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SIZ1/SIZ2 Control of Chromosome Transmission Fidelity Is Mediated by the Sumoylation of Topoisomerase II

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ABSTRACT

The Smt3 (SUMO) protein is conjugated to substrate proteins through a cascade of E1, E2, and E3 enzymes. In budding yeast, the E3 step in sumoylation is largely controlled by Siz1p and Siz2p. Analysis of Siz$^-$ cells shows that SUMO E3 is required for minichromosome segregation and thus has a positive role in maintaining the fidelity of mitotic transmission of genetic information. Sumoylation of the carboxy-terminus of Top2p, a known SUMO target, is mediated by Siz1p and Siz2p both *in vivo* and *in vitro*. Sumoylation *in vitro* reveals that Top2p is an extremely potent substrate for Smt3p conjugation and that chromatin-bound Top2p can still be sumoylated, unlike many other SUMO substrates. By combining mutations in the *TOP2* sumoylation sites and the *SIZ1* and *SIZ2* genes we demonstrate that the minichromosome segregation defect and dicentric minichromosome stabilization, both characteristic for Smt3p–E3-deficient cells, are mediated by the lack of Top2p sumoylation in these cells. A role for Smt3p-modification as a signal for Top2p targeting to pericentromeric regions was suggested by an analysis of Top2p–Smt3p fusion. We propose a model for the positive control of the centromeric pool of Top2p, required for high segregation fidelity, by Smt3p modification.

SUMO (*s*mall *u*biquitin-like *mo*difier) is a member of a growing family of ubiquitin-related proteins and is known to conjugate with RanGAP1, PML, IκBα, p53, yeast septin components, and other proteins (Hay *et al.* 1999; Hochstrasser 2000; Jentsch and Pyrowolakis 2000; Muller *et al.* 2001; Weissman 2001). The cells of higher eukaryotes have three SUMO paralogs: SUMO-1, SUMO-2, and SUMO-3 (Johnson 2004). In budding yeast, the sole SUMO-encoding gene *SMT3* is essential for cell viability (Meluh and Koshland 1995). Common E1 and E2 enzymes are required to conjugate all the SUMO variants. The E1 enzymes Uba2p/Aos1p in *Saccharomyces cerevisiae* and SAE1/SAE2 in mammals form a transient thioester bond between the C-terminal glycine of SUMO and SAE2/Uba2p (Dohmen *et al.* 1995; Desterro *et al.* 1997; Johnson *et al.* 1997). SUMO is then transferred to the E2 conjugating enzyme Ubc9p (Johnson and Blobel 1997; Schwarz *et al.* 1998).

The SUMO E3 proteins have been characterized as cofactors required for substrate recognition by Ubc9p (Hochstrasser 2001; Jackson 2001). The *S. cerevisiae* Siz1p/Ull1p (Strunnikov *et al.* 2001; Takahashi *et al.* 2001a), has been shown to be the E3 factor specific for septin sumoylation (Johnson and Gupta 2001; Takahashi *et al.* 2001a,b). Several additional types of E3 factors have been found in mammalian cells (Pichler *et al.* 2002; Kagey *et al.* 2003), but their counterparts are not present in *S. cerevisiae*. Yeast cells lacking E3 (*siz1/siz2* double deletion mutants) lose the bulk of detectable Smt3p conjugation, yet retain a wild-type growth rate (Johnson and Gupta 2001; Takahashi *et al.* 2003) after the 2μ plasmid is lost due to E3-dependent overmodification of its partition factors (Chen *et al.* 2005). The fact that the massive loss of SUMO conjugates in *siz1/siz2* double mutants results in only negligible phenotypic changes underscores the technical difficulty of identifying physiologically important Smt3p substrates and unraveling the essential role of Smt3p for cell viability. As *SMT3* is an essential gene, it is generally believed that some protein targets modified in the absence of Siz1p and Siz2p allow cells to survive. This modification either can potentially proceed by the conjugating activity of the E2 enzyme itself (Okuma *et al.* 1999) or may be catalyzed by some narrowly specialized SUMO E3 factors, such as the recently characterized Mms21p (Zhao and Blobel 2005).

The numerous biological roles of SUMO modification are dependent on the functions of the target proteins (Muller *et al.* 2001; Johnson 2004; Ulrich 2004). The pattern of Smt3p localization in yeast cells indicates that conjugated proteins are present in the nucleus (many targets) and in the bud neck (septins) (Johnson and Blobel 1999; Takahashi *et al.* 1999). Nuclear localization of Smt3p and the role of the SUMO-conjugation pathway in chromosome transmission fidelity (Biggins *et al.* 2001; Azuma *et al.* 2003) suggest that

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critically important targets of SUMO modification could be nuclear proteins. Indeed, recent identification of Smt3p substrates *in vivo* using proteomic approaches (Panse *et al.* 2004; Wohlschlegel *et al.* 2004; Zhou *et al.* 2004; Hannich *et al.* 2005) have demonstrated that numerous essential nuclear proteins are modified by SUMO.

The fact that mutations in enzymes removing SUMO from the conjugated targets result in severe loss of viability (Li and Hochstrasser 2000; Strunnikov *et al.* 2001) demonstrates that removal of Smt3p from its target is as important as conjugation. Budding yeast have two specialized SUMO isopeptidases: intranuclear, Smt4p(Ulp2p) (Li and Hochstrasser 2000; Strunnikov *et al.* 2001), and extranuclear, Ulp1p (Li and Hochstrasser 2003). These two enzymes are apparently strictly compartmentalized in the cell, as mistargeting of Ulp1p to the nucleus results in a severe phenotype (Panse *et al.* 2003). While the Ulp1p is an essential enzyme (Li and Hochstrasser 1999), carrying the bulk of Smt3p processing (Li and Hochstrasser 2003), the *smt4* mutants are able to survive (Li and Hochstrasser 2000; Strunnikov *et al.* 2001), but probably due only to a trace of Ulp1p activity reaching the nucleus (Li and Hochstrasser 2003). Characterization of the *SMT4* gene revealed a number of pathways controlled by Smt3p conjugation: *SMT4* is a dosage suppressor of mutations in the genes encoding the chromosomal proteins *MIF2*, *SMC2*, *PDS5* (Meluh and Koshland 1995; Strunnikov *et al.* 2001; Stead *et al.* 2003), and *smt4* mutations are synthetically lethal with DNA-replication arrest (Bachant *et al.* 2002). The severe cellular defects of *smt4* mutants can be attributed to over-sumoylation of many Smp3p targets. Thus, at present the negative impact of SUMO modification (Strunnikov *et al.* 2001; Bachant *et al.* 2002; Chen *et al.* 2005) has been documented to a much greater degree than its positive regulatory role.

Previously, we characterized *SIZ1* and *SIZ2* genes (Strunnikov *et al.* 2001) shown to encode the major SUMO E3 activity in yeast (Johnson and Gupta 2001). As double *siz1/siz2* deletion results in elimination of approximately 99% of the SUMO conjugates (Johnson and Gupta 2001) and many chromosomal proteins are sumoylated in Siz$^+$ cells (Zhou *et al.* 2004; Hannich *et al.* 2005), we became interested in assessing the potential role these two genes may play in chromosome segregation. Upon analysis of a number of potential substrates we found that Top2p modification is controlled by both Siz1p and Siz2p. While a previous study on Top2p in *S. cerevisiae* (Bachant *et al.* 2002) has uncovered that Top2p over-sumoylation results in precocious sister chromatid separation in kinetochore vicinity by an as yet unidentified mechanism (Bachant *et al.* 2002), the role Top2p sumoylation plays in the wild-type cells remains unknown. In higher eukaryotes and wild-type yeast cells only a very small fraction of topoisomerase II is sumoylated (Bachant *et al.* 2002; Azuma *et al.* 2003), making any direct analysis of this pool rather challenging. However, using a combination of *siz1*, *siz2*, and *top2* mutants we show that SUMO E3 machinery specifically facilitates Top2p–Smt3p conjugation and demonstrate that both Top2p sumoylation and Siz1p/Siz2p activity have a previously uncharacterized positive regulatory role in transmission of genetic information. We demonstrate that the critical role of SUMO E3 in minichromosome segregation is likely limited to the Smt3p modification of the COOH-terminal tail of Top2p.

MATERIALS AND METHODS

Microbiological and genetic methods: *Escherichia coli* strains Top10 and BL21(DE3) were used for plasmid propagation and recombinant protein production, respectively. Yeast media and genetic techniques were performed as described (Guthrie and Fink 1991). The *S. cerevisiae* strains were of S288C and W303 backgrounds (Table 1). For all genetic tests an isogenic set of strains was used and experiments were repeated for both S288C and W303. Minichromosome stability, *i.e.*, the fraction of cells in the population containing minichromosomes under selective conditions, was assayed essentially as described (Strunnikov *et al.* 1993). Briefly, exponential cultures of the strains harboring the YCplac111 (*CEN4*, *LEU2*), YCplac33 (*CEN4, URA3*) (Gietz and Sugino 1988), pPRS425 (2μ replication origin, *LEU2*), pRS415 (*CEN6, LEU2*) (Sikorski and Hieter 1989), pAS255 (*cen3-BCT1*, *ARS1 TRP1 URA3*), or pIA1 (*URA3*, 2μ) (P. Hieter, personal communication) plasmids and bearing different combinations of *siz1/siz2* and/or *top2* mutations were grown in minimal medium lacking uracil or leucine, respectively. Culture aliquots were plated on four YPD plates. The resulting colonies were analyzed for minichromosome presence by replica plating onto synthetic medium lacking uracil or leucine. The transmission efficiency (stability) of the conditional dicentric minichromosome pAS72 (*LEU2 URA3 ARS CEN6 pGAL:CEN3*, A. Strunnikov, unpublished data) was determined in a similar way, except that the log-phase cultures were first grown at 30° in selective medium containing 2% raffinose, 1% galactose (v/v) as a carbon source and then incubated in YPD for 4 hr before being plated.

Chromosome III loss rate assay was based on the ability of diploid strains to mate with both *MAT*α and *MAT***a** tester strains if chromosome segregation was impaired. Loss of heterozygosity of both the *MAT***a***/MAT*α and *leu2/LEU2* loci was considered a chromosome loss event. The details of the assay are as described (Gerring *et al.* 1990).

DNA constructs: The *SIZ1* overexpression construct was created by amplifying the galactose-controlled promoter and the marker from pFA6a–His3MX6–pGAL1–3HA (Longtine *et al.* 1998) with the specific primers fusing *SIZ1* ORF to pGAL. The PCR product was directly used in yeast transformation to replace the genomic copy of the *SIZ1* promoter.

The integrative construct to replace the *SMT3* gene with the polyhistidine and FLAG-tagged *SMT3* (HF-*SMT3*) expressed from the native promoter was constructed on the basis of the pHF-SMT3 plasmid (Johnson *et al.* 1997). The *LEU2* marker was inserted into the unique *Mlu*I site in the *SMT3* promoter and the resulting vector pAS924 was used to transform yeast after digestion with *Nco*I and *Bgl*II. The *smt4*, *siz1*, and *siz2* deletions were as reported (Takahashi *et al.* 2000, 2001b).

To generate the tagged and mutagenized *TOP2* replacement vectors a genomic copy of *TOP2* gene was amplified by PCR (primers: GCAACTGCAGTACCTAACGGTGCTTTCGG

TABLE 1

***S. cerevisiae* strains**

Strains	Relevant genotype	Source
BY4733	*MAT***a** *his3 leu2 met15 trp1 ura3*	ATCC
4bAS399	*MAT***a** *his3 leu2 lys2 met15 ura3 siz1-Δ::kanMX siz2-Δ::kanMX*	This work
12cAS399	*MATα his3 leu2 lys2 ura3 siz1-Δ::kanMX siz2-Δ::kanMX*	This work
924–YPH499	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 HF:SMT3::LEU2*	This work
924–4bAS399	*MAT***a** *his3 leu2 lys2 met15 ura3 siz1–Δ::kanMX siz2-HF:SMT3::LEU2*	This work
W303–1A	*MAT***a** *ade2 ura3 trp1 leu2 his3 can1*	R. Rothstein
YPH499	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3*	P. Hieter
YPH499bp1	*MAT***a** *ade2 his3 leu2 lys2 trp1 ura3 bar1-Δ pep4::HIS3 SMC4:6His:3HA::URA3*	This work
BY4733bp5	*MAT***a** *his3 leu2 met15 trp1 ura3 pep4::HIS3 bar1-Δ::LEU2 PDS5:6His:3HA::URA3*	This work
EY0987/*SPC42*:mRFP	*MATα his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 SPC42:mRFP::kanMX*	E. O'Shea

ATCC, American Type Culture Collection.

and GCGCGTCGACATCCTCTTCATTGAACGAAAC) and the PCR products were cloned into the PstI-SalI restriction sites of pTS901IU (5xHA URA3) (Sasaki *et al.* 2000) to produce pYT1033 (*URA3 TOP2:HA*). The *top2:HA* (ΔC) vector pYT1035, was constructed similarly, but using a truncated primer (GCG CGTCGACAATTTTTTTGCCCTTTCTAGCA). The ΔC allele was designated *top2–Δ200*. The *top2* 3xKR triple mutant allele (*top2–201*) encoding the substitution mutations of K1220R, K1246R, K1277R was obtained by PCR-based site-directed mutagenesis (primer pairs: CAAAAAAATTAGGTTAGAGGATAA/TTATCCTCTAACCTAATTTTTTTG, CTACAAAGATTAGAAAAGAGAAAAC/GTTTTCTCTTTTCTAATCTTTGTAG, TTTCGACATAAGGAAAGAAGATA/TATCTTCTTTCCTTATGTCGAAA) and the PCR product was cloned into pTS901IU to produce pYT1034 (*URA3 top2:HA* 3xKR). Plasmid pML251 (*top2-SNM:HA::KanMX*) was used to replace a single genomic copy of *TOP2* gene (Bachant *et al.* 2002) after the marker was changed to *URA3* to give pYT1032 (*URA3 top2-SNM:HA*). The plasmids for the COOH-terminal GFP-tagging of the different versions of *TOP2* pYT1026, pYT1027, and pYT1028 were analogous to (respectively) pYT1033, pYT1034, and pYT1032, except they had the COOH-terminal fusions to the GFP-encoding sequence and *LEU2* markers. The *TOP2:SMT3:HA* fusion plasmid pYT1051 (Figures 2B and 5D) was constructed by inserting the *SMT3* gene in-frame into the *Spe*I site in pYT1033. The *TOP2:SMT3:GFP* fusion plasmid pYT1101 (Figure 5A) was constructed by inserting the *SMT3* gene in-frame into the *Spe*I site in pYT1026.

The dicentric minichromosome pAS72 was constructed from pRS415 (*CEN6*, *LEU2*) by inserting pGAL:*CEN3* and *URA3* into the polylinker region. The resulting minichromosome behaves as dicentric in dextrose-containing media and is functionally monocentric in galactose media.

Biochemical methods: All chromatin fractions were prepared and verified by micrococcal nuclease digestion as described by Liang and Stillman (1997). For preparation of the yeast lysates and immunoblot analysis, the cells were collected, washed, resuspended in 2% SDS, and disrupted with glass beads using a TOMY shaker. The resulting lysates were boiled, supplemented with the LDS loading buffer (Invitrogen), and separated onto 4–12% Bis–Tris or 3–8% Tris–acetate NUPAGE gradient gels (Invitrogen). After Western blotting the specific protein reactive bands were visualized with ECL (Amersham Pharmacia).

The SUMO conjugation assay was performed as described by Takahashi *et al.* (2003). Briefly, the components of the conjugation reaction: 6xHis–Smt3p, GST–Uba2p, GST–Aos1p, Ubc9p, and Siz1p–Δ440 proteins were expressed and purified from *E. coli* and then used in the reaction mixture containing substrate. In most cases ~5 μl of chromatin (Liang and Stillman 1997) was prepared from the strains with the HA-tagged target protein. The reaction mixture (20 μl) was incubated in the presence of 10 mm ATP at 37° (or on ice, as a control) for 1 or 2 hr. Top2p–HA chromatin was used in each experiment with other protein as a positive control. The Top2p modification reaction was saturated after 2 hr. Reaction aliquots of 5 μl were boiled and subjected to immunoblotting. The untagged Top2p for Figure 2B was purified in J. L. Nitiss's laboratory, as described (Vaughn *et al.* 2005). It was detected on Western blots using specific anti-Top2p antibody (TopoGEN).

To purify the *in vivo* Smt3p-conjugated proteins by IMAC, a 50-ml culture of yeast cells with a genomic copy HF-*SMT3* was harvested, cells were disrupted by glass beads (10 min) in 500 μl of lysis buffer (0.1 m Tris pH 8.0, 6 m guanidine chloride, 0.5 n NaCl), and the extract was clarified by centrifugation at 20,000 × *g* for 30 min. The clarified protein extract was incubated in the batch mode with nickel-charged NTA resin (QIAGEN) for 6 hr at room temperature. The resin was then packed into a 2-ml Bio-Rad disposable column and the extract was passed one more time through the open column. The column was washed once with 10-column volumes of 0.1 m Tris pH 7, 6 m guanidine chloride, 0.5 m NaCl, and once with 0.1 m Tris pH 6, 6 m urea, 0.5 m NaCl. Then the bound proteins were eluted with stripping buffer (20 mm Tris pH 7, 40 mm EDTA, 2% SDS). The flow-through fraction was diluted 10-fold with water and proteins were precipitated with 10% TCA.

Microscopy: To generate strains expressing the GFP-tagged Top2p, the corresponding GFP-fusion plasmids were digested with *Spe*I or *Avr*II/*Bln*I and transformed into the W303-1A, YPH499bp, and BY4733 strains. The transformed cells were grown at low density in selective medium at 23°, washed in 0.5% PBS, and analyzed by fluorescent microscopy using a Zeiss AxioVert fluorescent microscope with a cooled CCD camera and Z-axis scanning capability. Coexpression of Top2p–GFP and Spc42p–mRFP fusions was achieved by crossing *MAT***a** strains expressing the Top2p fusions to EY0987/*SPC42*:mRFP (Huh *et al.* 2003).

RESULTS

The Smt3p E3 is required for minichromosome transmission fidelity: We previously obtained evidence that implicated the *SIZ1* and *SIZ2* genes in chromosome metabolism (Strunnikov *et al.* 2001). In addition, as chromatin proteins were shown to compose a significant fraction of all the SUMO targets in yeast (Wohlschlegel

et al. 2004; Zhou *et al.* 2004; Hannich *et al.* 2005) and Siz1p/Siz2p are responsible for the bulk of sumoylation in yeast (Johnson and Gupta 2001; Takahashi *et al.* 2001b), analysis of Siz1p/Siz2p function in chromatin might uncover the mechanism facilitating SUMO control of chromosome segregation in mitosis (Biggins *et al.* 2001). Although Siz1p, a SUMO E3, was previously found to be important for sumoylation of septins (Johnson and Gupta 2001; Takahashi *et al.* 2001a, 2001b), the physiological importance of septin modification was found to be negligibly small (Johnson and Blobel 1999). The role of Siz1p and Siz2p, as SUMO E3, in repressing amplification of 2μ plasmid has recently been documented (Chen *et al.* 2005), but their role in chromosome segregation is as yet uncharacterized.

To investigate whether Siz1p and Siz2p may potentially play a role in the chromosome cycle in budding yeast, we first analyzed the intracellular localization of Siz1p and Siz2p using chromatin fractionation. Both Siz1p and Siz2p were found to be enriched in chromatin after fractionation (Liang and Stillman 1997) (data not shown). Thus, chromatin proteins may be the primary target of Siz1p and Siz2p E3 activity. Therefore, we assessed chromosome transmission fidelity in Siz$^-$ (*siz1*-Δ *siz2*-Δ) cells. Using diploid strains heterozygous in both the *MAT***a**/*MAT*α and *LEU2/leu2* loci of chromosome III, we established that chromosome III loss in a Siz$^-$ diploid strain is indistinguishable from a Siz$^+$ diploid strain (data not shown). We also did not find any destabilization of chromosome III harboring translocation of rDNA (Freeman *et al.* 2000) in Siz$^-$ diploids. At the same time, we detected a notable destabilization of circular centromeric plasmids (minichromosomes) in the *siz1*-Δ *siz2*-Δ cells: the Siz$^-$ strains had a 30% decrease in minichromosome transmission fidelity, as compared to Siz$^+$ cells (Figure 1A). To determine whether this minichromosome loss was a result of missegregation or impaired replication, we assessed the stability of noncentromeric plasmids in both the Siz$^+$ and Siz$^-$ strains. The Siz$^-$ strains showed no difference in the stability of acentric ARS plasmids (Figure 1B). While plasmids containing the 2μ plasmid origin were extremely unstable in Siz$^-$ cells, this was due to the lack of endogenous 2μ plasmid (data not shown), which is lost in *siz1*-Δ *siz2*-Δ cells as a result of deregulation of the *FLP* gene (Chen *et al.* 2005). When a full-length Flp$^-$ 2μ plasmid was used, no difference was observed between its stability in Siz$^+$ and Siz$^-$ strains (Figure 1D). Therefore, minichromosome destabilization in Siz$^-$ is likely due to missegregation. The fact that loss of E3 activity (and the ensuing massive loss of SUMO conjugation) has a negative impact on segregation of minichromosomes indicates that SUMO E3 has a previously unknown positive role in chromosome transmission fidelity. This positive regulatory pathway could bear greater physiological relevance than the previously reported negative role of over-sumoylation in chromosome segregation (Li and

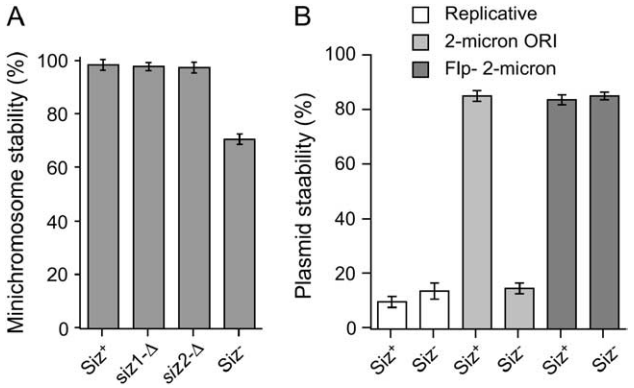

Figure 1.—Minichromosome maintenance phenotype of SUMO E3 mutants. (A) Siz$^-$ mutants destabilize mitotic transmission of minichromosomes. YCplac111 stability was determined in the Siz$^+$ (BY4733) and Siz$^-$ (4bAS399) strains at 30° as described in materials and methods. (B) Siz$^-$ mutants do not destabilize mitotic transmission of acentric plasmids. The stability of pAS255 (replicative), pRS426 (2μ ORI), and pIA1 (Flp$^-$ 2μ) plasmids in the Siz$^+$ (BY4733) and Siz$^-$ (4bAS399) strains was determined at 30° as described in materials and methods.

Hochstrasser 2000; Biggins *et al.* 2001; Strunnikov *et al.* 2001; Bachant *et al.* 2002).

Top2p modification by Smt3p is promoted by E3 both *in vivo* and *in vitro*: The data in Figure 1 can be interpreted to indicate E3 activity is required for the Smt3p modification of a chromatin protein, with a role in accurate segregation of sister chromatids. Thus, we tested a sample of putative Smt3p targets, including Pol30p and all the tagged subunits of cohesin (Kagansky *et al.* 2004) and condensin (Freeman *et al.* 2000) in an *in vitro* sumoylation system composed of recombinant Smt3p, E1, E2, and E3 enzymes (Takahashi *et al.* 2003). In addition, Top2p over-sumoylation was previously shown to have a negative impact on pericentromeric cohesion (Bachant *et al.* 2002), prompting us to analyze Top2p as a potential target of the SUMO E3 activity responsible for the Siz$^-$ segregation defect (Figure 1A). Most of the proteins we tested showed no propensity for Smt3p modification, while being bound to chromatin, in the presence of either Siz1p or Siz2p in the reaction mix, with the exception of Top2p (Figure 2A and data not shown). The Smt3p modification of chromatin-bound Top2p was readily detected (Figure 2A), suggesting that Top2p may be a potential mediator of the E3 role in chromatin.

The use of an *in vitro* SUMO modification system allowed us to circumvent the low abundance of Top2p–Smt3p conjugates *in vivo* (Bachant *et al.* 2002). While the soluble Top2 protein was also shown to be a potent SUMO substrate *in vitro* (Figure 2B), the Smt3p modification of Top2p did not alter the Top2p affinity to chromatin (Figure 2C) as judged by unchanged resistance to salt extraction. Thus, we routinely used Top2p-containing chromatin as a substrate to make the *in vitro*

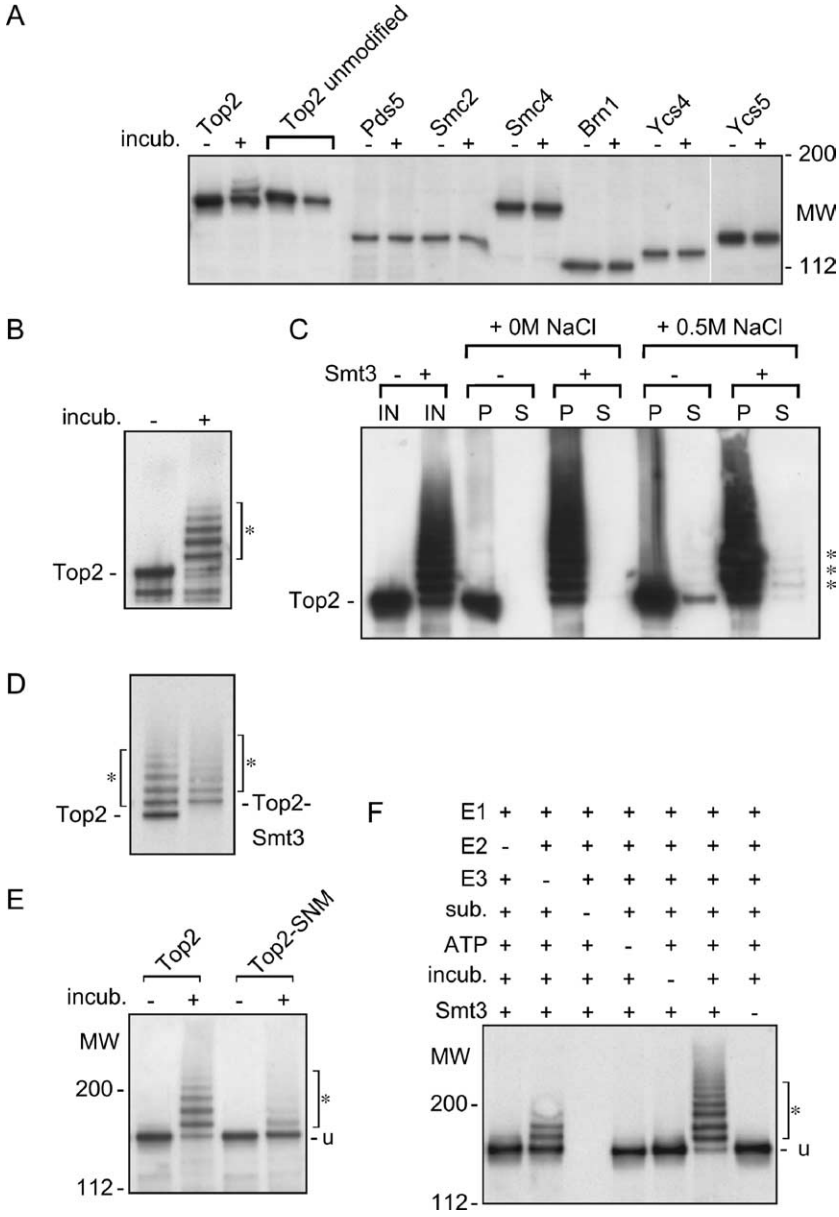

FIGURE 2.—Top2p is modified in a SUMO E3 dependent manner. (A) Chromatin-bound Top2p is a sumoylation substrate *in vitro*. All putative targets are HA-tagged. Reaction mixtures with 1 μl of chromatin purified from yeast cells BY4733/pYT1033 (Top2), BY4733bp5 (Pds5), YPH499bp1 (Smc4), YPH499bp2 (Smc2), YPH499bp6 (Brn1), BY4733bp4 (Ycs4), and YPH499bp5 (Ycs5) were incubated at 37° for 120 min (incub. +) and subjected to immunoblotting with anti-HA antibodies. Identical reaction mixtures held on ice (incub. −) were used as negative controls. (B) Purified Top2p is modified by Smt3p *in vitro*. A total of 4.4 μg purified Top2p (VAUGHN *et al.* 2005) was subjected to sumoylation *in vitro* (incub. +) or left on ice (incub. −) (see MATERIALS AND METHODS) at 37° for 60 min. Western blotting was done with anti-Top2p antibodies. (C) Smt3p-modified Top2p remains strongly associated with chromatin. Top2p was modified in chromatin context *in vitro* as described in MATERIALS AND METHODS. The mock reaction (Smt3 −) was carried out in the absence of recombinant Smt3p. IN, reaction before extraction. Extraction of Top2p after sumoylation reaction was performed for 30 min at 4° with EBX or EBX + 0.5 M NaCl buffers. Chromatin (P) and soluble fractions (S) were separated by centrifugation and analyzed by Western blotting. The Top2p–Smt3p conjugates are marked with an asterisk. (D) Top2p is modified by Smt3p *in vitro*. A total of 5 μl of chromatin from BY4733/pYT1033 (Top2) or BY4733/pYT1051 (Top2–Smt3) were incubated with the *in vitro* sumoylation reaction mix (see MATERIALS AND METHODS) at 37° for 60 min and subjected to immunoblotting with anti-HA antibodies. The characteristic mobility shift caused by sumoylation corresponds to the shift generated by Smt3p fusion. The Top2p–Smt3p conjugates are marked with an asterisk. (E) The COOH-terminal consensus sumoylation sites of Top2p are the primary targets of Smt3p conjugation *in vitro*. Reaction mixtures with 5 μl of chromatin purified from yeast cells BY4733/pYT1033 (Top2) and BY4733/pYT1032 (Top2–SNM), containing HA-tagged Top2p, were incubated at 37° for 60 min and subjected to immunoblotting with anti-HA antibodies. (F) Top2p–Smt3p conjugation *in vitro* is stimulated by SUMO E3. A total of 5 μl of chromatin (sub.) from 4bAS399/pYT1033 (Siz$^-$) was incubated (incub.) with the *in vitro* sumoylation reaction mix (see MATERIALS AND METHODS) at 37° for 60 min and subjected to immunoblotting with anti-HA antibodies. Combinations of the following proteins and cofactors were used: E1, 4.5 μg Uba2p, 5.2 μg Aos1p; E2, 0.75 μg Ubc9p; E3, 4.5 μg Siz1p$^{\Delta 440}$; ATP (10 mM) and 2.9 μg of 6xHis–Smt3p. The unmodified Top2p band is indicated by "u." The Top2p–Smt3p conjugates are indicated with an asterisk. Multiple modified forms of Top2p were formed only in the presence of Smt3p.

sumoylation system a better approximation of the *in vivo* situation. The identity of the modified Top2p bands as Smt3p conjugates was confirmed by two experiments. First, we showed that direct fusion of Top2p to Smt3p produces the same electrophoretic shift (Figure 2D). Second, the "sumo-no-more mutant" *top2-SNM* (BACHANT *et al.* 2002), lacking consensus sumoylation sites in the Top2p tail, showed only marginal modification by Smt3p upon prolonged incubation *in vitro* (Figure 2E).

Chromatin-associated Top2p, even when isolated from *siz1*-Δ *siz2*-Δ cells, was found to be modified by Smt3p to some extent even in the absence of E3 (Figure 2F). However, the addition of recombinant Siz1p (or Siz2p, data not shown) to the system allowed conversion of virtually all Top2p into Smt3p-modified forms in an ATP-dependent fashion (Figure 2F). As the SUMO E2 Ubc9p is known to support limited E3-independent Smt3p conjugation *in vitro* (OKUMA *et al.* 1999), we investigated the role of E3 in Top2p modification *in vivo*. We generated replacements of the wild-type *TOP2* gene with the HA-tagged wild-type gene, the *top2*-3xKR, and *top2*-ΔC alleles (Figure 3A). The mutant alleles of *TOP2*

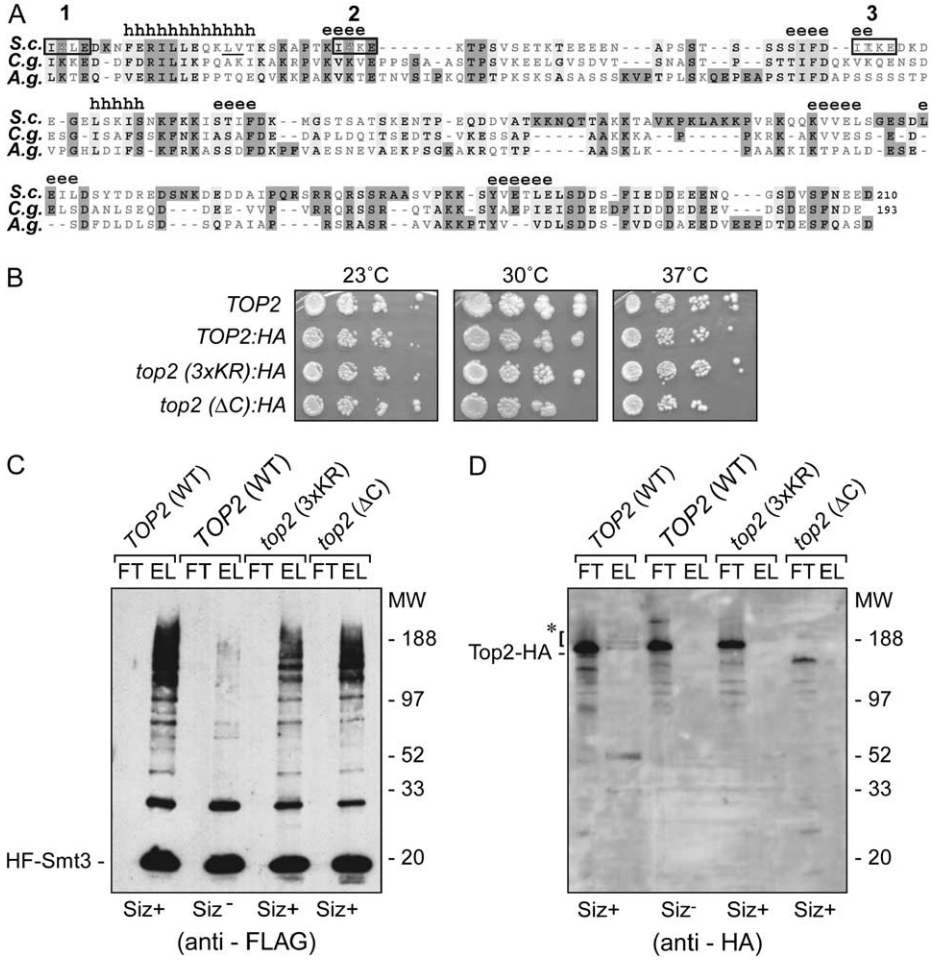

FIGURE 3.—Top2p tail is modified *in vivo* in a SUMO E3-dependent manner. (A) The COOH-terminal region of Top2p and the consensus sumoylation sites have limited conservation among yeast species. The *S. cerevisiae* Top2p tail region (residues 1219–1428) was aligned with the *Candida albicans* (*C.g.*) and *Ashbya gossypii* (*A.g.*) topoisomerases II and the secondary structure was predicted using the JPred package (CUFF and BARTON 2000). h, alpha helix; e, beta-sheet. The SUMO consensus sites are marked with open boxes and numbered. The acceptor lysine residues are outlined. (B) Cell growth is not perturbed by the tagged *top2* alleles ΔC and 3KR. Ten-fold serial dilutions from overnight cultures of wild-type (*TOP2, TOP2*:HA) and different *top2* mutants were spotted onto YPD plates and incubated at the temperatures indicated. The tagged *TOP2* allele replacement strains (W303-1A/pYT1033, W303-1A/pYT1034, and W303-1A/pYT1035) were generated after transformation with plasmids as described in MATERIALS AND METHODS. The corresponding GFP-tagged strains (YPH499/pYT1026, YPH499/pYT1027, and YPH499/pYT1028) were also generated and showed no interference with cell proliferation or Top2p nuclear localization (data not shown). (C and D) Western blot analysis of Top2p sumoylation *in vivo*. Extracts from the strains 924-YPH499 (Siz^+) or 924-4bAS399 (Siz^-) expressing physiological levels of HF-Smt3p and carrying different *top2* alleles were fractionated by IMAC. FT, flow through; EL, eluate. The wild type *TOP2* gene and all *top2* alleles were HA-tagged. HF–Smt3p conjugates eluted from the column were analyzed by Western blotting using anti-FLAG (C) and anti-HA (D) antibodies. The conjugated forms of Top2p (present only in Siz^+ *TOP2* cells) are indicated by an asterisk.

had no notable impact on overall cell growth or viability (Figure 3B). The 3xKR (*top2–201*) allele was a triple substitution of the SUMO-acceptor lysine residue (sites 1, 2, and 3, Figure 3A). This allele is different from the *top2*–SNM allele (BACHANT *et al.* 2002), in which all the lysine residues (not just the acceptor residues) in the consensus sites were mutated, raising the possibility that the *top2*–SNM allele is defective in more than just Smt3p conjugation interference. The ΔC allele (*top2–200*) lacks the whole region encoding the COOH-terminal Top2p tail, as shown in Figure 3A, and the corresponding Top2p–ΔC protein was completely refractory to sumoylation *in vitro* (data not shown).

Only a small fraction of Top2p is sumoylated in wild-type cells (BACHANT *et al.* 2002), making analysis of this pool quite challenging. To facilitate enrichment for SUMO conjugates we replaced the native *SMT3* gene with a construct expressing the polyhistidine and FLAG-tagged Smt3 protein (HF–Smp3p), and introduced either a double *siz1 siz2* deletion with the wild-type *TOP2*, with an HA tag (Siz^- in Figure 3, C and D), or the HA-tagged *top2* ΔC and 3xKR alleles (Siz+). IMAC fractionation, allowing enrichment for the HF–Smt3p conjugates, indicated that the combined deletions of *SIZ1* and *SIZ2* genes resulted in the loss of Top2p sumoylation (Figure 3, C and D). Elimination of a single *SIZ* gene did not result in a notable reduction of Top2p sumoylation (data not shown). E3-dependent SUMO modification of Top2p *in vivo* was limited to the COOH-terminal tail (Figure 3A) and, in particular, to the predicted consensus site lysines (K1220, K1246, and K1277) as shown by IMAC in the *top2 cis* mutants ΔC and 3xKR, which phenocopied the loss of Top2p sumoylation in the Siz^- cells (Figure 3, C and D). Even though the Top2p–ΔC protein had a lower abundance than wild-type Top2p or Top2p–3xKR (Figure 3D), scaled up purifications did not reveal any sumoylation of this truncated protein (data not shown). Thus, the Top2p is modified by Smt3p *in vivo* predominately at the COOH-terminal sites, and the Siz1p and Siz2p play a critical, but mutually redundant role in catalyzing this modification.

The positive regulatory role of E3 in minichromosome transmission is mediated by Top2p: To test whether the role of E3 in minichromosome segregation (Figure 1) is linked to the Top2p function we analyzed minichromosome transmission in the *top2* 3xKR and ΔC mutants and compared it with *SIZ1* and *SIZ2* double deletion strains. Analysis of minichromosome stability revealed that both *top2* mutants had a decrease in segregation fidelity, which was similar to the minichromosome destabilization in the *siz1*-Δ/*siz2*-Δ (Siz⁻) strain (Figure 4). Moreover, we found that the Siz⁻ and *top2* mutations were epistatic for minichromosome stability in triple-mutant strains (combining both *top2* and *siz* mutations), as no additive decrease in minichromosome stability was observed in these mutants (Figure 4). Thus, we demonstrated that the positive regulatory role of the Smt3p E3 in chromosome stability is to modify the consensus sumoylation sites in the COOH tail of topoisomerase II.

As the *siz1*-Δ/*siz2*-Δ, *top2* 3xKR, and ΔC mutations destabilized only the centromere-containing minichromosomes (Figures 1 and 4 and data not shown), the chromatin immunoprecipitation (ChIP) analysis showed that the Top2p protein is present in yeast centromeres (see Figure 5), and Top2p over-sumoylation has been shown to disrupt centromere cohesion (Bachant *et al.* 2002), we investigated whether the E3-dependent Top2p sumoylation is involved in kinetochore function. Therefore, we tested the same (Figure 4A) set of mutants for stable maintenance of dicentric minichromosomes. Stabilization of dicentric chromosomes has been shown to be a reliable and sensitive genetic assay for testing kinetochore proficiency, as dicentric minichromosomes are not stably maintained in yeast cells unless kinetochore function is compromised by *cis* or *trans* mutations (Mythreye and Bloom 2003). To eliminate minichromosome rearrangement as a possible pathway allowing dicentric minichromosomes to be stabilized, we used a conditional dicentric minichromosome, where one of the kinetochores is inactivated by potently inducible transcription prior to the experiment. As shown in Figure 4B, the *top2* 3xKR and the *siz1*-Δ/*siz2*-Δ mutations were epistatic in their ability to stabilize dicentric minichromosomes, as well as in the monocentric minichromosome segregation phenotype (Figure 4A). Thus it is likely that the SUMO E3-dependent regulatory pathway, which facilitates positive regulation of Top2p function in minichromosome segregation, mainly controls the function of the Top2p pool located at the centromeres.

Chromosomal address of sumoylated Top2p: We hypothesized that a specific physiological level of Top2p sumoylation is required to target it to centromeric regions. Testing this hypothesis is, however, technically challenging due to the above-mentioned difficulties in locating a small sumoylated fraction of a given protein in the cell. To overcome this technical problem we utilized a Top2p–Smt3p fusion as an *in situ* model of

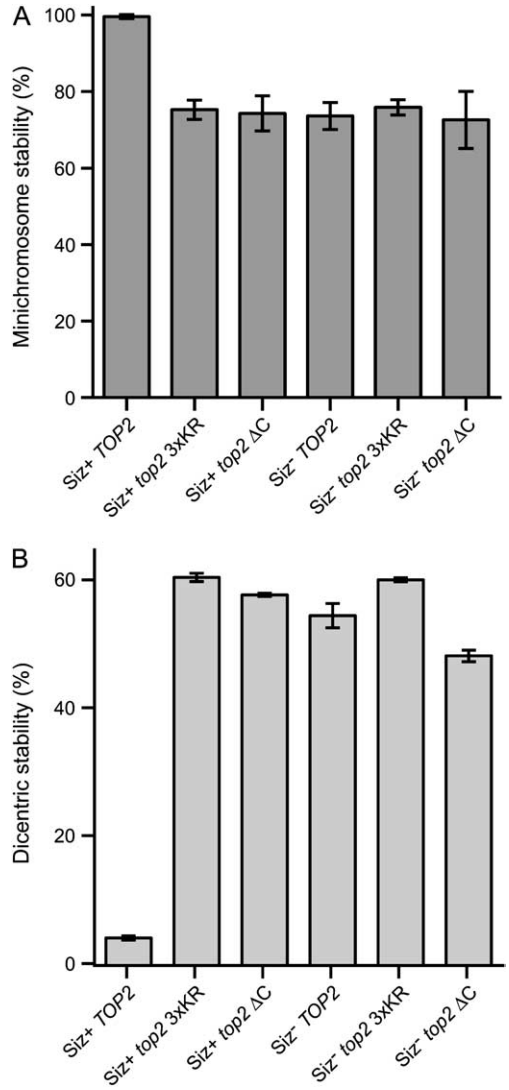

Figure 4.—Epistatic interaction between the Smt3p-conjugation-deficient top2 mutations and SUMO E3 deficiency in the control of minichromosome stability. (A) Transmission efficiency of the pRS415 minichromosome. Minichromosome stability was measured (see materials and methods) at 30° in the wild-type (BY4733) and *siz1/siz2* mutant (4bAS399) strains with different *top2* variants (pYT1033, pYT1034, or pYT1035). (B) SUMO E3 mutants and *top2* Smt3p-conjugation-deficient mutations stabilize dicentric minichromosomes. Transmission efficiency of pAS72, a conditional dicentric minichromosome, was measured at 30° in the wild-type (BY4733) and *siz1/siz2* (4bAS399) strains with *top2* variants (pYT1033, pYT1034, or pYT1035) as described in materials and methods.

sumoylated Top2p. This approach is based on recent data on physiologically relevant replacement of the isopeptide bond-conjugated ubiquitin with peptide-bond ubiquitin fusions (Ciechanover and Ben-Saadon 2004; Saeki *et al.* 2004). Thus, we inserted the *SMT3* ORF into integrative *TOP2* constructs so that an in-frame Top2p(core)–Smt3p–Top2(tail) fusion is produced, with Smt3p inserted between Leu-1235 and Val-1236 of the native Top2p sequence (underlined in Figure 3A). This

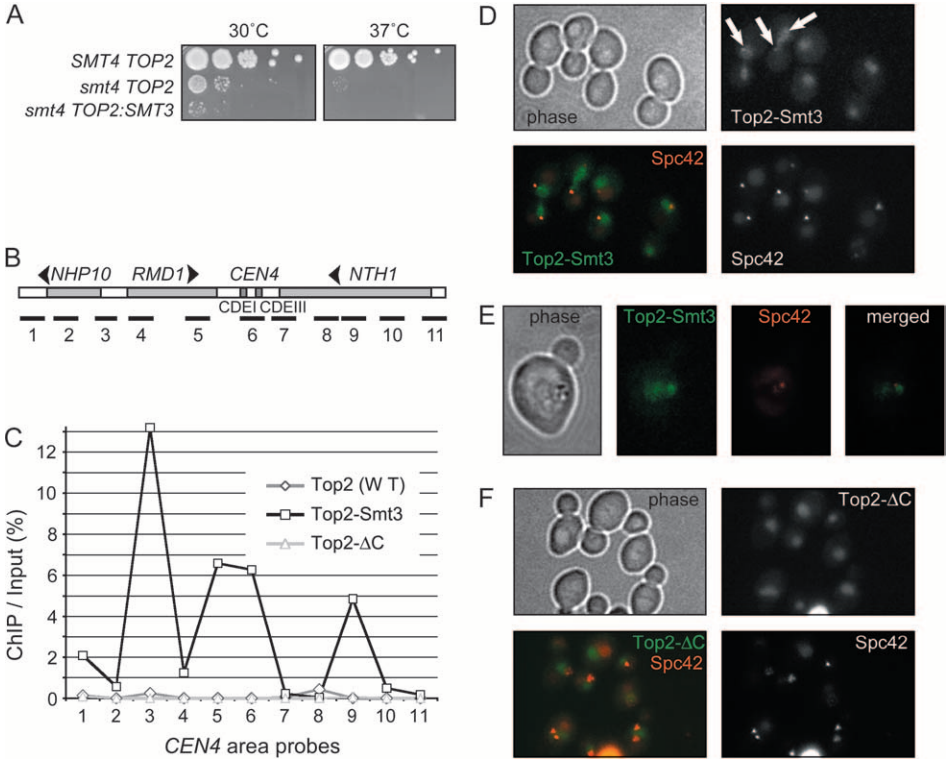

FIGURE 5.—Constitutive sumoylation results in pericentromeric targeting of Top2p. (A) Synthetic interaction between *smt4*-Δ and *TOP3:SMT3* fusion. The same-concentration (10^6 cells/ml) cultures of three strains were plated on YPD plates in serial 10-fold dilutions and incubated at 30° (permissive for *smt4*-Δ) and 37° (nonpermissive for *smt4*-Δ) temperatures for 48 hr. Integrated *TOP2* variants produce Top2p–HA fusions. *SMT4 TOP2*: W303 with the wild-type *SMT4* gene transformed with pYT1033. *smt4 TOP2* and *smt4 TOP2:SMT3* are W303 with *smt4* deletions, transformed with pYT1033 or pYT1051, respectively. As *smt4*-Δ results in massive lethality, even at permissive temperature, the starting dilutions have different number of growing colonies, as compared to $Smt4^+$. (B) Layout of the PCR probes used for ChIP analysis of Top2p binding to the *CEN4* region. (C) Smt3p fusion to Top2p tail results in Top2p enrichment at the *CEN4* pericentromeric region. The W303 strains transformed with pYT1033 (*TOP2*:HA), pYT1051 (*TOP2*:*SMT3*:HA) or pYT1035 (*TOP2*-ΔC:HA), all replacing the wild-type *TOP2* gene, were subjected to ChIP analysis using the PCR probes shown in B. ChIP analysis was as described (STRUNNIKOV *et al.* 2001; WANG *et al.* 2004). (D–F) Fusion to Smt3p changes localization of Top2p–GFP in the nucleus. Spc42p-mRFP was used to mark SPB in diploid strains expressing both the wild-type Top2p and a corresponding Top2p–GFP fusion: *TOP2*:*SMT3*:GFP (D and E show maximal resolution) and *TOP2*-ΔC:GFP (E). Twenty optical Z-sections with 0.2-μm spacing were combined to compose the images (2 × 2 binning, 1-sec exposure per frame, except in E: no binning, 3-sec exposure per frame). Arrows point to clustered Top2p–Smt3p–GFP staining in mitotic cells.

fusion places the Smt3p constitutively between the first and the second sumoylation sites in the Top2p tail. Haploid strains carrying the integrated HA-tagged and GFP-tagged versions of *TOP2*:*SMT3* fusions were viable and the Top2p–Smt3p–GFP fusions localized throughout the nucleus (data not shown). Moreover, the Top2p–Smt3p appear to be mimicking the sumoylated Top2p *in vivo*, as such a fusion resulted in almost complete growth inhibition in the *smt4*-Δ background (Figure 5A). Both HA and GFP-tagged *TOP2*:*SMT3* fusions also resulted in a notable mitotic delay (Figure 5D and data not shown) in the corresponding cell populations, indicating that Smt3p fusion to all of the Top2p molecules in the cell may be detrimental to proliferation.

To test whether Top2p–Smt3p fusion is enriched at the centromeres, as predicted by genetic analysis (Figures 1 and 4) for sumoylated Top2p, we conducted ChIP analysis of the HA-tagged Top2p–Smt3p. Chromatin extracted from the strains expressing Top2p–Smt3p–HA, Top2p–HA and Top2p–ΔC–HA was subjected to immunoprecipitation with anti-HA antibody and analyzed by PCR as described (STRUNNIKOV *et al.* 2001; WANG *et al.* 2004). The PCR probes were designed to tile the 5.5-kb region centered at the *CEN4* core sequence (Figure 5B). While Top2p–HA displayed only minimal enrichment at the pericentromeric loci compared to Top2p–ΔC–HA, the Top2p–Smt3p-HA fusion was significantly and reproducibly enriched in the *CEN4* vicinity (Figure 5C). As it is not known whether Top2p has specific enrichment sites in the genome, it is difficult to determine whether the observed enrichment of binding represents a strictly pericentromeric phenomenon or whether the whole Top2p–Smt3p pool becomes more concentrated at the defined genomic loci. However, ChIP analysis of a randomly selected set of genomic sites (according to WANG *et al.* 2005) did not reveal any enrichment for Top2p–Smt3p (data not shown), suggesting that sumoylated Top2p likely has a propensity to be enriched at the centromeric regions, as compared to the unmodified form, consistent with our genetic results (Figure 4).

To address the above mentioned caveat and to mimic the wild-type situation, where only a fraction of Top2p is sumoylated, we crossed the haploid strains with integrated HA-tagged and GFP-tagged *TOP2*:*SMT3* fusions to the wild-type *TOP2* strains. The resulting strains had both the constitutively modified (fused to Smt3p) Top2p and the wild-type Top2p, with only the fusion form detectable by either GFP or HA tags. Analysis of Top2p–Smt3p–GFP localization in the strain expressing Spc42p-mRFP, an SPB marker, revealed that the fusion,

while still diffusely localized to the nucleus, forms distinctive areas of concentration next to spindle pole bodies in mitotic cells (Figure 5D, arrows, and 5E). This localization of the modified Top2p pool is consistent with it being enriched around the centromeric regions. The peri-SPB GFP enrichment was not observed, however, when Top2p–ΔC–GFP (Figure 5F) or wild-type Top2p–GFP (not shown) fusions were investigated in the similarly constructed diploid strains. This result suggests that the peri-SPB enrichment of Top2p–Smt3p–GFP is mediated by Smt3p.

DISCUSSION

Chromosomal function of Siz1p/Siz2p: The SUMO E3 proteins appear to serve as specificity factors directing the sumoylation event to specific targets in eukaryotic cells (Johnson 2004; Muller *et al.* 2004). In budding yeast, the Siz1 and Siz2 proteins are localized in the nucleus and are required for the bulk of sumoylation (Johnson and Gupta 2001; Takahashi *et al.* 2001b). However, the *siz1 siz2* double mutants are viable, indicating that the majority of Smt3p-conjugation events are not required for the essential housekeeping functions of the cell. In this work we demonstrated that SUMO E3 is required for minichromosome transmission fidelity. This suggests that certain proteins required for normal chromosome dynamics may be functionally impaired by the lack of E3-dependent sumoylation. Even though we were unable to detect a significant destabilization of the relatively short chromosome III or a longer, rDNA-containing, chromosome, we did find a notable increase in mitotic recombination for distal chromosomal markers (not shown), which suggests an additional role of SUMO E3 in chromatin.

A concurrent study has established that the Siz1 and Siz2 proteins are involved in inhibiting amplification of 2μ plasmids by virtue of promoting the inhibitory sumoylation of two plasmid-encoded proteins (Chen *et al.* 2005). This exemplifies the negative regulatory role of Smt3p in chromatin. The Loc$^-$ chromosomal phenotype (with an inability to separate sister chromatids in mitosis) of the *smt3–331* mutation (Biggins *et al.* 2001) also suggests that Smt3p hyperconjugation has a negative impact on chromosome segregation, as this mutant displays accumulation of sumoylated proteins (A. Strunnikov, unpublished data) in a manner similar to *smt4* mutants (Li and Hochstrasser 2000; Strunnikov *et al.* 2001) lacking isopeptidase activity. In contrast, we can view destabilization of minichromosome transmission in *siz1/siz2* as a demonstration of a positive regulatory role of Smt3p in segregation of chromosomal material in budding yeast. Establishing the fact that SUMO E3 factors are required for the fidelity of minichromosome transmission allowed us to identify Top2p as an Smt3p target protein likely mediating this E3 role in chromosome segregation.

Top2p tail is a potent SUMO E3 substrate: Several essential chromosomal proteins in budding yeast have been reported to have SUMO-modifications *in vivo*: Top2p, Pol30p, Pds5p, and Ycs4p (Bachant *et al.* 2002; Hoege *et al.* 2002; Stead *et al.* 2003; D'amours *et al.* 2004). However, the experimental evidence for the biological role of sumoylation in most of these cases has proven to be inconclusive. Recent proteome-wide analyses (Panse *et al.* 2004; Wohlschlegel *et al.* 2004; Zhou *et al.* 2004; Hannich *et al.* 2005) of Smt3p targets in *S. cerevisiae* suggest that modifications of some of the previously reported targets (*e.g.*, Pds5p and Ycs4p) cannot be detected by these techniques (Wohlschlegel *et al.* 2004). This agrees with our data *in vitro* (Figure 2A), suggesting that these proteins are poor substrates for Smt3p conjugation. Many other chromosomal proteins, including the condensin subunits Brn1p, Smc4p, and Smc2p and some cohesin subunits, have been shown by proteomic approaches to be Smt3p substrates *in vivo* (Wohlschlegel *et al.* 2004). Retesting these proteins for modification *in vitro* (Figure 2A) and *in vivo* (not shown), using the Smt3p "fingerprint" technique (Panse *et al.* 2004), failed to detect significant modifications, suggesting that these proteins are also poor substrates. In the case of PCNA (Pol30p) the E3-dependence of its sumoylation (Hoege *et al.* 2002; Haracska *et al.* 2004) was demonstrated *in vitro* (Stelter and Ulrich 2003) and confirmed by us (Y. Takahashi, unpublished data), yet in the chromatin context Pol30p showed no modification *in vitro* (data not shown). These data suggest that while many chromosome proteins can be modified by SUMO *in vivo*, such a modification, in many cases, is incompatible with the chromatin association of these proteins. Thus, one can hypothesize that for many proteins sumoylation serves as an inhibitor of chromatin association.

In contrast, our analysis of chromatin-bound Top2p indicates that it is by far the most potent acceptor of Smt3p conjugation among the chromosomal SUMO targets tested. We demonstrated that Top2p can be sumoylated *in vitro* and elucidated the key role of SUMO E3 in this modification (Figures 2 and 3). We established that Top2p sumoylation is not inhibited in the chromatin-bound forms, making this substrate unique among other SUMO targets, particularly PCNA, and suggesting a high degree of functional specialization of Top2p sumoylation. Mutations of the three consensus-site lysine residues in the Top2p tail largely abolish the ability of Top2p to be modified by Smt3p *in vivo* and greatly inhibit the *in vitro* modification reaction (Figure 2E). Deletion of the whole Top2p tail (Top2p–ΔC) eliminates the residual nonspecific modification *in vitro* (data not shown). These results confirm the role of these sites in Top2p modification (Bachant *et al.* 2002) and establish that the modification of the Top2p tail is mediated by SUMO E3.

The function of Siz1p/Siz2p in minichromosome transmission is to modify Top2p: While in higher

eukaryotes the effect of *UBC9* depletion on chromosome segregation is evident *in vitro* (Azuma *et al.* 2003) but not *in vivo* (Hayashi *et al.* 2002), disruption of SUMO E2 function in yeast cells impairs mitotic chromosome segregation (Dieckhoff *et al.* 2004). We established that depletion of the major SUMO E3 activity (*Siz1p* and *Siz2p*) also results in an *in vivo* segregation defect (Figures 1 and 4). Moreover, we found that the Top2p tail deletion and the triple lysine-to-arginine residue mutation at the SUMO acceptor sites have a destabilizing effect on minichromosome transmission similar and epistatic to the SUMO E3 double mutants. While previously overmodification of the Top2p SUMO-target sites in *smt4* mutants was shown to impair pericentromeric cohesion, the elimination of these modification sites by point mutations resulted in a practically undetectable cohesion phenotype (Bachant *et al.* 2002). Thus, the role of Smt3p modification of Top2p in wild-type yeast cells remains obscure.

The results showing that the simultaneous loss of Siz1p and Siz2p activity is epistatic to the *top2* SNM alleles and yields an unmodified Top2p suggest that the role of SUMO E3 in chromosome segregation could be limited to the Smt3p modification of a specific Top2p subpopulation. As sumoylation of topoisomerase II in *S. cerevisiae* (data not shown) and vertebrates (by SUMO-2) (Azuma *et al.* 2003) peaks in mitosis, it is conceivable that the sumoylated pool of Top2p plays an important role in mitotic chromosome segregation. As the sumoylated pool of Top2p is very small (Figure 3C), it is likely that this subset of Top2p molecules participates in centromere–kinetochore dynamics. Indeed, utilizing a novel approach of modeling sumoylated proteins by direct fusion of targets to SUMO (constitutive SUMO modification), we were able to show that the modified pool of Top2p is enriched at the centromeres (Figure 5C). The exact function of this pool is still unknown, but our genetic data (Figure 4) and disruption of pericentromeric sister chromatid cohesion by hypersumoylation of Top2p (Bachant *et al.* 2002) suggest that Top2p, when sumoylated at the physiological level, is involved in establishing or maintaining the bipolar kinetochore orientation.

What molecular mechanism can be responsible for the role played by sumoylated Top2p both at the centromere in general and in sister chromatid cohesion in particular? As chromatin-bound Top2p can be readily modified *in vitro* (Figure 2). it is conceivable that mitotic activation (Johnson and Gupta 2001; Takahashi *et al.* 2001a) of Siz1p and/or Siz2p activity results in a localized Top2p sumoylation at the centromeric regions. In turn, the SUMO moiety at the Top2p tail could contribute to cohesion by stabilizing the Top2p dimer at the loci that hold two sister chromatids together. As the sumoylation sites in the Top2p tail are situated close to the DNA-release gate in the dimer (Champoux 2001), one can speculate that SUMO-modified tails may delay release of the DNA strands after the enzymatic topoisomerase II reaction is complete; thus, allowing a cohesion mechanism alternative to cohesin clamp.

In this report we demonstrate that the major *S. cerevisiae* SUMO E3 function in minichromosome transmission is in the same pathway as Smt3p modification of the Top2p tail. While it is formally possible that *in vivo* there is another Siz1p/Siz2p substrate protein that bridges the Siz1p/Siz2p E3 activity and Top2p modification, all of these data can be explained by direct sumoylation of Top2p by Siz1p/Siz2p, as occurs *in vitro* (Figure 2). Thus, it is concluded that the important role played by Siz1p and Siz2p in mitotic segregation is embodied by the small pool of Smt3p-modifid Top2p, probably localized in the vicinity of the centromere.

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