chan SR, Blackburn EH.** 2004. Telomeres and telomerase. Philos Trans R Soc Lond B Biol Sci **359:**109–121. http://dx.doi.org/10.1098/rstb.2003.1370.
17. **Duharcourt S, Yao MC.** 2002. Role of histone deacetylation in developmentally programmed DNA rearrangements in Tetrahymena thermophila. Eukaryot Cell **1:**293–303. http://dx.doi.org/10.1128/EC.1.2.293-303.2002.
18. **Altschuler MI, Yao M-C.** 1985. Macronuclear DNA of Tetrahymena thermophila exists as defined subchromosomal-sized molecules. Nucleic Acids Res **13:**5817–5831. http://dx.doi.org/10.1093/nar/13.16.5817.
19. **Coyne RS, Chalker DL, Yao MC.** 1996. Genome downsizing during ciliate development: nuclear division of labor through chromosome restructuring. Annu Rev Genet **30:**557–578. http://dx.doi.org/10.1146/annurev.genet.30.1.557.
20. **Matsuda A, Forney JD.** 2006. The SUMO pathway is developmentally regulated and required for programmed DNA elimination in Paramecium tetraurelia. Eukaryot Cell **5:**806–815. http://dx.doi.org/10.1128/EC.5.5.806-815.2006.
21. **Shang Y, Song X, Bowen J, Corstanje R, Gao Y, Gaertig J, Gorovsky MA.** 2002. A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in Tetrahymena thermophila. Proc Natl Acad Sci U S A **99:**3734–3739. http://dx.doi.org/10.1073/pnas.052016199.
22. **Bruns PJ, Cassidy-Hanley D.** 2000. Biolistic transformation of macro- and micronuclei. Methods Cell Biol **62:**501–512. http://dx.doi.org/10.1016/S0091-679X(08)61553-8.
23. **Hai B, Gaertig J, Gorovsky MA.** 2000. Knockout heterokaryons enable facile mutagenic analysis of essential genes in Tetrahymena. Methods Cell Biol **62:**513–531. http://dx.doi.org/10.1016/S0091-679X(08)61554-X.
24. **Matsuda A, Shieh AW, Chalker DL, Forney JD.** 2010. The conjugation-specific Die5 protein is required for development of the somatic nucleus in both Paramecium and Tetrahymena. Eukaryot Cell **9:**1087–1099. http://dx.doi.org/10.1128/EC.00379-09.
25. **Gaertig J, Thatcher TH, Gu L, Gorovsky MA.** 1994. Electroporation-mediated replacement of a positively and negatively selectable beta-tubulin gene in Tetrahymena thermophila. Proc Natl Acad Sci U S A **91:**4549–4553. http://dx.doi.org/10.1073/pnas.91.10.4549.
26. **Stover NA, Krieger CJ, Binkley G, Dong Q, Fisk DG, Nash R, Sethuraman A, Weng S, Cherry JM.** 2006. Tetrahymena Genome Database (TGD): a new genomic resource for Tetrahymena thermophila research. Nucleic Acids Res **34:**D500–D503. http://dx.doi.org/10.1093/nar/gkj054.
27. **Miao W, Xiong J, Bowen J, Wang W, Liu Y, Braguinets O, Grigull J, Pearlman RE, Orias E, Gorovsky MA.** 2009. Microarray analyses of gene expression during the Tetrahymena thermophila life cycle. PLoS One **4:**e4429. http://dx.doi.org/10.1371/journal.pone.0004429.
28. **Evdokimov E, Sharma P, Lockett SJ, Lualdi M, Kuehn MR.** 2008. Loss of SUMO1 in mice affects RanGAP1 localization and formation of PML nuclear bodies, but is not lethal as it can be compensated by SUMO2 or SUMO3. J Cell Sci **121:**4106–4113. http://dx.doi.org/10.1242/jcs.038570.
29. **La Salle S, Sun F, Zhang XD, Matunis MJ, Handel MA.** 2008. Developmental control of sumoylation pathway proteins in mouse male germ cells. Dev Biol **321:**227–237. http://dx.doi.org/10.1016/j.ydbio.2008.06.020.
30. **Nie M, Xie Y, Loo JA, Courey AJ.** 2009. Genetic and proteomic evidence for roles of Drosophila SUMO in cell cycle control, Ras signaling, and early pattern formation. PLoS One **4:**e5905. http://dx.doi.org/10.1371/journal.pone.0005905.
31. **Donaghue C, Bates H, Cotterill S.** 2001. Identification and characterisation of the Drosophila homologue of the yeast Uba2 gene. Biochim Biophys Acta **1518:** 210–214. http://dx.doi.org/10.1016/S0167-4781(01)00185-3.
32. **Dohmen RJ, Stappen R, McGrath JP, Forrova H, Kolarov J, Goffeau A, Varshavsky A.** 1995. An essential yeast gene encoding a homolog of ubiq-

uitin-activating enzyme. J Biol Chem **270:**18099–18109. http://dx.doi.org/10.1074/jbc.270.30.18099.

33. **Johnson ES.** 2004. Protein modification by SUMO. Annu Rev Biochem **73:** 355–382. http://dx.doi.org/10.1146/annurev.biochem.73.011303.074118.

34. **Jones D, Crowe E, Stevens TA, Candido EP.** 2002. Functional and phylogenetic analysis of the ubiquitylation system in Caenorhabditis elegans: ubiquitin-conjugating enzymes, ubiquitin-activating enzymes, and ubiquitin-like proteins. Genome Biol **3:**RESEARCH0002. http://dx.doi.org/10.1186/gb-2001-3-1-research0002.

35. **Johnson ES, Schwienhorst I, Dohmen RJ, Blobel G.** 1997. The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. EMBO J **16:**5509–5519. http://dx.doi.org/10.1093/emboj/16.18.5509.

36. **Saracco SA, Miller MJ, Kurepa J, Vierstra RD.** 2007. Genetic analysis of SUMOylation in Arabidopsis: conjugation of SUMO1 and SUMO2 to nuclear proteins is essential. Plant Physiol **145:**119–134. http://dx.doi.org/10.1104/pp.107.102285.

37. **Nacerddine K, Lehembre F, Bhaumik M, Artus J, Cohen-Tannoudji M, Babinet C, Pandolfi PP, Dejean A.** 2005. The SUMO pathway is essential for nuclear integrity and chromosome segregation in mice. Dev Cell **9:**769–779. http://dx.doi.org/10.1016/j.devcel.2005.10.007.

38. **Chalker DL, Fuller P, Yao MC.** 2005. Communication between parental and developing genomes during tetrahymena nuclear differentiation is likely mediated by homologous RNAs. Genetics **169:**149–160. http://dx.doi.org/10.1534/genetics.104.032300.

39. **Ho JC, Warr NJ, Shimizu H, Watts FZ.** 2001. SUMO modification of Rad22, the Schizosaccharomyces pombe homologue of the recombination protein Rad52. Nucleic Acids Res **29:**4179–4186. http://dx.doi.org/10.1093/nar/29.20.4179.

40. **Hoege C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S.** 2002. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. Nature **419:**135–141. http://dx.doi.org/10.1038/nature00991.

41. **Maeda D, Seki M, Onoda F, Branzei D, Kawabe Y, Enomoto T.** 2004. Ubc9 is required for damage-tolerance and damage-induced interchromosomal homologous recombination in S. cerevisiae. DNA Repair **3:**335–341. http://dx.doi.org/10.1016/j.dnarep.2003.11.011.

42. **Galanty Y, Belotserkovskaya R, Coates J, Polo S, Miller KM, Jackson SP.** 2009. Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. Nature **462:**935–939. http://dx.doi.org/10.1038/nature08657.

43. **Morris JR, Boutell C, Keppler M, Densham R, Weekes D, Alamshah A, Butler L, Galanty Y, Pangon L, Kiuchi T, Ng T, Solomon E.** 2009. The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress. Nature **462:**886–890. http://dx.doi.org/10.1038/nature08593.

44. **Frankenberg-Schwager M, Kirchermeier D, Greif G, Baer K, Becker M, Frankenberg D.** 2005. Cisplatin-mediated DNA double-strand breaks in replicating but not in quiescent cells of the yeast Saccharomyces cerevisiae. Toxicology **212:**175–184. http://dx.doi.org/10.1016/j.tox.2005.04.015.

45. **Cunha D, Cunha R, Corte-Real M, Chaves SR.** 2013. Cisplatin-induced cell death in Saccharomyces cerevisiae is programmed and rescued by proteasome inhibition. DNA Repair **12:**444–449. http://dx.doi.org/10.1016/j.dnarep.2013.02.005.

46. **Ormerod MG, Orr RM, Peacock JH.** 1994. The role of apoptosis in cell killing by cisplatin: a flow cytometric study. Br J Cancer **69:**93–100. http://dx.doi.org/10.1038/bjc.1994.14.

47. **Golebiowski F, Matic I, Tatham MH, Cole C, Yin Y, Nakamura A, Cox J, Barton GJ, Mann M, Hay RT.** 2009. System-wide changes to SUMO modifications in response to heat shock. Sci Signal **2:**ra24. http://dx.doi.org/10.1126/scisignal.2000282.

48. **Sydorskyy Y, Srikumar T, Jeram SM, Wheaton S, Vizeacoumar FJ, Makhnevych T, Chong YT, Gingras AC, Raught B.** 2010. A novel mechanism for SUMO system control: regulated Ulp1 nucleolar sequestration. Mol Cell Biol **30:**4452–4462. http://dx.doi.org/10.1128/MCB.00335-10.

49. **Chalker DL.** 2008. Dynamic nuclear reorganization during genome remodeling of Tetrahymena. Biochim Biophys Acta **1783:**2130–2136. http://dx.doi.org/10.1016/j.bbamcr.2008.07.012.

50. **Deyrieux AF, Rosas-Acosta G, Ozbun MA, Wilson VG.** 2007. Sumoylation dynamics during keratinocyte differentiation. J Cell Sci **120:**125–136. http://dx.doi.org/10.1242/jcs.03317.

51. **Johnson ES, Blobel G.** 1997. Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. J Biol Chem **272:**26799–26802. http://dx.doi.org/10.1074/jbc.272.43.26799.

52. **Yurchenko V, Xue Z, Sadofsky MJ.** 2006. SUMO modification of human XRCC4 regulates its localization and function in DNA double-strand break repair. Mol Cell Biol **26:**1786–1794. http://dx.doi.org/10.1128/MCB.26.5.1786-1794.2006.

53. **Kapusta A, Matsuda A, Marmignon A, Ku M, Silve A, Meyer E, Forney JD, Malinsky S, Betermier M.** 2011. Highly precise and developmentally programmed genome assembly in Paramecium requires ligase IV-dependent end joining. PLoS Genet **7:**e1002049. http://dx.doi.org/10.1371/journal.pgen.1002049.

54. **Wang Y, Dohlman HG.** 2006. Pheromone-regulated sumoylation of transcription factors that mediate the invasive to mating developmental switch in yeast. J Biol Chem **281:**1964–1969. http://dx.doi.org/10.1074/jbc.M508985200.

55. **Nixon CE, Wilcox AJ, Laney JD.** 2010. Degradation of the Saccharomyces cerevisiae mating-type regulator α1: genetic dissection of cis-determinants and trans-acting pathways. Genetics **185:**497–511. http://dx.doi.org/10.1534/genetics.110.115907.

56. **Cervantes MD, Hamilton EP, Xiong J, Lawson MJ, Yuan D, Hadjithomas M, Miao W, Orias E.** 2013. Selecting one of several mating types through gene segment joining and deletion in Tetrahymena thermophila. PLoS Biol **11:**e1001518. http://dx.doi.org/10.1371/journal.pbio.1001518.