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Defects in SUMO (Small Ubiquitin-related Modifier) Conjugation and Deconjugation Alter Cell Sensitivity to DNA Topoisomerase I-induced DNA Damage*

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Eukaryotic DNA topoisomerase I (Top1p) has important functions in DNA replication, transcription, and recombination. This enzyme also constitutes the cellular target of camptothecin (CPT), which induces S-phase-dependent cytotoxicity. To define cellular pathways that regulate cell sensitivity to Top1p-induced DNA lesions, we described a yeast genetic screen for conditional *tah* (*top1*T722A-hypersensitive) mutants with enhanced sensitivity to low levels of the CPT mimetic mutant *top1T722A* (Reid, R. J., Fiorani, P., Sugawara, M., and Bjornsti, M. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 11440–11445; Fiorani, P., Reid, R. J., Schepis, A., Jacquiau, H. R., Guo, H., Thimmaiah, P., Benedetti, P., and Bjornsti, M. A. (2004) *J. Biol. Chem.* 279, 21271–21281). Here we report that *tah* mutant *ubc9–10* harbors a hypomorphic allele of *UBC9*, which encodes the essential SUMO (small ubiquitin-related modifier) E2-conjugating enzyme. The same conditional *ubc9P123L* mutant was also isolated in an independent screen for enhanced sensitivity to a distinct Top1p poison, Top1N726Hp. The *ubc9–10* mutant exhibited a decrease in global protein sumoylation and increased sensitivity to a wide range of DNA-damaging agents independent of Top1p. Deletion of the Ulp2 SUMO protease failed to restore *ubc9–10* cell resistance to Top1p poisons or hydroxyurea yet adversely affected wild-type *TOP1* cell genetic stability and sensitivity to hydroxyurea. Moreover, although mutation of different consensus SUMO sites in the N terminus and linker region of yeast Top1p failed to recapitulate *ubc9–10* mutant phenotypes, they revealed distinct and subtle effects on cell sensitivity to CPT. These results provide insights into the complexities of SUMO conjugation and the confounding effects of SUMO modification on Top1p function and cell sensitivity to genotoxic agents.

In eukaryotes, DNA topoisomerase I (Top1p)[1] is a highly conserved enzyme that catalyzes the relaxation of positively and negatively supercoiled DNA (1–4). This nuclear enzyme is encoded by a single gene and plays critical roles in DNA replication, recombination, transcription, and chromosome condensation. Top1p is also the cellular target of several anticancer agents, including camptothecin (CPT) analogs, topotecan, and irinotecan (CPT-11) approved by the Food and Drug Administration, as well as additional compounds in preclinical and clinical development (5–9).

DNA topoisomerase I transiently cleaves a single strand of duplex DNA, creating a protein-linked nick in the DNA, which allows strand rotation to effect changes in DNA topology (1–4). The nucleophilic attack of the 5′-OH end of the cleaved DNA strand on the phosphotyrosyl linkage between Top1p and the 3′-DNA end restores the integrity of the DNA and liberates the enzyme. Camptothecin targets Top1p by reversibly stabilizing the covalent enzyme-DNA intermediate (10, 11). Although drug-stabilized Top1p-DNA ternary complexes are detected throughout the cell cycle, the cytotoxic activity of CPT is S-phase-dependent. The collision of advancing DNA polymerases with CPT·Top1p·DNA complexes converts readily reversible DNA nicks into irreversible DNA lesions, which trigger checkpoint activation and cell death. Several studies have established the increased CPT sensitivity of cells defective for various components of DNA damage and replication checkpoints such as yeast Rad9p, Rad53p, Mec1p, Tel1p, and human ATR and SMC1 (12–16). Additional studies in yeast indicate that processive DNA replication (*CDC45*, *DPB11*) (17) and the repair of stalled replication forks (*RAD52*, *SGS1*, and *SRS2*) (18–20) are also critical determinants of CPT cytotoxicity.

Post-translational modification of Top1p has also been implicated in modulating enzyme activity and sensitivity to CPT. For instance, the covalent attachment of ubiquitin to lysine residues in Top1p following exposure of mammalian cells to CPT coincides with a rapid down-regulation of the enzyme and, in some cases, drug resistance (5, 21). In yeast, the deubiquitinating enzyme, Doa4p, functions to maintain free ubiquitin pools by recycling ubiquitin from proteins destined for vacuolar or proteasomal degradation (22, 23). Dysregulation of ubiquitin homeostasis in *doa4* mutant strains results in enhanced sen-

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[1] The abbreviations used are: Top1p, DNA topoisomerase I; SUMO, small ubiquitin-related modifier; E1, activating enzyme; E2, conjugating enzyme; E3, ubiquitin-like protein isopeptide ligase; CPT, camptothecin; HU, hydroxyurea; *ts*, temperature-sensitive; Me_2SO, dimethyl sulfoxide; DAPI, 4′,6-diamidino-2-phenylindole; *tah*, *top1*T722A-hypersensitive; *nhh*, *top1*N726H-hypersensitive; MOPS, 4-morpholinepropanesulfonic acid.

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TABLE I
Yeast strains

Strain	Genotype	Ref.
FY250	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63*	61
FY251	*MAT***a** *ura3–52, his3Δ200, leu2Δ1, trp1Δ63*	61
EKY2	*MAT***a***, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::HIS3*	61
EKY3	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1*	61
RRY82	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1, ubc9–10*	This work
RRY82a	*MAT***a***, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1, ubc9–10*	This work
PTY30	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1, ubc9Δ::his5+, YCpUBC9*	This work
RWY10	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1, ulp2Δ::his5+*	This work
RWY11	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, top1Δ::TRP1, ubc9–10, ulp2Δ::his5+*	This work
HJY13	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, ubc9–10*	This work
HJY14	*MAT***a***, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, ubc9–10*	This work
HJY15	*MAT***a***, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, ubc9–10, doa4–10*	This work
HJY16	*MAT***a***, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, ubc9–10, doa4–10*	This work
HJY19	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, ulp2Δ::his5+*	This work
HJY21	*MAT***a***, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, ulp2Δ::his5+*	This work
HJY22	*MATα, ura3–52, his3Δ200, leu2Δ1, trp1Δ63, ubc9–10, ulp2Δ::his5+*	This work

sitivity to Top1p poisons and other DNA-damaging agents independent of detectable alterations in Top1 protein levels or activity (16). Rather, genetic evidence implicates *doa4* mutant-induced defects in checkpoint function where global alterations in ubiquitin conjugation adversely impact cell survival in response to a variety of genotoxic stresses (16).

SUMO (small ubiquitin-related modifier) polypeptides are also covalently attached to ε-amino groups of lysine residues in target proteins through the action of a cascade of enzymes, similar to those required for ubiquitin conjugation (reviewed in Refs. 24, 25). In humans, four SUMO family members have been described, whereas in yeast, a single essential gene encodes the related Smt3 protein. As with ubiquitin, proteolytic processing of inactive SUMO/Smt3p precursors by a SUMO-specific protease generates the mature GG C terminus. Mature SUMO forms a thiolester linkage with a heterodimeric E1-activating enzyme (Aos1p/Uba2p in yeast) in an ATP-dependent reaction, which is then transferred to Ubc9p. SUMO conjugation to substrate proteins may be catalyzed by Ubc9p alone or in concert with an E3 ligase. Ubc9p is the sole SUMO E2-conjugating enzyme and is essential in yeast and mammalian cells. Sumoylation is also reversible due to the isopeptidase activity of SUMO proteases, such as yeast Ulp1p and Ulp2p (26–28).

A SUMO consensus site (ΨK*X*E) has been defined, where Ψ is a large hydrophobic residue and *X* is any amino acid (29). However, recent studies including proteomic approaches to analyze global protein sumoylation have defined lysine modifications in noncanonical SUMO sequences (30, 31).

Sumoylation of human Top1p has been demonstrated in response to high concentrations of CPT and other agents that poison Top1p and has been suggested to regulate the partitioning of the enzyme between the nucleus and nucleolus (6, 32–35). However, these results have generated some debate with a recent report concluding that sumoylation of human Top1 does not affect CPT-induced nucleolar clearance of the enzyme (36). Yeast Top1p was also identified in recent global analyses of sumoylated proteins based on extremely sensitive detection methods (37, 38). These studies support the widely held view that sumoylation is a dynamic process with only a small percentage of target proteins sumoylated at a given time. The expression of a dominant negative UBC9 mutant in human breast cancer MCF7 cells has also been shown to increase cell sensitivity to the CPT analog, topotecan (39). However, other studies indicate that sumoylation of Top1p correlates with increased CPT cytotoxicity (35). These somewhat contradictory findings may be, in part, a consequence of the myriad of genetic alterations that attend the malignant transformation of the cancer cell lines used or reflect the complications of using high drug concentrations.

To investigate cellular pathways regulating cell sensitivity to CPT in an experimental system that avoids some of these issues, we developed a yeast genetic screen to isolate conditional mutants exhibiting enhanced sensitivity to low levels of a self-poisoning Top1T722A mutant enzyme (17, 40). A panel of conditional *tah* (*top1*T722A-hypersensitive) mutants were unable to tolerate low levels of CPT-induced DNA damage at the nonpermissive temperature of 36 °C because of alterations in processive DNA polymerization (*cdc45–10*, *dpb11–10*), ubiquitin homeostasis (*doa4–10*), or actin cytoskeletal architecture (*sla1–10*, *sla2–10*) (16, 17, 40). As with CPT poisoning of wild-type Top1p, Top1T722Ap exhibits reduced rates of DNA religation without obvious effects on DNA binding or cleavage (41). This cytotoxic mechanism contrasts with that of other self-poisoning mutant enzymes, such as Top1N726Hp, where increased rates of DNA cleavage produce elevated covalent complexes (42). We recently reported that some *tah* mutants (*cdc45–10*, *dpb11–10* and *doa4–10*) exhibit varying patterns of sensitivity to these distinct Top1p poisons (41). These results prompted a second screen for temperature sensitive (*ts*) mutants with enhanced sensitivity to DNA lesions resulting from elevated rates of DNA cleavage by Top1N726Hp, termed *nhh* for *top1*N726H-hypersensitive. Here we report the identification of the same *ubc9–10* mutation in these two independent screens and discuss the function of Ubc9p in protecting cells from diverse Top1p poisons and DNA-damaging agents.

EXPERIMENTAL PROCEDURES

Chemicals, Yeast Strains, and Plasmids—CPT and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma. Stock solutions of CPT (4 mg/ml in Me_2SO) were stored at −20 °C. Ethyl methanesulfonate was from Kodak (Eastman Kodak Company, Rochester, NY), whereas 5-fluoroorotic acid was purchased from U. S. Biological (Swampscott, MA).

Saccharomyces cerevisiae strains, listed in Table I, were isogenic with FY250 (*MATα*, *ura3–52*, *his3Δ200*, *leu2Δ1*, *trp1Δ63*), which was kindly provided by Fred Winston (Harvard Medical School, Cambridge, MA). Gene deletions were made by PCR (43). The *URA3*, *ARS*/*CEN* plasmids YCpScTOP1, YCpSctop1T722A, and YCpSctop1N726H and the corresponding *LEU2* vectors, YCpScTOP1·L and YCpSctop1T722A·L, which constitutively express the indicated *TOP1* allele from the yeast *TOP1* promoter, have been described previously (16, 44). The inclusion of an N-terminal FLAG epitope is indicated by an e prefix in the *URA3* vectors, YCpSceTOP1 and YCpScetop1T722A (44, 45). Plasmids pRS416 and pRS415 (46) served as controls. A *URA3*, *ARS*/*CEN* plasmid-based yeast genomic DNA library, referred to as YCp-FY250, was described (17). Substituting Arg for Lys^{600} (in the consensus sumoylation site $LK^{600}KE$) in YCpSctop1K600R was accomplished by oligonucleotide-directed mutagenesis with the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). The mutation of Lys residues within consensus sumoylation sites $IK^{65}TE$ and $IK^{91}K^{92}E$ to Arg was accomplished by PCR-based substitution of genomic *TOP1* DNA. PCR-amplified sequences then replaced the corresponding wild-

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type sequences in plasmid encoded *top1* alleles to yield YCpSctop1K65,91,92R and YCpSctop1K65,91,92R,T722A. Mutations were confirmed by DNA sequencing.

tah and nhh Mutant Isolation—A yeast genetic screen to isolate *tah* mutants exhibiting temperature-sensitive growth in the presence of low levels of Top1T722Ap has been described (17). An independent screen for *nhh* mutants that were nonviable at the nonpermissive temperature of 36 °C when expressing *top1N726H* was also performed. In each case, *top1*Δ cells transformed with YCpSctop1T722A or YCpSctop1N726H, respectively, were ethyl methanesulfonate-mutagenized and screened for colonies exhibiting *ts* growth at 36 °C. Subsequent plating on 5-fluoroorotic acid eliminated mutants exhibiting *ts* growth in the absence of plasmid-encoded Top1T722Ap or Top1N726Hp. Backcrossing to unmutagenized *top1*Δ strains identified individual *tah* or *nhh1* mutants where a single recessive gene defect was linked to *top1T722A* or *top1N726H* hypersensitivity, respectively.

Cloning UBC9 by Complementation—*tah12* cells co-transformed with plasmid YCpSctop1T722A·L and YCp-FY250 library DNA were screened for growth at 36 °C on SC-uracil/-leucine medium. Two *TAH12* clones contained overlapping fragments of chromosome IV encompassing *UBC9*, *YDL063*, *YDL062*, and *RPS29B*. Subsequent subcloning into pRS416 to yield YCpUBC9 confirmed that *UBC9* complemented the *top1T722A* and HU hypersensitivity of *tah12* cells.

For the *nhh1* mutant, cells transformed with YCp-FY250 library DNA were screened for plasmid-dependent viability at 35 °C on SC-uracil medium supplemented with 7 mg/ml HU. Sequencing and subcloning of the insert within the complementing clone also identified *UBC9*.

To confirm the genetic identity of *TAH12/NHH1* as *UBC9*, a selectable *URA3* (or *LEU2*) marker was integrated into the genomic sequences flanking *UBC9*. After mating with respective *tah12* or *nhh1* mutants, the meiotic products were assessed for segregation of uracil (or leucine) prototrophy and the *tah* or *nhh* phenotype. YIpUBC9·U and YIpUBC9·L integration constructs were made by ligating a 1.8-kbp HindIII/SacI DNA fragment from one *TAH12* clone into pRS406 and pRS405, respectively. Digestion with BglII targeted *URA3* or *LEU2* integration to sequences 3′ to *UBC9*.

The mutant *ubc9* allele from *tah12* cells was recovered after targeted integration of YIpUBC9·U. Purified genomic DNA, restricted with XbaI, was size-selected, ligated, and transformed into *Escherichia coli*. DNA sequencing defined a Pro^{123} to Leu substitution in *ubc9–10* cells. The same P123L mutation was defined for *nhh1* cells by sequencing the entire *UBC9* coding region in PCR-amplified genomic DNA.

Mutation of Pro^{123} to Leu or Ala (YCpubc9P123L and YCpubc9P123A, respectively) was undertaken with the QuickChange mutagenesis kit using YCpUBC9 as template. After sequence verification, the *ubc9P123L* and *ubc9P123A* alleles were cloned into pRS415 and the multicopy vector, pRS425.

Cell Viability Assays—Exponential cultures of cells transformed with YCpScTOP1, YCpSctop1T722A, YCpSctop1N726H, or pRS416 were serially 10-fold diluted, and aliquots were spotted onto SC-uracil plates plus 25 mM HEPES, pH 7.2, 0.125% Me_2SO, and 0 or 5 μg/ml CPT. Cell viability was assessed following incubation at 26 or 36 °C. For a more quantitative measure of colony formation, serial dilutions of transformed cells were plated onto SC-uracil medium ± CPT and incubated at 26 or 36 °C. Wild-type *UBC9* and mutant *ubc9–10* cell sensitivity to methyl methanesulfonate (0.0125 or 0.025%), HU (5–10 mg/ml), or UV (10 or 20 $\mu J/m^2$) was assessed as described previously (17). Sensitivity to bleomycin was assessed by treating exponentially growing wild-type or *ubc9–10* cells with 0, 25, or 50 μg/ml bleomycin at 36 °C. At various times, aliquots were serially diluted and the number of viable cells forming colonies was determined after incubation at 26 °C.

Yeast Cell Microscopy—Exponential cultures of wild-type and *ubc9–10* cells transformed with YCpSctop1 vectors were shifted from 26 to 36 °C for 6 h or retained at 26 °C. Aliquots of cells were fixed with 70% ethanol and stored at −20 °C for subsequent DAPI staining and microscopy. 3–5-μl aliquots of fixed cells were applied to individual wells of poly-L-lysine-coated Teflon-masked slides (Polysciences Inc., Warrington, PA). After 15 min, 5 μl of 1 μg/ml DAPI and 2 μl of Prolong® Antifade (Molecular Probes Inc., Eugene, OR) were added. Cells were viewed with a Zeiss Axioskop 2 microscope equipped with Differential interference contrast, epifluorescence, and UV-blocking filter sets. Images were acquired with a Micromax CCD camera (Princeton Instruments Inc., Monmouth Junction, NJ) and IPLab software (Scanalytics). For each sample, at least 100 cells were counted, noting the number of unbudded cells, cells with small buds (less than half of the diameter of the mother cell), and cells with large buds. DAPI staining further distinguished cells with a single nuclear mass from those with segregated nuclei.

DNA Topoisomerase I Activity—Top1 protein levels in wild-type and *ubc9–10* cells transformed with YCpScetop1 vectors were assessed in crude extracts prepared from exponential cultures grown at 26 or shifted to 36 °C for 6 h. As described previously (16, 42), cells were harvested by centrifugation and resuspended in 2 ml/g cells of TEEG buffer (20 mM Tris, pH 7.5, 0.2 M KCl, 10 mM EDTA, 10 mM EGTA, 10% glycerol) supplemented with phosphatase inhibitor cocktails I and II (Sigma) and protease inhibitor Complete™ mixture (Roche Diagnostics GmbH, Mannheim, Germany). Following a freeze-thaw cycle at −80 °C, the cells were lysed by vortexing with glass beads and extracts were clarified by centrifugation. After correcting for total protein, DNA topoisomerase I catalytic activity was assessed in a plasmid DNA relaxation assay (47). Top1 protein levels and integrity were assessed in immunoblots using either monoclonal M2 antibody specific for the N-terminal FLAG epitope or a polyclonal antibody specific for yeast Top1p followed by chemiluminescence (Amersham Biosciences).

Yeast Ubc9 Antibodies and Western Blotting—Antibodies were raised in rabbits against a synthetic peptide spanning residues 134–145 of yeast Ubc9p. The resultant polyclonal antibodies were affinity-purified on Sepharose-4B conjugated to the same peptide using standard procedures (48). Antibody fractions were concentrated by Centriplus Amicon filtration and tested for their reactivity against purified yeast and human Ubc9 protein (the generous gift of Brenda Schulman, St. Jude Children's Research Hospital).

To assess Ubc9 protein levels, exponential cultures of untransformed or transformed cells grown at 26 °C were split in half and incubated for an additional 6 h at 26 or 36 °C. Equivalent numbers of cells were then pelleted by centrifugation and lysed with NaOH and trichloroacetic acid as described (49). $\sim 10^7$ cells, resuspended in 1 ml of ice-cold H_2O, were incubated with 150 μl of 34.5 mM NaOH, 5.5% β-mercaptoethanol on ice for 10 min followed by 150 μl of 50% trichloroacetic acid for 10 min, all with occasional vortexing. Following centrifugation, the precipitated proteins were resuspended in 100 μl of SDS-PAGE sample buffer and boiled after adjusting the pH with 1 M Tris, pH 8.5. The proteins were resolved in 12% Bis-Tris NuPAGE gels with MOPS running buffer (Invitrogen) and blotted onto activated polyvinylidene difluoride membranes (PerkinElmer Life Sciences), as per the manufacturer's instructions. Yeast Ubc9 proteins were detected in immunoblots with the polyclonal yUbc9 antibody and chemiluminescence.

To assess global steady-state levels of sumoylated proteins, NaOH/trichloroacetic acid extracts of wild-type *UBC9* and *ubc9–10* cells were resolved in 4–12% Bis-Tris PAGE gels (Invitrogen) and immunoblotted with polyclonal rabbit antibodies specific for yeast Smt3p (50) as described above. In all of the cases, immunostaining with tubulin-specific antibodies served as loading controls.

RESULTS

Ubc9p Affects Cell Sensitivity to DNA Topoisomerase I Poisons and Other DNA-damaging Agents—We previously described a yeast genetic screen to isolate *ts* mutants exhibiting enhanced sensitivity to CPT, using the self-poisoning *top1T722A* mutant as a CPT mimetic (16, 17, 40). Conditional *tah* (top1T722A-hypersensitive) mutants could not tolerate low levels of CPT-induced DNA damage at the nonpermissive temperature of 36 °C. However, several *tah* mutants exhibited varying patterns of temperature sensitivity to another self-poisoning *top1N726H* mutant where elevated rates of DNA cleavage produce increased levels of covalent complexes (41). Thus, we initiated a second genetic screen for conditional *nhh* mutants. Here we report that the same mutant allele of *UBC9* was independently isolated in both the *tah* and *nhh* screens.

As detailed under "Experimental Procedures," the *ts* phenotypes of the *tah12* and *nhh1* mutants segregated as recessive single gene defects in backcrosses with wild-type strains. Subsequent complementation and genetic analyses identified *UBC9* as *TAH12* and *NHH1*, whereas DNA sequencing identified the same Pro^{123} to Leu substitution for each mutant strain (Fig. 1). For ease of presentation, hereafter we refer to either strain as the *ubc9–10* mutant. Our initial analyses were carried out in *top1*Δ strain backgrounds to facilitate the analysis of cell sensitivity to CPT in the presence of plasmid-encoded *TOP1* or various *top1* mutants.

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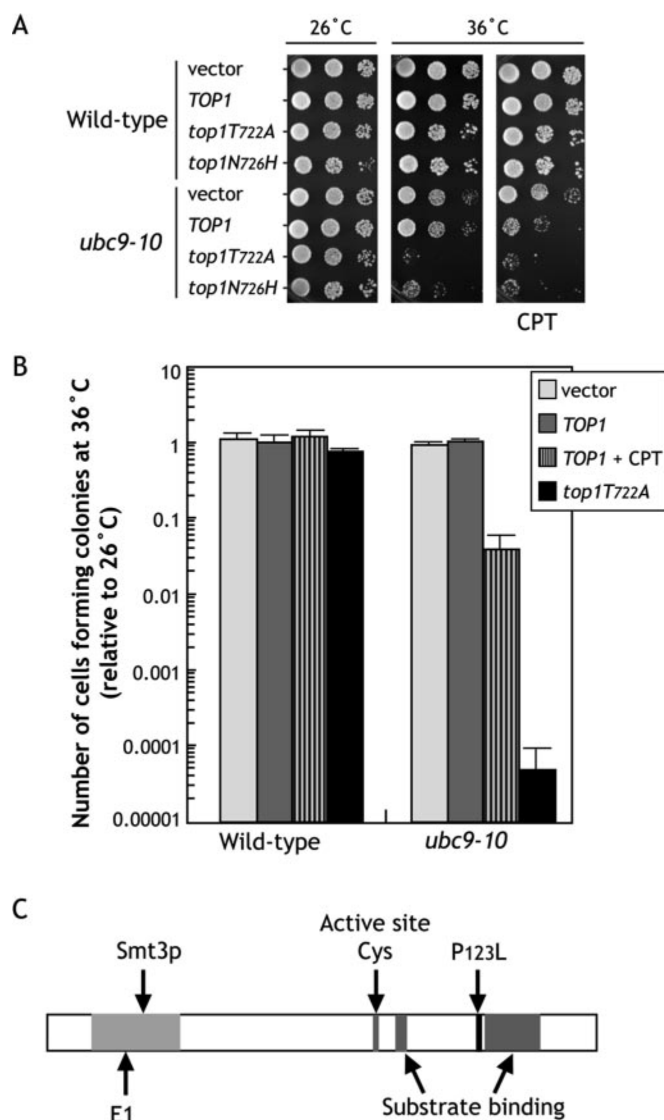

FIG. 1. **A *ubc9P123L* mutant enhances *ubc9–10* cell sensitivity to Top1p poisons.** *A*, exponential cultures of wild-type (*UBC9*) or *ubc9–10* cells transformed with *ARS/CEN* plasmids pRS416 (*vector*), YCpScTOP1 (*TOP1*), YCpSctop1T722A (*top1T722A*), and YCpSctop1N726H (*top1N726H*) and grown at 26 °C were serially 10-fold diluted and spotted onto SC-uracil medium plus or minus 5 μg/ml CPT, 25 mM HEPES, pH 7.2, and 0.125% Me_2SO. Cell viability was assessed following incubation at 26 or 36 °C. *B*, exponential cultures of wild-type (*UBC9*) or *ubc9–10* cells transformed with pRS416 (*vector*), YCpScTOP1 (*TOP1*), or YCpSctop1T722A (*top1T722A*) were serially diluted and plated on SC-uracil medium ± CPT. The number of viable cells forming colonies was determined following incubation of duplicate plates at 36 °C and plotted relative to the number obtained on duplicate plates at 26 °C ($n = 3$). *C*, in *ubc9–10*, the codon encoding Pro^{123} has been mutated to code for Leu. Residues known to affect yeast Ubc9 binding to the Aos1/Uba2 E1 enzyme and Smt3p span N-terminal residues 14–37 (60). The corresponding residues shown to be involved in human Ubc9 binding to the RanGAP1 substrate (100–101 and 125–140) (51) are also indicated. The conserved active site residue is Cys^{93}.

Ubc9p is a highly conserved, essential E2 SUMO-conjugating enzyme that catalyzes the formation of an isopeptide linkage between a C-terminal Gly in SUMO (yeast Smt3p) and the ε-amino group of lysine residues in target proteins (24). The Pro^{123} residue mutated in *ubc9–10* is also conserved and, in human Ubc9p crystal structures, lies in a loop positioned over a cleft containing the active site Cys^{93} residue (51, 52). Pro^{123} is also N-terminal to an α-helix implicated in binding consensus SUMO sites in target proteins (51).

As shown in Fig. 1, *A* and *B*, and summarized in Table II, low level expression of wild-type *TOP1* or the self-poisoning *top1T722A* or *top1N726H* mutants had little effect on wild-type *UBC9* cell viability, either in the presence or absence of CPT. In contrast, *ubc9–10* mutant cells were unable to tolerate either self-poisoning Top1 enzyme or CPT in the presence of wild-type Top1p at 36 °C. As with other *tah* mutants (16, 17, 53), *ubc9–10* cells exhibited increased sensitivity to the ribonucleotide reductase inhibitor, HU, at 36 °C. However, the *ubc9–10* mutant was unique among *tah* mutants in its conditional hypersensitivity to a wide range of DNA-damaging agents (alkylating agent methyl methanesulfonate, UV light, and bleomycin) (Table II and Fig. 2) with no detectable alterations in cell growth under a wide range of other environmental conditions (oligomycin, cycloheximide, high salt, or glycerol) (data not shown). With the exception of X-rays (data not shown), *ubc9–10* cells exhibited increased sensitivity to all of the DNA-damaging agents tested but not to other environmental stresses. Because *UBC9* is an essential gene, these findings further indicate that the alterations in Ubc9P123Lp function, sufficient to sensitize cells to genotoxic stress, were insufficient to induce cell lethality in the absence of DNA damage.

Given the somewhat confounding reports that both increased sumoylation of human Top1p and decreased global sumoylation (induced by a dominant negative human *UBC9* mutant) enhance the cytotoxic activity of CPT (35, 39), we next asked whether alterations in yeast Top1p activity or sumoylation could be detected in isogenic wild-type *UBC9* and *ubc9–10* mutant strains. To address this possibility, extracts were made of *UBC9* and *ubc9–10* cells transformed with YCpScTOP1 or YCpSctop1T722A vectors and cultured at 26 °C or shifted to 36 °C for 6 h. The specific activities of wild-type Top1p and mutant Top1T722Ap were unaffected by the temperature shift (Fig. 3), consistent with the lack of any alterations in Top1 protein levels detected in Western blots (data not shown). Moreover, under these conditions, we were unable to detect SUMO (Smt3p) modification of wild-type or mutant Top1 proteins, either in the immunoprecipitates of epitope-tagged Top1 proteins or whole cell extracts, using a polyclonal Smt3p antibody (data not shown). These results are not surprising, because recent proteomic analyses of sumoylated yeast proteins indicate that only a small percentage of a SUMO target may be sumoylated at any given time, necessitating more sensitive multidimensional protein identification technology and instrumentation to detect Top1p-Smt3p conjugates (37, 38). However, when cells overexpress wild-type Top1p or the self-poisoning Top1T722Ap from *GAL1*-promoted constructs, similar levels of Top1p sumoylation were detected.[2] Thus, the extent of Top1p sumoylation did not change with the self-poisoning mutant enzyme.

Low constitutive expression of Top1T722Ap in *ubc9–10* cells at 36 °C was, nevertheless, sufficient to induce a terminal phenotype of large-budded cells with an undivided nucleus (Table III), consistent with the cytotoxic activity of this self-poisoning enzyme in S-phase. In the absence of Top1p poisons, *ubc9–10* cells also exhibited an increase in large budded cells containing a single nuclear mass relative to wild-type *UBC9* cells at 36 °C (42 *versus* 17%), albeit at lower levels than the 70% observed in *ubc9–10* cells expressing Top1T722Ap at the nonpermissive temperature (Table III). However, as *ubc9–10* cells expressing Top1p remained viable at 36 °C, the accumulation of cells with this morphology indicated a decreased rate of S-phase transit. One possibility was the induction of sublethal levels of DNA damage sufficient to trigger S-phase checkpoints and slow the advance of replication forks. Indeed, as

[2] X. L. Chen and E. S. Johnson, unpublished results.

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TABLE II
ubc9–10 cell sensitivity to DNA-damaging agents

Yeast strain[a]	Cell viability at the nonpermissive temperature[b]					
	top1T722A[c]	CPT[c]	HU[d]	MMS[d]	Bleo[e]	UV[f]
Wild-type	+++	++++	++++	++++	+++	++++
ubc9–10 (tah12)	+	++	−	++	+	++

[a] Isogenic *top1*Δ yeast strains, wild-type for *UBC9* or containing the *ubc9–10* mutant allele, were used.
[b] For the conditions indicated, exponentially growing cells adjusted to an OD_{595} = 0.3 were serially 10-fold diluted and 4-μl aliquots were spotted onto plates. After 3 days at 26 or 36°C, viability was scored as ++++ for colonies at 10^{-3} dilution, +++ for colonies at 10^{-2}, ++ for colonies at 10^{-1}, + for colonies in undiluted samples, and − for no growth at 36°C.
[c] To assess cell sensitivity to Top1 poisons, cells were transformed with plasmids YCpSctop1T$_{722}$A, YCpSctop1N726H, or YCpScTOP1. Individual transformants were spotted on SC-uracil medium and incubated at 26 or 36°C. Similar results were obtained for Top1N726H or Top1T722A-expressing cells. For CPT sensitivity, YCpScTOP1 transformants were plated on selective medium with 25 mM HEPES, pH 7.2, and 0 or 5 μg/ml CPT in a final 0.125% Me_2SO.
[d] Sensitivity to HU (5 mg/ml) and methyl methane sulfonate (MMS) (0.0125%) was assessed on YPD plates.
[e] Sensitivity to bleomycin (Bleo) was assessed by colony formation at 26°C following exposure to 50 μg/ml Bleo for 24 h at 36°C.
[f] UV sensitivity was assessed by colony formation on YPD plates following irradiation with 0, 10 or 20 μJ/m^2 UV and growth at 36°C.

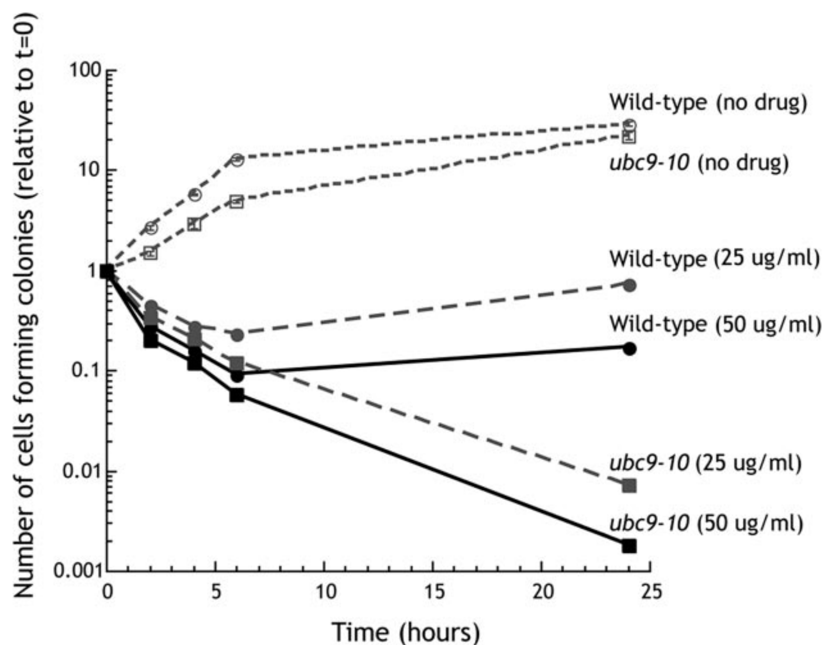

FIG. 2. ***ubc9–10* cells are hypersensitive to bleomycin.** Exponential cultures of isogenic *top1*Δ strains (wild-type for *UBC9* or *ubc9–10*) were treated with 0 (no drug), 25, or 50 μg/ml bleomycin at 36 °C. At the times indicated, aliquots were serially diluted and plated on YPD medium at 26 °C. The number of viable cells forming colonies was plotted relative to that obtained at t = 0. *Error bars* from three independent experiments are smaller than the symbols used.

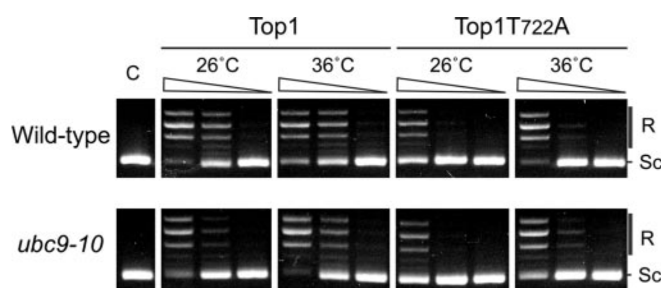

FIG. 3. **DNA topoisomerase I catalytic activity is unaltered in *ubc9–10* cells.** Exponential cultures of isogenic *top1*Δ strains, wild type for *UBC9* (wild type) or harboring the *ubc9–10* mutant, that constitutively express Top1p or Top1T722Ap were shifted to 36 °C for 6 h or maintained at 26 °C. Serial 10-fold dilutions of cell extracts prepared as described (16) and corrected for protein concentration were incubated in plasmid DNA relaxation assays. The reaction products were resolved by agarose gel electrophoresis and visualized following ethidium bromide staining. For each series, the first lane contained samples derived from the 10^{-1} dilution. The images were taken from the same exposure of a single gel. *Sc* marks the position of negatively supercoiled plasmid DNA, *R* refers to the ladder of relaxed DNA topoisomer bands, and *C* is control DNA alone.

with wild-type *UBC9* cells, *ubc9–10* cells treated with HU for 3–4 h exhibited no loss of viability at 26 or 36 °C, indicating an intact S-phase checkpoint (data not shown). However, when the *RAD9* DNA damage checkpoint was deleted in *ubc9–10*

TABLE III
Morphology of ubc9–10 cells

Isogenic haploid *top1*Δ strains, wild-type for *UBC9* or harboring the *ubc9–10* mutant, were transformed with YCpSctop1 vectors that constitutively express the indicated *top1* allele from the *TOP1* promoter. Exponential cultures of individual transformants grown at 26°C, were shifted to 36°C for 6 h or maintained at 26°C. The cells were then fixed, stained with DAPI, and visualized with a fluorescent microscope equipped with differential interference contrast optics, as described under "Experimental Procedures."

Strain	°C	*top1* allele	% unbudded, small budded cells[a]	% large budded cells[b] (single DNA mass)	% large budded cells[b] (segregated DNA masses)
UBC9	26	*TOP1*	70	10	20
	36	*TOP1*	54	17	29
	26	*top1T722A*	66	14	20
	36	*top1T722A*	53	22	25
ubc9-10	26	*TOP1*	69	9	22
	36	*TOP1*	41	42	17
	26	*top1T722A*	64	21	15
	36	*top1T722A*	19	70	11

[a] Based on the analysis of a minimum of 100 cells/sample, cells were scored as either having no small buds or large buds (where the diameter of the bud was greater than half of that of the mother cell) and tabulated as a percent of the total.
[b] Large budded cells were further distinguished as having a single DNA mass or segregated DNA masses.

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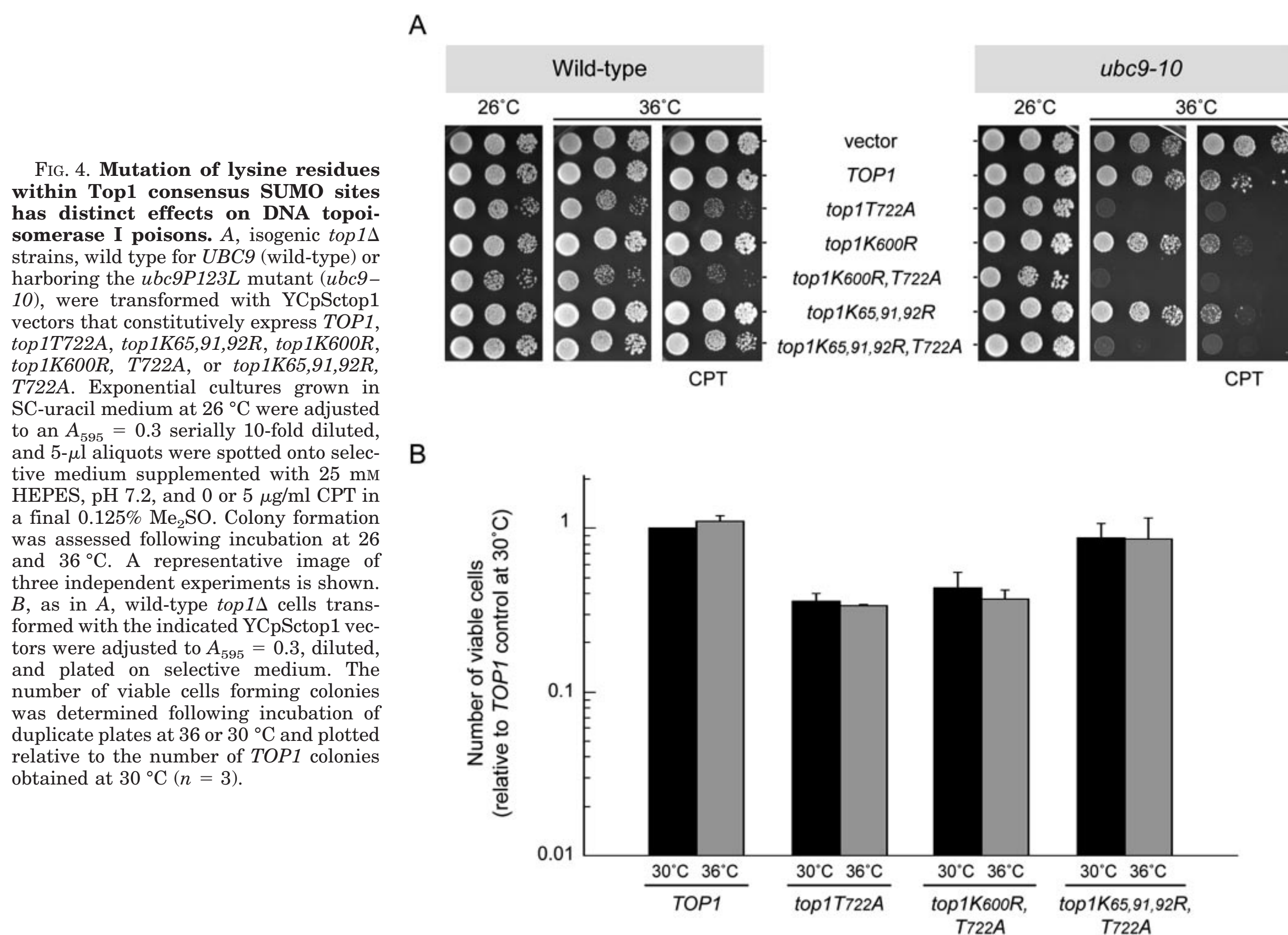

FIG. 4. **Mutation of lysine residues within Top1 consensus SUMO sites has distinct effects on DNA topoisomerase I poisons.** *A*, isogenic *top1Δ* strains, wild type for *UBC9* (wild-type) or harboring the *ubc9P123L* mutant (*ubc9–10*), were transformed with YCpSctop1 vectors that constitutively express *TOP1*, *top1T722A*, *top1K65,91,92R*, *top1K600R*, *top1K600R, T722A*, or *top1K65,91,92R, T722A*. Exponential cultures grown in SC-uracil medium at 26 °C were adjusted to an A_{595} = 0.3 serially 10-fold diluted, and 5-μl aliquots were spotted onto selective medium supplemented with 25 mM HEPES, pH 7.2, and 0 or 5 μg/ml CPT in a final 0.125% Me_2SO. Colony formation was assessed following incubation at 26 and 36 °C. A representative image of three independent experiments is shown. *B*, as in *A*, wild-type *top1Δ* cells transformed with the indicated YCpSctop1 vectors were adjusted to A_{595} = 0.3, diluted, and plated on selective medium. The number of viable cells forming colonies was determined following incubation of duplicate plates at 36 or 30 °C and plotted relative to the number of *TOP1* colonies obtained at 30 °C (*n* = 3).

cells, the double mutant remained viable at 36 °C. These results contrast with the synthetic lethal interactions observed between a *rad9* null mutation and two other *tah* mutants, *cdc45–10* and *dpb11–10*, both of which exhibit defects in Okazaki fragment maturation at 36 °C (17). These findings suggest that the defects in Ubc9P123Lp function, *per se*, did not induce lesions recognized by the Rad9 DNA damage checkpoint.

Even though steady-state levels of sumoylated Top1p were difficult to detect, we reasoned that mutation of lysine residues within consensus SUMO sites might alter transient cycles of Top1p SUMO conjugation and cell sensitivity to Top1p poisons. As reported for human Top1p (34), potential sumoylation sites exist in the nonconserved N-terminal domain (IK^{65}TE, IK^{91}KE) and in the extended pair of α-helices that comprise the linker domain (LK^{600}KE), which connects the C-terminal active site tyrosine domain with the Top1 protein clamp. Because these domains have been implicated in protein-protein interactions and/or in DNA binding by the enzyme (54–56), we asked whether mutating these lysines to arginine would either suppress or enhance *ubc9–10* cell sensitivity to Top1p poisons. Mutation of Lys^{65}, Lys^{91}, Lys^{92} (Lys^{65,91,92}), or Lys^{600} to Arg had no effect on the cytotoxic activity of the *top1T722A* mutant in *ubc9–10* cells at 36 °C (Fig. 4*A*) or in wild-type UBC9 cells (Fig. 4, *A* and *B*). Indeed, in wild-type *UBC9* cells, mutation of the N-terminal SUMO sites partially suppressed, rather than enhanced, the cytotoxic activity of Top1T722A in the presence and absence of CPT (Fig. 4, *A* and *B*, respectively). In the context of wild-type Top1p, the same mutations induced a slight but reproducible decrease in *ubc9–10* cell viability when exposed to CPT at 36 °C (Fig. 4*A*). However, when enzyme activity in cell extracts was assessed in a plasmid DNA relaxation assay, no alterations in enzyme-specific activity or protein levels were detected (data not shown). Thus, whereas mutations of consensus SUMO sites had subtle and contrary effects on cell sensitivity to CPT, they failed to recapitulate the *tah* phenotype of *ubc9–10* cells. Although these experiments do not exclude alterations in Top1p localization or the modification of nonconsensus SUMO sites, we were unable to attribute the enhanced sensitivity of *ubc9–10* cells to Top1p poisons to any direct alterations in Top1 protein sumoylation.

Ubc9P123Lp Activity and Protein Stability Is Thermolabile—To further investigate the defects in Ubc9p activity induced by mutation of Pro^{123} to Leu, global patterns of protein sumoylation were analyzed in extracts of isogenic wild-type *UBC9* and mutant *ubc9–10* strains expressing either wild-type Top1p or Top1T722Ap. In Fig. 5*A*, similar levels of Smt3p-protein conjugates were detected when cells were cultured at the permissive temperature of 26 °C. However, a 6-h shift of *ubc9–10* cells to 36 °C resulted in a dramatic decrease in global sumoylation, whether cells expressed wild-type or self-poisoning Top1 proteins. The same results were obtained in a *top1* null background (data not shown). This dramatic decrease in overall protein sumoylation coincided with a decrease in Ubc9P123L protein levels upon shift to the nonpermissive temperature (Fig. 5*B*) independent of Top1p. Although Ubc9P123L protein and catalytic activity were significantly reduced at 36 °C, they sufficed to maintain cell viability in the absence of DNA damage. The synthetic lethal phenotype of *ubc9–10* cells exposed to low levels of DNA damage might either 1) be a consequence of lower thresholds of global sumoylation necessary to maintain essential cellular function(s) than to effect DNA repair, or 2) derive from alterations in Ubc9P123L substrate specificity.

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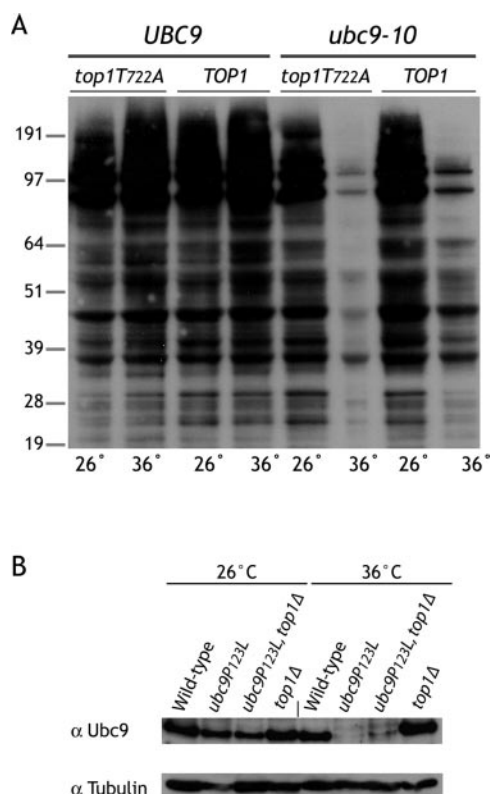

FIG. 5. **Decreased levels of SUMO conjugates and Ubc9 protein are detected in *ubc9–10* cells at the nonpermissive temperature.** *A*, exponential cultures of wild-type *UBC9, top1*Δ and *ubc9–10, top1*Δ strains transformed with YCpScTOP1 or YCpSctop1T722A were shifted to 36 °C for 6 h or maintained at 26 °C. Following cell lysis with NaOH/trichloroacetic acid (49), SUMO-protein conjugates were resolved in 4–12% polyacrylamide Bis-Tris gels and visualized in immunoblots with a polyclonal Smt3 antibody and chemiluminescence. Molecular weight markers are indicated. *B*, extracts of exponential cultures of the indicated *top1*Δ and *TOP1* strains (wild-type *UBC9* or the *ubc9P123L* mutant) grown at 26 or 36 °C were prepared by NaOH/trichloroacetic acid lysis and resolved in 12% polyacrylamide Bis-Tris gels. Ubc9 protein levels were detected by chemiluminescence in immunoblots stained with affinity-purified yeast Ubc9 antibodies. Tubulin staining served as a loading control.

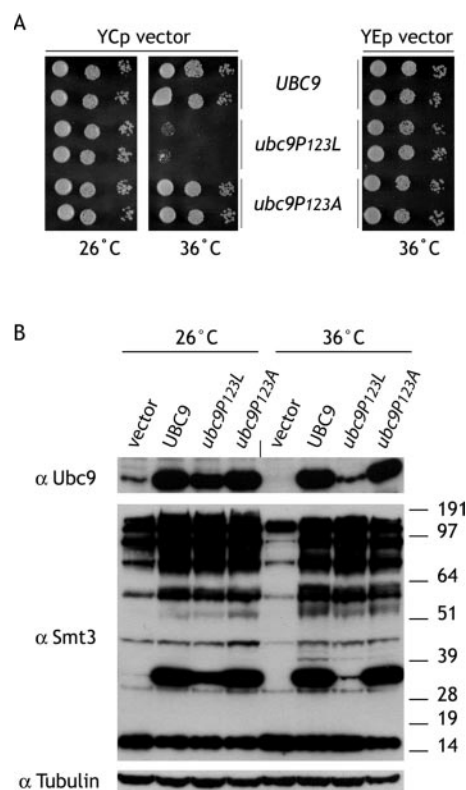

FIG. 6. **Increased expression of Ubc9P123L suppresses the *tah* phenotype of *ubc9–10* cells.** *A*, as detailed under "Experimental Procedures," multicopy YEp-LEU2 vectors expressing wild-type *UBC9*, *ubc9P123L* or *ubc9P123A* from the *UBC9* promoter were introduced into *ubc9*Δ, *top1*Δ cells containing a *URA3*-marked YCpUBC9 vector. After curing the transformants of the YCpUBC9 vector on 5-fluoroorotic acid plates, exponential cultures were serially 10-fold diluted and 5-μl aliquots were spotted onto SC-leucine plates supplemented with 5 mg/ml HU and incubated at 26 or 36 °C. *B*, extracts of exponential cultures of *ubc9–10* cells, transformed with the indicated YEpUBC9 vector and shifted to 26 or 36 °C for 6 h, were prepared by NaOH/trichloroacetic acid lysis. The levels of Ubc9 protein, SUMO-protein conjugates, and tubulin were assessed following SDS-PAGE and immunoblotting as described in the legend to Fig. 5.

To begin addressing these possibilities, we first asked whether the thermoinstability of Ubc9P123Lp resulted from a loss in structural rigidity imparted by the proline at this position or from the insertion of a large hydrophobic leucine residue. As shown in Fig. 6, the latter seems to be the case because mutating Pro^{123} to Ala had no detectable effect on cell viability. When expressed from low copy YCp vectors, both *ubc9P123L* and *ubc9P123A* were able to maintain *ubc9*Δ viability and resistance to HU at 26 °C (Fig. 6*A*). However, in contrast to Ubc9P123Lp, Ubc9P123Ap maintained cell resistance to HU at 36 °C. Ubc9P123Ap also restored *ubc9–10* cell resistance to low levels of Top1T722Ap at 36 °C (data not shown) and exhibited the same steady-state protein levels as wild-type Ubc9p in cells cultured at 26 and 36 °C (Fig. 6*B*). Thus, it appears that the introduction of Leu at position 123, rather than the loss of Pro, reduces Ubc9P123Lp stability at 36 °C. However, this *ts* phenotype could also be complemented by increased expression of *ubc9P123L* from a high copy YEp vector, which typically maintains in excess of 50 plasmids per cell. Under these conditions, the cells remained resistant to low concentrations of HU or Top1p poisons at 36 °C (Fig. 6*A*, data not shown) and the steady-state levels of Ubc9P123Lp at 36 °C approached those observed in *ubc9–10* cells transformed with vector alone at 26 °C (Fig. 6*B*).

Protein sumoylation is a function of SUMO conjugation by Ubc9p and desumoylation by SUMO-specific peptidases (24). The Ulp1p protease is essential and functions in the maturation of Smt3p as well as in catalyzing SUMO deconjugation (27). Ulp2p function, on the other hand, is only required at high temperature and appears to be restricted to deconjugation reactions in the nuclear compartment of the cell (28). Based on the gene dosage results obtained in Fig. 6, we asked whether *ulp2*Δ could complement the *tah* phenotype of *ubc9–10* cells by restoring the equilibrium of SUMO conjugation dysregulated by Ubc9P123Lp instability at 36 °C. As has previously been reported, *ulp2*Δ strains were inviable at 36 °C (28) but the *ulp2*Δ, *ubc9–10* double mutant was viable at all of the temperatures (Fig. 7). Thus, the decrease in Ubc9P123Lp-catalyzed conjugation at 36 °C restored the viability of cells deleted for the Ulp2p SUMO-protease. Similar results were reported for the *ulp2*Δ, *ubc9–1* double mutant, where the *ubc9–1* mutant is also inviable at 36 °C (28). However, the *ulp2* null mutant failed to complement the *tah* phenotype of *ubc9–10* cells. The double mutant exhibited the same hypersensitivity to HU and Top1p poisons as the single *ubc9–10* mutant at 36 °C (Fig. 7 and data not shown). Along similar lines, overexpression of Smt3p from the *GAL1* promoter also failed to complement the *tah* phenotypes of *ubc9–10* cells or to increase the CPT resistance of wild-type cells expressing Top1p (data not shown).

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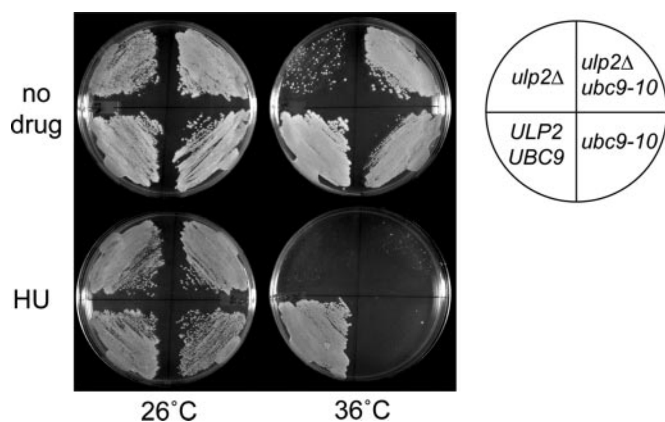

FIG. 7. **Deletion of *ULP2* does not restore *ubc9–10* cell resistance to HU.** Isogenic *top1Δ* strains harboring wild-type alleles of *ULP2*, *UBC9*, single *ubc9–10* or *ulp2Δ* mutations, or the double *ulp2Δ*, *ubc9–10* mutations were streaked on YPD in the presence or absence of 5 mg/ml HU and incubated at 26 and 36 °C.

Deletion of ULP2 Enhanced Cell Sensitivity to Increased TOP1 Gene Dosage and to HU in the Presence of Wild-type Top1p—Our analyses of *ulp2Δ* cell sensitivity to the self-poisoning Top1 mutant enzymes were complicated by the genetic instability of strains lacking Ulp2p. Consistent with previous reports of chromosomal instability (57), our *ulp2Δ* strains quickly acquired spontaneous revertants. For example, note the large number of papillae in the *ulp2Δ* strain plated on YPD at 36 °C in Fig. 7. This high background complicated our analysis of cells transformed with the various YCpTOP1 vectors. In contrast to the original *ulp2* null mutants, *ulp2Δ* cells transformed with various YCp-based vectors and then cultured in selective medium at 26 °C were viable when plated at 36 °C. Surprisingly, however, this effect appeared to be exacerbated in *ulp2Δ* strains that were wild type for *TOP1*, suggesting that the defects in chromosome and plasmid stability evident in *ulp2* null cells could be partially suppressed by deletion of *TOP1*. These findings prompted further study of possible genetic interactions between *ulp2Δ* and *TOP1*.

First, *ulp2* deletion mutants of isogenic wild-type *TOP1* and *top1Δ* strains were generated by PCR-based gene disruptions. Second, as shown in Fig. 8, under conditions where a low background of spontaneous revertants was evident at 36 °C, *i.e.* early passage of the mutant strains, the *ulp2Δ*, *TOP1* strain was hypersensitive to HU at all of the temperatures, whereas the double *ulp2Δ*, *top1Δ* mutant was only inviable at 36 °C. Thus, the deletion of *TOP1* suppressed the HU hypersensitivity of cells lacking Ulp2p.

To assess *ulp2Δ* cell sensitivity to self-poisoning Top1 mutants, we reasoned that short-term incubation of the resultant transformants might also avoid some of the genetic instability attendant with long term culturing. Surprisingly, we found that *ulp2Δ*, *TOP1* strains were unable to tolerate any increase in *TOP1* gene dosage at 26 °C, as evidenced by our inability to recover viable transformation colonies (Table IV). In contrast to *ulp2Δ*, *top1Δ* strains, which were readily transformed with all of the indicated YCpScTOP1 vectors, *ulp2Δ*, *TOP1* strains could not tolerate low copy *ARS*/*CEN* vectors expressing catalytically active Top1 or To1pT722A enzymes. Even increased dosage of the catalytically inactive Top1Y727Fp induced a slow growth phenotype of *ulp2Δ*, *TOP1* cells at 26 °C. Moreover, this increased sensitivity to Top1 protein dosage was marginally suppressed by the mutation of the active site tyrosine (*top1Y727F*) or consensus SUMO sites (*top1K65,91,92R* or *top1K600R*). Transformants were obtained; however, they exhibited a slow growth phenotype when cultured at 26 °C (Table IV). Similar phenotypes were obtained with the *ulp2Δ*, *ubc9–10* double mutant, although in this genetic background, mutation of consensus SUMO sites in Top1T722A also improved cell viability at 26 °C. Thus, although the decreased stability and activity of Ubc9P123Lp at 36 °C enhanced cell sensitivity to a wide range of DNA-damaging agents independent of *TOP1* or Top1p sumoylation, the enhanced sensitivity of *ulp*2 null mutants to HU and Top1 protein dosage was, in part, dependent on Top1p sumoylation.

DISCUSSION

Independent yeast genetic screens for conditional hypersensitivity to distinct DNA topoisomerase I poisons defined the same Pro[123] to Leu substitution in the essential SUMO E2-conjugating enzyme, Ubc9p. Alterations in the SUMO conjugation machinery have previously been reported to enhance cell sensitivity to DNA-damaging agents and in cells expressing human Top1p to CPT (5, 32, 34, 35, 39, 58). However, the results of our studies are significant for several reasons. First, the *ubc9–10* mutant uncoupled the essential function of Ubc9p in maintaining cell viability at 36 °C from catalytic activities necessary to protect cells from a wide range of DNA-damaging agents. Mutation of Pro[123] to a hydrophobic bulky residue Leu but not Ala impaired protein stability at 36 °C, resulting in a dramatic reduction in global sumoylation and enhanced cell sensitivity to Top1p poisons and genotoxic stress. The *ubc9–10* mutant was viable at all temperatures in the absence of DNA damage yet exhibited at a 2–3 log drop in cell viability when exposed to low levels of Top1p poisons, UV light, bleomycin, or methyl methanesulfonate. Indeed, the *ubc9–10* mutant was unique among previously reported *tah* mutants in exhibiting enhanced sensitivity to Top1T722Ap, which acts as a CPT mimetic, and to Top1N726Hp, which acts as a Top1p poison by virtue of enhanced rates of DNA cleavage. However, this synthetic lethality was restricted to DNA-damaging agents because cell viability was unaffected by other environmental stress, such as high salt, growth on glycerol, or cycloheximide treatment. These findings suggest that cells can tolerate rather dramatic decreases in sumoylation and still retain viability. However, a higher threshold of SUMO conjugation is required for cells to tolerate genotoxic stresses.

The defect in global sumoylation imparted by Ubc9P123Lp sufficed to restore the viability of *ulp2Δ* cells at 36 °C. However, our studies also demonstrated that deletion of the Ulp2p SUMO-deconjugating enzyme failed to complement the *tah* phenotype or HU sensitivity of *ubc9–10* cells. Thus, these data indicate that specific alterations in Ubc9P123Lp substrate specificity may also contribute to the increased sensitivity of *ubc9–10* cells to DNA-damaging agents. Earlier studies demonstrated that Ulp2p functions to edit SUMO chains produced by the sumoylation of Smt3 polypeptides covalently attached to lysines in target proteins (59). Although Smt3p chains are not required for cell viability, their accumulation in *ulp2Δ* strains induced cell death at 36 °C and correlated with increased sensitivity to HU (59). *ulp2Δ* cell viability was restored by mutation of SUMO sites in DNA topoisomerase II, consistent with the role for Top2p in chromosome segregation (57, 59). Here we show that the genetic instability and HU sensitivity of *ulp2Δ* strains were also enhanced in the presence of wild-type Top1p. Deletion of *TOP1* suppressed both phenotypes. However, increased dosage of Top1 protein severely compromised cell viability, which was partially eliminated by mutation of consensus N-terminal SUMO sites in Top1p. Taken together, these findings suggest transient cycles of Top1p sumoylation and desumoylation contribute to cell genetic stability and regulate replication stress induced by HU.

In contrast, our mutational analysis of consensus SUMO

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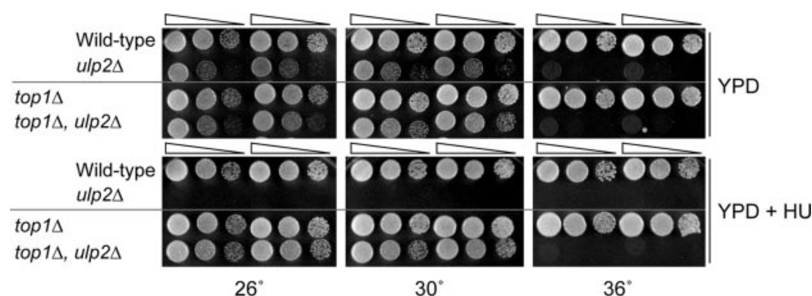

FIG. 8. ***ulp2Δ* cells expressing wild-type Top1p exhibit enhanced sensitivity to HU.** Two independent cultures of isogenic strains, wild-type for *TOP1* and *ULP2* or harboring the indicated *top1Δ* and *ulp2Δ* mutants, were adjusted to an A_{595} = 0.3 serially 10-fold diluted and spotted onto YPD plates or YPD + 5 mg/ml HU plates. Cell viability was assessed following incubation at 26, 30, and 36 °C.

TABLE IV
Transformation assay of ulp2Δ strains

Yeast strain	Plasmid transformation efficiency[a]							
	Vector	TOP	top1T722A	top1Y727F	top1K600R	top1K600R T722A	top1K65,91,92R	top1K65,91,92R T722A
Wild type[b]	+++	+++	+++	+++	+++	+++	+++	+++
ubc9–10[b]	+++	+++	+++	+++	+++	+++	+++	+++
ulp2Δ, top1Δ	+++	+++	+++	+++	+++	+++	+++	+++
ulp2Δ	+++	+/−	−	(++)[c]	(+)[c]	−	(++)[c]	−
ulp2Δ, ubc9–10, top1Δ	+++	+++	+++	+++	+++	+++	+++	+++
ulp2Δ, ubc9–10	+++	+/−	+/−	(+++)[c]	(++)[c]	(++)[c]	(++)[c]	(+)[c]

[a] Isogenic yeast strains wild-type for *TOP1, UBC9,* and *ULP2* or harboring the indicated mutant allele of these genes were transformed with *URA3* marker, *ARS/CEN* vectors that constitutively express wild-type *TOP1* or the designated *top1* mutant from the yeast *TOP1* promoter. The average number of transformants obtained following selection on SC-uracil plates at 26°C were determined in ≥3 independent experiments. +++ indicates greater than 1,000 colonies/transformation, ++ indicates between 100 and 1,000 transformants, +/− indicates <10 transformants, and − indicates no colonies.

[b] Similar results were obtained with isogenic *top1Δ* strains (data not shown).

[c] Parentheses indicate very small colonies, and cells exhibited a slow growth phenotype when cultured at 26°C.

sites in Top1p or Top1T722Ap failed to recapitulate the increased sensitivity of *ubc9–10* cells to low levels of Top1p poisons. Although the N-terminal Top1p SUMO site mutations did induce contrary, albeit subtle effects on the sensitivity of isogenic *UBC9* and *ubc9–10* strains to Top1p poisons, the pronounced *tah* phenotype of *ubc9–10* cells cannot be ascribed to a direct effect on Top1p sumoylation. Although we cannot exclude SUMO conjugation of noncanonical SUMO sites, the N-terminal sites examined in these studies correspond to similar consensus sites examined in human Top1p. Indeed, contrary effects of SUMO conjugation on human cell sensitivity to Top1p poisons have also been reported (33, 35). Consistent with the *tah* phenotype of *ubc9–10* cells at 36 °C, the expression of a dominant negative UBC9 mutant enhanced human cancer cell sensitivity to topotecan (39), although in another study (35), sumoylation of Top1p actually correlated with increased CPT cytotoxicity.

Recent proteomic screens using highly sensitive methodologies identified hundreds of sumoylated proteins in yeast, including Top1p, in the absence of DNA damage (37, 38). This large number is staggering and underscores both the complexity and transient nature of SUMO modification. Because the majority of these proteins are nuclear, these data further support an emerging view in the field that coupled cycles of SUMO conjugation and deconjugation may profoundly affect protein function in various multi-protein complexes. Indeed, this is consistent with 1) the rather subtle yet contradictory effects of SUMO site mutations on Top1p poisoning in isogenic wild-type *UBC9* and *ubc9–10* strains, 2) the more profound sensitivity of *ubc9–10* cells to DNA-damaging agents, and 3) the dramatic effects of Top1p dosage on *ulp2Δ* cell genetic stability and sensitivity to HU.

Taken together, our studies refute a simple model where the balance of SUMO conjugation and deconjugation suffice to regulate Top1p activity and sensitivity to CPT. Rather, SUMO modification of different sites within the enzyme apparently have diverse and contradictory effects on Top1p activity and drug sensitivity. Given the function of Top1p in DNA replication, transcription, and recombination as well as chromosome condensation, it is tempting to speculate that transient cycles of Top1p sumoylation at a variety of sites may regulate the dynamic association of this protein with different multi-protein complexes to affect these processes. The challenge is to devise strategies with which to discern these associations. Toward this end, the effects of SUMO pathway alterations on Top1 protein interactions and intracellular localization are being investigated. Several genetic approaches have also been pursued to address questions of Ubc9p substrate specificity and how this impacts cell sensitivity to Top1p poisons and gene dosage.

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DNA: Replication, Repair, and Recombination:

Defects in SUMO (Small Ubiquitin-related Modifier) Conjugation and Deconjugation Alter Cell Sensitivity to DNA Topoisomerase I-induced DNA Damage

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