Saccharomyces cerevisiae SMT4 Encodes an Evolutionarily Conserved Protease With a Role in Chromosome Condensation Regulation

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ABSTRACT

In a search for regulatory genes affecting the targeting of the condensin complex to chromatin in *Saccharomyces cerevisiae*, we identified a member of the adenovirus protease family, *SMT4*. *SMT4* overexpression suppresses the temperature-sensitive conditional lethal phenotype of *smc2-6*, but not *smc2-8* or *smc4-1*. A disruption allele of *SMT4* has a prominent chromosome phenotype: impaired targeting of Smc4p-GFP to rDNA chromatin. Site-specific mutagenesis of the predicted protease active site cysteine and histidine residues of Smt4p abolishes the *SMT4* function *in vivo*. The previously uncharacterized *SIZ1* (*S*AP and M*iz*) gene, which encodes a protein containing a predicted DNA-binding SAP module and a Miz finger, is identified as a bypass suppressor of the growth defect associated with the *SMT4* disruption. The *SIZ1* gene disruption is synthetically lethal with the *SIZ2* deletion. We propose that *SMT4*, *SIZ1*, and *SIZ2* are involved in a novel pathway of chromosome maintenance.

THE condensin complex plays an essential role in chromosome condensation in all eukaryotes studied so far (Kimura and Hirano 1997; Sutani *et al.* 1999; Freeman *et al.* 2000). The activity of the purified condensin complex from *Xenopus laevis* embryos has been recently characterized *in vitro* (Kimura *et al.* 1999). The complex introduces a positive writhe in DNA in an ATP-dependent fashion, an activity believed to be central to its chromosome condensation function *in vivo*. In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* condensin is crucial for mitotic chromosome condensation and segregation. The *S. cerevisiae* condensin subunits are encoded by five genes, *SMC2*, *SMC4*, *BRN1*, *YCS4*, and *YCS5/YCG1* (Freeman *et al.* 2000; Lavoie *et al.* 2000; Ouspenski *et al.* 2000).

The SMC components of condensin belong to the SMC family of ABC-class ATPases, a group of proteins that is nearly ubiquitous and highly conserved in evolution (Hirano 1999). In *S. cerevisiae*, the Smc2 and Smc4 proteins are bound to chromosomes throughout the cell cycle, with unique binding characteristics coinciding with the G2/M phase of the cell cycle (Freeman *et al.* 2000). Mutations in these genes impair chromosome condensation and lead to incorrect chromosome transmission in anaphase. Chromosomes containing rRNA genes (rDNA) are especially sensitive to condensation defects (Freeman *et al.* 2000).

In contrast to the apparent high degree of conservation of the mechanism of condensin activity throughout Eukaryota, the regulation of condensin is not as conserved. In *X. laevis* embryonic extracts, mitosis-specific activity of condensin is triggered by the cdc2-dependent phosphorylation of three non-SMC condensin subunits, XCAP-H, XCAP-D2, and XCAP-G (Kimura *et al.* 1998). Mitosis-specific targeting of condensin to chromatin sites is one possible regulatory mechanism of chromosome condensation. Targeting of condensin to chromosomes in human cells is mediated by a kinase-anchoring protein AKAP95 (Collas *et al.* 1999). In *S. pombe*, mitosis-specific phosphorylation of cut3, the Smc4p ortholog, is required for condensin activity and proper targeting *in vivo* (Sutani *et al.* 1999). In *S. cerevisiae*, the phosphorylation sites identified in *X. laevis* and in *S. pombe* are not conserved, and phosphorylation of condensin subunits has not yet been demonstrated. Thus the mechanism of condensin regulation, particularly targeting to chromatin in mitosis in *S. cerevisiae*, remains unknown.

We used a genetic approach to identify the potential regulatory factors that affect condensin activity and chromatin targeting in *S. cerevisiae*. A gene dosage suppressor screen was used to isolate the genes that, when overexpressed, suppress mutations in the genes encoding the SMC proteins, the core condensin subunits. The isolated suppressor of the *smc2-6* allele, *SMT4*, encodes a member of the adenovirus protease (AVP) family (Stephens *et al.* 1998). We show that predicted catalytic residues of this protease are required for the *smc2-6* suppressor activity. The only paralog of Smt4p in *S. cerevisiae*, Ulp1p, is an isopeptidase involved in removal of small ubiquitin-like protein (SUMO; Saitoh *et al.* 1997; Kretz-Remy and Tanguay 1999), encoded by

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the *SMT3* gene (Meluh and Koshland 1995; Johnson *et al.* 1997), from SUMO-conjugated intracellular proteins (Li and Hochstrasser 1999). Smt4p also has a SUMO-cleaving activity *in vitro* (Li and Hochstrasser 2000) and possibly *in vivo*, which raises the possibility of SUMO-mediated regulation of condensin or other proteins required for the condensin function in *S. cerevisiae*. The requirement for *SMT4* is bypassed when a previously uncharacterized gene *SIZ1*, which encodes a predicted DNA-binding protein, is overexpressed, suggesting a possible mechanism integrating higher order chromatin structure and *SMT4* function.

MATERIALS AND METHODS

Cloning, DNA sequencing, and sequence analysis: The *SMT4* and *SIZ1* genes were isolated as dosage-suppressors essentially as described previously (Guacci *et al.* 1997). The strain 3aAS283 (*MAT*α *ade2 his3 leu2 lys2 ura3 smc2-6*) was transformed with a multicopy genomic library containing either *LEU2* or *URA3* markers (gifts of P. Hieter and J. Boeke) and transformants growing at 36° were selected. Two independent overlapping clones contained the *SMT4* gene (pAS322 and pAS323) and one contained the *SSD1/SRK1* gene (pAS321). Cloning of *SIZ1* was done with the strain 4aAS320 (Table 1) using the same approach. Two independent clones were isolated, one containing the *SMT4* gene, and the other one encompassing three open reading frames (ORFs), *YDR409w*, *ADE8*, and truncated *YDR407c* (pAS358). A series of deletions confirmed that YDR409w encodes a bypass suppressor of the *smt4-Δ2* allele. The DNA sequence of *SMT4* was determined by partial clone sequencing (ABI Prism 377 dye-terminator method; Applied Biosystems, Foster City, CA) and from the Yeast Genome Project (Galibert *et al.* 1996; Johnston *et al.* 1997).

Protein sequence database searches were performed using the gapped BLAST program or the position-specific iterating BLAST (PSI-BLAST) program (Altschul *et al.* 1997). Multiple alignments of protein sequences were constructed using the Clustal_X (Thompson *et al.* 1997) or MacVector (Oxford Molecular) programs. Structural models were constructed using the ProMod program (Peitsch 1996) and visualized using the MolScript program (Kraulis 1991).

Strains, plasmids, and genetic techniques: Genotypes of yeast strains are shown in Table 1. To disrupt the chromosomal copy of the *SMT4* gene, AS260 (Strunnikov *et al.* 1995b) was transformed with the *Sph*I-*Nhe*I fragment of pAS334 containing the *ADE2* marker inserted between the *Bam*HI sites of *SMT4*. The resulting diploid AS320 (Table 1) was subjected to genetic analysis yielding 2:2 segregation of the slow growth and temperature-sensitive (ts) phenotypes, cosegregating with Ade^+. The *SIZ1* ORF was replaced by *HIS3* (*Bam*HI-*Bam*HI) in plasmid pAS358 digested with *Bam*HI and *Bgl*II. A *Xho*I-*Eco*RI fragment of the resulting plasmid was transformed into AS260, giving AS417. Haploid *siz1-Δ1::HIS3* strains were isolated as meiotic progeny. Alternative deletion of SIZ1, *siz1-Δ0::kanMX*, and *SIZ1* deletion *siz1-Δ0::kanMX* were generated by the systematic ORF deletion project (Winzeler *et al.* 1999) and obtained from Research Genetics. Strains 14245 and 2412 were crossed to form the AS399 diploid (Table 1).

SMT4 was tagged with HA and MYC epitopes using the following approach. Plasmid pAS337 containing the full-length *SMT4* was digested with *Bgl*II and the 6MYC *Bam*HI fragment or 3HA *Bgl*II fragment was inserted to generate pAS337/1 and pAS356, respectively.

SMT4 mutagenesis was performed with two sets of overlapping mutagenic primers: AACATAAGTTACGCGTGGTT TAGTTGCATTATAACAAAC/GCAACTAAACCACGCGTA ACTTATGTTAATTGGTATAAC (*smt4-H531A*, *Mlu*I marker site) and AATATGAGCGATATCGGTGTTCATGTTATTTTGA ATATT/AACATGAACACCGATATCGCTCATATTAGGTT GTTGTGG (*smt4-C624I*, *Eco*RV marker site) using PCR with Pfu polymerase. For each mutation two overlapping PCR prod-

TABLE 1

Yeast strains

Strain	Genotype	Source
3aAS283	*MAT*α *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52 smc2-6*	Strunnikov *et al.* (1995a)
AS260	*MAT***a**/α *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63/TRP1 ura3-52*	Guacci *et al.* (1997)
AS320	*MAT***a**/α *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63/TRP1 ura3-52 SMT4/smt4-Δ2::ADE2*	This work
4aAS320	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52 smt4-Δ2::ADE2*	This work
4aAS320b	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52 smt4-Δ2::ADE2 bar1-Δ::hisG*	This work
YPH499b	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 bar1-Δ::hisG*	Freeman *et al.* (2000)
YPH499bp	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52 bar1-Δ::hisG pep4-Δ::HIS3*	Freeman *et al.* (2000)
AS417	*MAT***a**/α *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63/TRP1 ura3-52 SIZ1/siz1-Δ1::HIS3*	This work
14245	*MAT*α *his3-Δ1 leu2-Δ0 lys2-Δ0 ura3-Δ0 siz1-Δ0::kanMX*	Research Genetics
2412	*MAT***a** *his3-Δ1 leu2-Δ0 met15-Δ0 ura3-Δ0 siz2-Δ0::kanMX*	Research Genetics
AS399	*MAT***a**/α *his3-Δ1 leu2-Δ0 LYS2/lys2-Δ0 MET15/met15-Δ0 ura3-Δ0 SIZ1/siz1-Δ0::kanMX SIZ2/siz2-Δ0::kanMX*	This work
BY4733bp4	*MAT***a** *his3-Δ200 leu2-Δ0 met15-Δ0 trp1-Δ63 ura3-Δ0 bar1-Δ::LEU2 pep4::HIS3 YCS4:12His:6HA::URA3*	Freeman *et al.* (2000)
1-4aAS320b/pAS622	*MAT***a** *ade2-101 his3-Δ200 leu2-Δ1 lys2-801 ura3-52 smt4-Δ2::ADE2 bar1-Δ SIR2:GFP::HIS3 SIR4-42::URA3*	This work

ucts were joined in the second-round PCR and cloned into *Eag*I and *Age*I sites of a *SMT4-HA* plasmid (pAS356), resulting in pA637 (*smt4-H531A*) and pAS637/1 (*smt4-C624I*).

Yeast cultures were maintained following standard techniques (Rose *et al.* 1990). Cell-cycle experiments were conducted as described previously (Strunnikov *et al.* 1995a; Guacci *et al.* 1997). Due to high lethality of the *smt4-Δ* cells, full synchronization was not achievable. Chromosome and minichromosome loss rates were measured as described previously (Strunnikov *et al.* 1993).

Antibodies and microscopy: All commercial antibodies were used according to manufacturer recommendations. Chromatin-binding assays were performed according to Liang and Stillman (1997), except cells were disrupted at 4° with five 2-min rounds of glass-bead beating due to extreme instability of Smt4p in the course of proteolytic removal of the cell wall at 23°. Immunoprecipitations and immunofluorescent staining were performed as described (Freeman *et al.* 2000). Chromatin immunoprecipitation was done with an asynchronous cell population (grown at 23°) exactly as in Freeman *et al.* (2000), with one modification: PCR products from the input DNA were quantified and the chromatin immunoprecipitation (ChIP) results were expressed as ratios between immmunoprecipitated and total (input) DNA. This approach allows direct comparison of ChIP results obtained for different proteins. The strains 4aAS320bp/pAS337 (without an HA tag) and BY4733bp4 (Ycs4p-HA) were used as the negative and positive controls, respectively, in every ChIP experiment with 4aAS320bp/pAS356 (Smt4p-HA).

To stain cells with a double deletion of *SIZ1* and *SIZ2* (5dAS399) cells were collected from the surface of agar and resuspended in 200 μl YPD. After 1-day incubation at 23° they were fixed with 3.7% formaldehyde, washed three times with PBS, concentrated, and mounted for microscopy with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting media. Microscopy was done with a Zeiss AxioVert 135M microscope with epifluorescence. The images were collected at ×100 or ×250 magnification using a MicroMax cooled CCD camera (Princeton Instruments), Z-axis motor assembly (Ludl), and IP Lab software (Scanalytics). Ten 0.3-μm optical sections for each field were converted into a stacked image with IP-Lab software (Scanalytics).

RESULTS

Isolation of *SMT4*: We undertook a dosage suppressor screen for genes potentially interacting with ts mutations in the *SMC2* and *SMC4* genes, which encode subunits of *S. cerevisiae* condensin (Freeman *et al.* 2000), using two multicopy vector libraries. Of the three alleles used, *smc2-6*, *smc2-8*, and *smc4-1*, only *smc2-6*, with the weakest phenotype, was suppressed by genes other than corresponding wild-type genes. Two genes were isolated as dosage suppressors of the *smc2-6* allele (Figure 1A). These genes, however, showed no suppressor activity toward the *smc2-8* or *smc4-1* mutants. The first gene, *SSD1/SRK1*, was previously isolated as a suppressor of multiple ts alleles in several unrelated genes (Sutton *et al.* 1991; Wilson *et al.* 1991). This gene, albeit important for chromosome stability (Uesono *et al.* 1994), was not analyzed further in this study. The second gene, *SMT4*, was independently isolated as a high-copy suppressor of the *mif2-1* mutation (Meluh and Koshland 1995; Strunnikov 1998) and, hence, given its name (*Suppressor of Mif Two*). The *SMT4* gene is predicted to encode a 117-kD protein. The protein of the corresponding size was detected by Western blot analysis (not shown) when an epitope-tagged *SMT4* (Figure 1B; materials and methods) was introduced into yeast cells on a multicopy plasmid. Expression of a single copy of tagged *SMT4* under its own promoter was not detectable by Western blot, indicating that Smt4p is not an abundant protein. We used the 2μ plasmid vector with a tagged *SMT4* to localize the Smt4-HA protein inside the yeast cell by indirect immunofluorescence (Figure 2A). In all cells where staining was detected, the signal was predominantly nuclear, with some additional cytoplasmic staining.

Disruption of *SMT4* with the *ADE2* marker (Figure 1B) was engineered using a standard approach (see materials and methods). The meiotic progeny of the diploid heterozygous for *SMT4* disruption was viable, but spores containing the *smt4-Δ2::ADE2* allele germinated much later and grew slower than *SMT4* spores. The estimated doubling time of the *smt4-Δ2::ADE2* population was 5 hr at 23° *vs.* 2 hr for the $Smt4^+$ strains. This slow growth is likely attributed to high lethality of *smt4-Δ* cells. The *smt4-Δ2::ADE2* cells were also temperature sensitive and thus unable to grow at 37°, with 100% of the *smt4-Δ2* cells losing viability after a 6-hr incubation at 37°. Analysis of cell morphology in a mitotically growing population of *smt4-Δ2* cells revealed profound abnormalities in nuclear DNA transmission (Figure 2C), suggesting that mitotic chromosome segregation is impaired even at 23°. Up to 10% of anucleate cells were detected in the population after 4 hr of incubation at 37° (Figure 2D). Thus *SMT4* function is important for proper progression through mitosis in *S. cerevisiae*.

Loss of *SMT4* affects chromosome structure: The *smt4-Δ2* strains display a variety of morphological and genetic defects, including abnormal mitotic spindle structure and benomyl hypersensitivity (data not shown). The strains carrying the *smt4-Δ2* allele also displayed severely diminished minichromosome stability (<1%) and chromosome III segregation fidelity (loss rate 1.9×10^{-4}; see materials and methods). This phenotype of the *smt4-Δ2* mutant cell may be a result of improper centromere attachment to the mitotic spindle. Indeed, investigation of mitotic spindles visualized in *smt4-Δ2* cells with Tub3p-green fluorescent protein (GFP) and segregation of pericentromeric regions labeled with lacO/LacI-GFP tags (Straight *et al.* 1996) demonstrated that at least one quarter of *smt4-Δ2* cells have a morphology of spindle collapse (A. Strunnikov, unpublished data). However, analysis of the *smt4-Δ2* strain for the synthetic acentric phenotype (Strunnikov *et al.* 1995b) did not reveal any specific interaction between the *smt4* deletion and *cis* centromere mutations in *CDEI*, *CDEII*, and *CDEIII* (data not shown). The broad-peak DNA content determined by FACS analysis of the asynchronous *smt4-Δ2* population at 37° (Figure 2B) suggests that

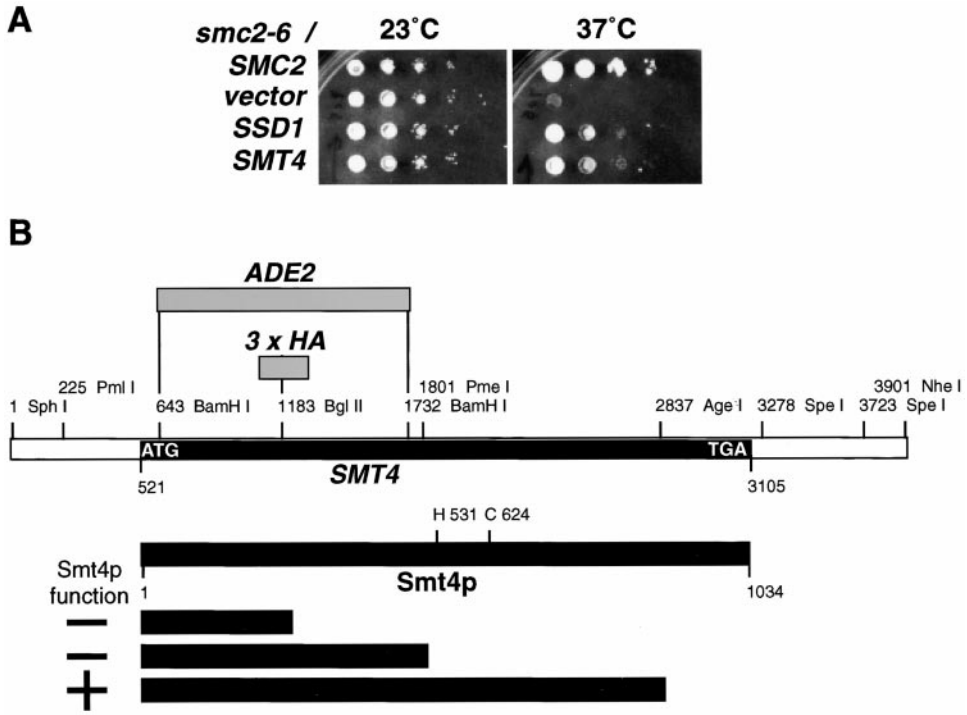

FIGURE 1.—Cloning and characterization of *SMT4*. (A) Suppressor activity of *SMT4*. The 3aAS283 strain was transformed with pAS406 (*SMC2*), pRS426 (*vector*), pAS321 (*SSD1*), and pAS337 (*SMT4*). Threefold serial dilutions were plated and analyzed at 23° and 37° after 3 days. (B) *SMT4* gene structure and deletion analysis. *SMT4* function was assayed as ability to complement the ts phenotype of *smt4-Δ2*. Position of restriction sites and predicted catalytic residues H531 and C624 are shown.

Smt4p could function in chromatin assembly or maturation, which in turn might affect chromosome condensation. Smt4p itself is associated with chromatin (Figure 2E), as shown by the chromatin-binding assay (LIANG and STILLMAN 1997). Moreover, Smt4p is detectable by ChIP analysis in rDNA chromatin, a preferred chromosomal site of yeast condensin (FREEMAN *et al.* 2000; Figure 2F). There is six to eight times less Smt4p than Ycs4p (a condensin subunit) in rDNA chromatin, yet the binding profile across the 9-kb repeat is similar. Considering that Smt4p binding to the chromosomal sites was not mapped yet at a genome-wide scale there is a distinct possibility that some other chromatin domains with a higher concentration of Smt4p can be found.

The *SMT4* gene is required for mitosis-specific targeting of condensin to the rDNA locus: As an excess of Smt4p suppresses the mutation in the Smc2 protein, a condensin subunit, and Smt4p itself is a chromatin component, we assessed the consequences of *SMT4* disruption on condensin targeting in yeast cells. In a recent study (FREEMAN *et al.* 2000), we showed that chromosome condensation in *S. cerevisiae* can be monitored in live cells using the mitosis-specific intranuclear redistribution of condensin visualized with Smc4p-GFP. We applied this assay to *smt4-Δ2* strains because high lethality of these cells prevents their synchronization and thus precludes a traditional assessment of chromosome condensation by fluorescent *in situ* hybridization (FISH; Figure 3A). At 23°, the *smt4-Δ2* strain displayed residual subnuclear concentration of Smc4p-GFP that reached neither full size nor the characteristic crescent shape of nucleolar chromatin in an isogenic wild-type strain (Figure 3B). Most of the Smc4p-GFP was diffusely distributed throughout the nucleus. We also monitored GFP signal in 600 budded *smt4-Δ2* cells incubated at 37° for 6 hr (Figure 3B). In all cases, no specific nucleolar staining was observed even in >50 cells displaying the clear morphology of anaphase cells, which in the wild-type strain manifest the most characteristic rDNA staining (Figure 3B, inset). All GFP signal was nuclear without any reproducible subnuclear concentration, indicating that targeting of condensin to rDNA and probably chromosome condensation did not occur. To test whether this *smt4-Δ2* phenotype is specific for condensin mitotic targeting we tested localization of another abundant nucleolar chromatin protein, Sir2p, in the *smt4-Δ2* strain. Sir2p-GFP (FREEMAN *et al.* 2000) was still effectively targeted to the nucleolus in the *smt4-Δ2* cells (Figure 3C). Thus *SMT4* is the first yeast gene that affects mitosis-specific targeting of condensin to rDNA and thus might be a regulator of condensin function in *S. cerevisiae*.

***SMT4* encodes a protease of the adenovirus protease family:** Deletion analysis of the *SMT4* gene (Figure 1B) showed that a central part of the protein is essential for its function. As was shown previously, this domain of Smt4p belongs to the family of experimentally characterized and predicted proteases whose structural prototype is the adenovirus protease (hereinafter adenovirus protease, or AVP, family; STEPHENS *et al.* 1998). An iterative database search using the PSI-BLAST program (cut off for inclusion of sequences in the profile $e = 0.01$) with the central region of Smt4p as the query showed statistically significant similarity to a number of eukaryo-

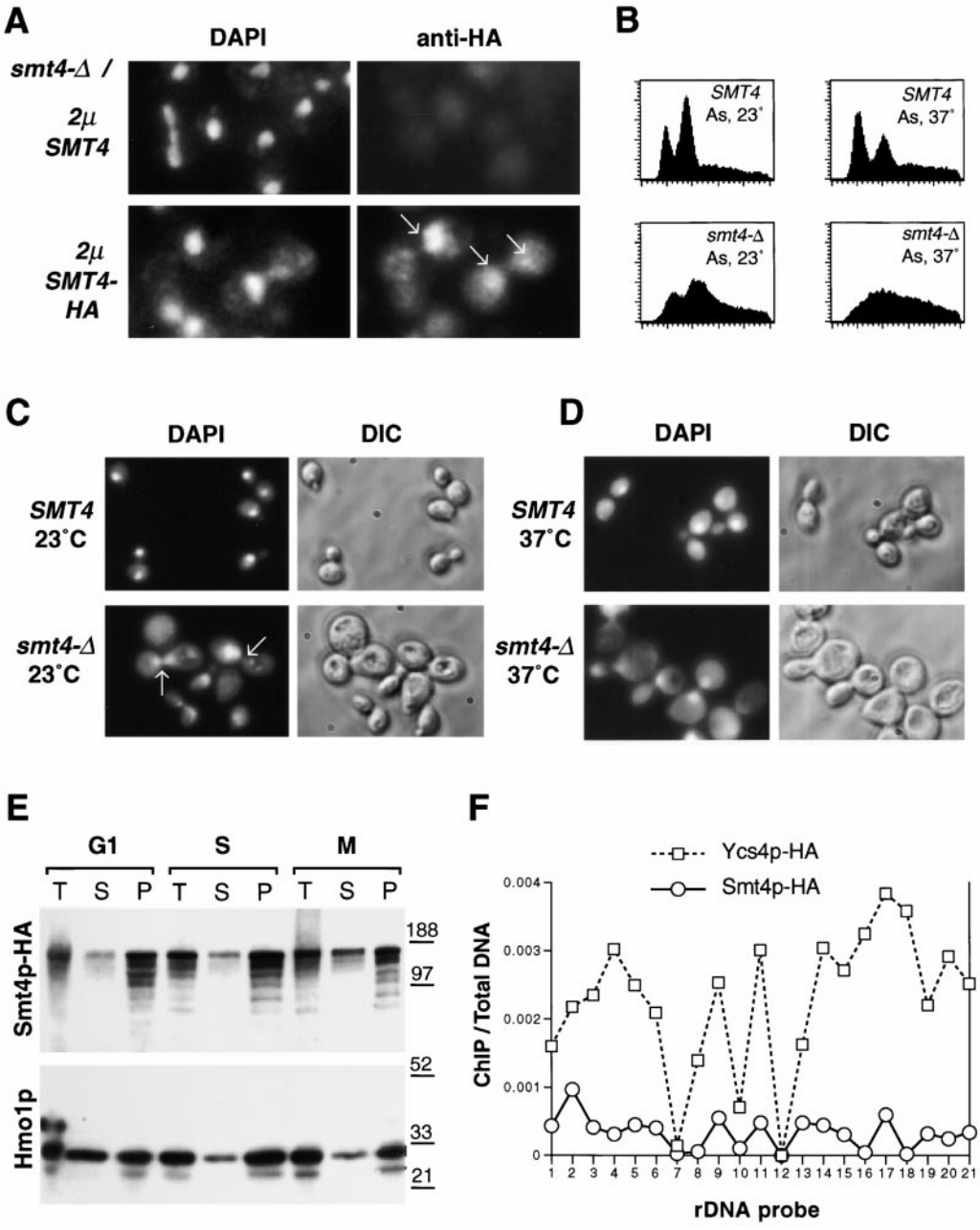

FIGURE 2.—Smt4p localization and *smt4-Δ2* phenotype. (A) Localization of Smt4p-HA as determined by 12CA5 staining of 4aAS320/pAS356 cells (*2μ SMT4-HA*). 4aAS320/pAS337 cells (*2μ SMT4*) were used as a negative control. (B) FACS analysis of $Smt4^{+}$ (YPH499) and $Smt4^{-}$ (4aAS320) strains. (C) Chromosome segregation defects in *smt4-Δ2* cells. Fixed rho^{0} YPH499 (*SMT4*) and 4aAS320 (*smt4-Δ*) cells were stained with DAPI at 23°. (D) The same as in C, 37°. (E) Chromatin-binding assay for 4aAS320bp/pAS356 (*Smt4pHA*) (LIANG and STILLMAN 1997). T, total extract; S, supernatant (protein fraction not bound to chromatin); P, chromatin pellet. Hmo1p is shown as a control chromatin protein in the same fractions. (F) rDNA ChIP of 4aAS320 bp/pAS356 (*Smt4p-HA*). BY-4733bp4 (*Ycs4p-HA*) ChIP results are shown as a positive control. rDNA PCR primers were as described in FREEMAN *et al.* (2000).

tic proteins from fungi, animals, and plants as well as limited similarity to adenovirus proteases and predicted proteases of poxviruses. It was, however, difficult to ascertain orthologous relationships between Smt4p and other eukaryotic proteins, beyond its counterpart in *S. pombe*, due to the limited sequence conservation in the predicted protease domain (20% identity with the most similar homologs in ~200-amino-acid alignment) and differences of the overall domain architectures. The regions of Smt4p located upstream and downstream of the protease domain contain long stretches of low-complexity sequence that are predicted to adapt a non-globular structure, but no recognizable globular domains (Figure 4).

All (predicted) proteases of the AVP family contain three conserved motifs (labeled motifs I–III in Figure 4) corresponding to the catalytic triad (histidine, aspartate, and cysteine) that can be identified from the crystal structure of the adenovirus endoprotease (PDB:1avp). Thiol proteases adapt at least two widespread structural scaffolds, the caspase/hemoglobinase and the papain/transglutaminase/UB-hydrolase folds (RAWLINGS and BARRETT 2000). While the linear arrangement of the catalytic histidine and cysteine in the AVP family resembles that of the caspase/hemoglobinase fold, the two folds share no structural similarity. Identification of the conserved sequence elements of the AVPs (Figure 4) and mapping of these onto the crystal structure of the adenoviral endoprotease (Figure 5A) show that they correspond to a core of three strands of a central β-sheet

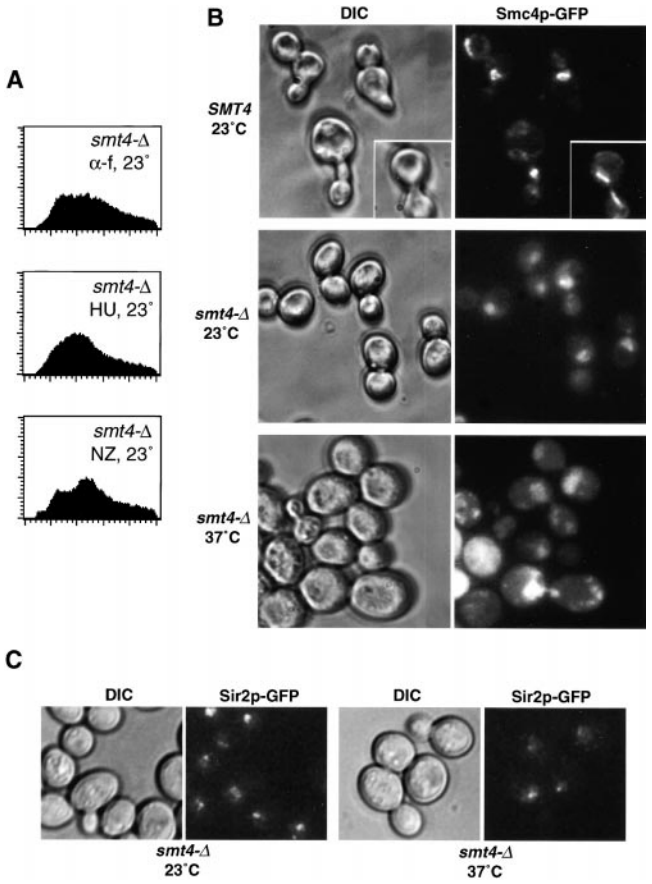

FIGURE 3.—*SMT4* deletion abolishes mitotic targeting of condensin to rDNA chromatin. (A) FACS analysis of 4aAS320b showing failure to form uniform arrest-specific peaks at 23°. Cells were treated with α-factor (α-f), hydroxyurea (HU), and nocodazole (NZ) for 7 hr. (B) Strains YPH499bp/pLF640 (*SMT4*) and 4aAS320b/pLF640 (*smt4*-Δ) expressing Smc4p-GFP at 23° and 37°. YPH499bp/pLF640 cells were presynchronized with α-factor to increase the proportion of mitotic cells. In the *smt4*-Δ strain the Smc4p-GFP fusion fails to properly localize to rDNA in a cell-cycle-specific manner. (C) The cells of 1-4aAS320b/pAS622 (*smt4*-Δ) expressing Sir2p-GFP at 23° and 37°.

and a C-terminal α-helix. This suggests that the AVPs could represent a circular permutation of the papain/transglutaminase/UB-hydrolase-like thiol protease fold wherein the helix encompassing the catalytic cysteine has moved to the C terminus. This predicts the typical papain-like catalytic mechanism for the AVPs within a similar structural framework, which is compatible with the spatial proximity of the residues that form the catalytic triad (Figure 4). A notable feature of the AVPs is the presence of a conserved aromatic residue (almost always tryptophan) in the position immediately C-terminal to the catalytic histidine. This residue, while not directly involved in the reaction, is likely to perform a critical steric role in properly orienting the ring of the catalytic histidine for catalysis.

To verify the importance of the predicted catalytic residues, histidine 531 and cysteine 624, for the Smt4p function, we constructed two substitution mutants, H531A and C624I. Both mutant alleles have lost the ability to complement *smt4*-Δ2 (Figure 5B), and their phenotypes were indistinguishable from the phenotype of the deletion allele. Thus, the predicted catalytic residues of the AVP protease domain of Smt4p are essential for the Smt4p function.

It was recently shown that Ulp1p, a Smt4p paralog, is the isopeptidase for the SUMO conjugates in *S. cerevisiae* (LI and HOCHSTRASSER 1999). In addition, the *SMT3* gene, encoding SUMO in budding yeast, was isolated in the same genetic screen as *SMT4* (MELUH and KOSHLAND 1995). In a concurrent study, Smt4p has been shown to possess a protease activity with the SUMO substrate *in vitro* (LI and HOCHSTRASSER 2000). It is not clear, however, what is the *in vivo* specificity of Smt4p, as *SMT4* disruption results in both increased and decreased Smt3p modification of cellular proteins (LI and HOCHSTRASSER 2000). We obtained similar results when an epitope-tagged Smt3p (FLAG-Smt3p; JOHNSON *et al.* 1997) was introduced into in $Smt4^+$ and $Smt4^-$ strains (data not shown). This suggests that the *SMT4* loss defect has a pleiotropic effect on SUMO modification and thus it is difficult to identify the specific *in vivo* target of Smt4p as a SUMO hydrolase biochemically. The fact that *SMT4* and *ULP1* are not redundant *in vivo* and localize to different cellular compartments (LI and HOCHSTRASSER 2000) also suggests that the substrates of these two peptidases are distinct. The components of the condensin complex were tested as candidates for modification by Smt3p *in vivo*, but SUMO modification was not detectable on any condensin subunit in the anti-HA tag immunoprecipitates prepared from extracts expressing both Smp3p-FLAG and Ycs5p-HA (data not shown). This may suggest that involvement of *SMT4* and *SMT3* in the condensation pathway is mediated by some other proteins, possibly chromatin proteins involved in condensin targeting. Thus, we applied a genetic screen to identify the *SMT4* target *in vivo*.

Requirement for *SMT4* function can be bypassed by overexpression of *YDR409w/SIZ1*: The finding that loss of *SMT4* function is not lethal, but leads to severe cell-cycle defects, may suggest that another gene with an overlapping function is responsible for the survival of *smt4*-Δ*2* cells. One candidate for this role could be *ULP1* (LI and HOCHSTRASSER 1999). However, the fact that *ULP1* is essential for viability and distinct localization patterns for the two proteins (LI and HOCHSTRASSER 2000) argue against the possibility that *ULP1* and *SMT4* functions are redundant. Such a protein could be also a hypothetical primary substrate of Smt4p proteolytic activity. If Smt4p is indeed a hydrolase involved in the removal of SUMO moieties from a distinct protein, overexpression of this target protein, presumably in the unmodified form, could mimic the *SMT4* activity. Thus, we performed a genetic screen for bypass dosage suppressors to uncover genes that could compensate for

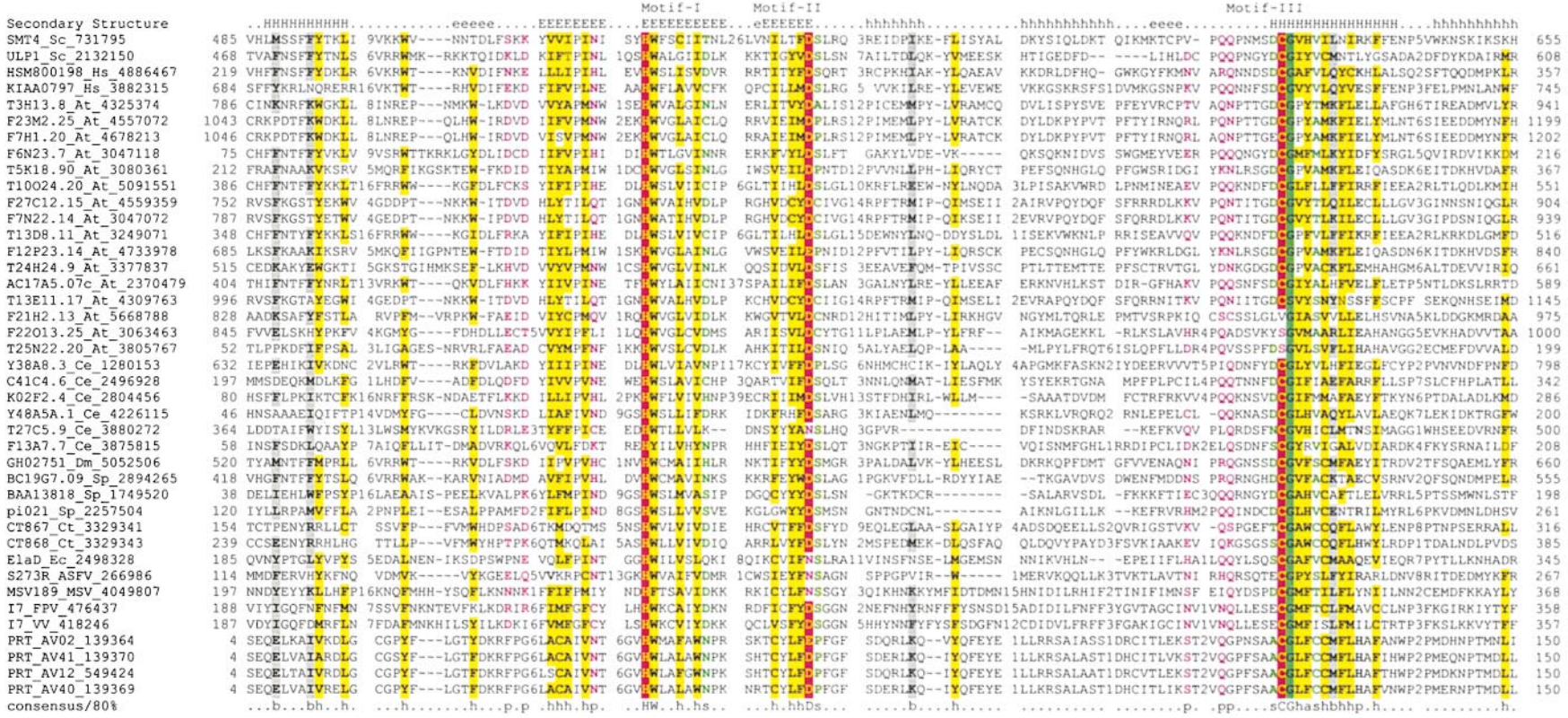

FIGURE 4.—Smt4p is a member of the adenovirus protease family. Conserved sequence elements of the AVP proteases are shown. The alignment, constructed using the Clustal_X program and manually adjusted on the basis of PSI-BLAST search results, is shown along with the superimposed secondary structure elements of the adenovirus endoprotease (1avp). Each protein is designated by the respective gene name, an abbreviated species name, and the gene identification (GI) number. The lengths of poorly conserved spacers between the aligned blocks are indicated by numbers. The numbers at the beginning and at the and of each sequence indicate the position of the first and the last residue of the aligned region in the respective protein. The coloring is according to the 80% consensus, which includes the following: h, hydrophobic residues (YFWLIVMAC); a, aromatic residues (FYW), shaded yellow; s, small residues, colored green (SAGTVPNHD); p, polar residues colored purple (STQNEDRKH); and b, bulky residues, shaded gray (KREQWFYLMI). The predicted catalytic residues (replaced in several proteins, which probably are inactivated) are shown by reverse shading. The species abbreviations are as follows: ASFV, African swine fever virus; At, *Arabidopsis thaliana*; AV, adenovirus (with the number indicating the strain); Ce, *Caenorhabditis elegans*; Ct, *Chlamydia trachomatis*; Dm, *Drosophila melanogaster*; Ec, *Escherichia coli*; FPV, fowlpox virus; Hs, *Homo sapiens*; MSV, Melanoplus sanguinus entomopoxvirus; Sp, *Schizosaccharomyces pombe*; Sc, *Saccharomyces cerevisiae*; VV, Vaccinia virus.

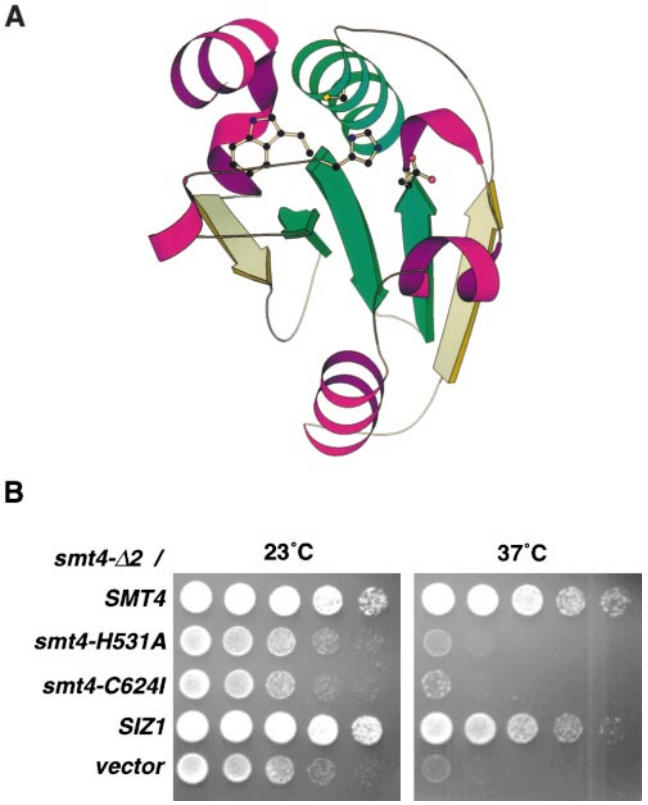

FIGURE 5.—*SMT4* mutagenesis and suppressor analysis. (A) Mapping of the conserved motifs of the AVP family protease fold onto the structure of the adenoviral endoprotease. The three predicted catalytic residues and the conserved tryptophan residue adjacent to the predicted catalytic histidine are shown as ball-and-stick models. (B) Inability of H531A and C624I mutants to complement *smt4*-Δ*2* and *SIZ1* suppressor phenotype. The strain 4aAS320 was transformed with pAS356 (*SMT4*), pAS637 (*H531A*), pAS637/1 (*C624I*), pAS358/1 (*smt4*-Δ/*SIZ1*), and pAS672 (*vector*). All plasmids are 2μ based. Threefold serial dilutions were plated and analyzed at 23° and 37° after 3 days.

the loss of *SMT4*. We used direct selection for increased growth rate of the *smt4*-Δ strain to identify potential suppressing clones. As a result of this screen for overexpression bypass suppressors, we isolated a clone with three ORFs, one of which, YDR409w, carried the ability to completely suppress growth defects of *smt4*-Δ*2* (Figure 5B).

The YDR409w gene encodes a predicted 100-kD protein that contains two distinct structural modules, namely the so-called Miz Zn-finger (WU *et al.* 1997; Figure 6A) and the recently described predicted DNA-binding motif designated SAP, after SAF-A/B, Acinus, and PIAS (Figure 6A; ARAVIND and KOONIN 2000). Therefore we designated this gene *SIZ1* after *S*AP and M*iz*. The Siz1p sequence showed extended similarity to a yeast paralog, *YOR156c*, its ortholog from *S. pombe*, and animal protein inhibitors of activated STAT (PIAS) proteins. The sole unpublished observation that the protein encoded by this gene interacts with Cdc12p in a two-hybrid assay (*S. cerevisiae* genome database) has never been verified by alternative means. Thus we designated the uncharacterized *YOR156c* gene *SIZ2*. All proteins with a similar arrangement of the SAP and Miz modules also have the moderately conserved sequence between them, but without any known functional motifs. Siz1p additionally contains a long C-terminal extension, which is enriched in low-complexity segments (including a poly-asparagine tract) and probably forms a nonglobular structure. The SAP module is likely to mediate sequence-specific DNA binding whereas the Miz finger could be involved in DNA binding or protein-protein interactions. The mouse Miz1 is a DNA-binding protein (WU *et al.* 1997). If Siz1p is indeed a target of Smt4p activity, the phenotype of *SIZ1* disruption should mimic the phenotype of *smt4*-Δ. To test this we initiated genetic analysis of the *SIZ1* gene.

***SIZ1* and *SIZ2* deletions display synthetic lethality:** To investigate the null phenotype of *SIZ1*, we constructed the disruption allele *siz1*-Δ*::HIS3* (see MATERIALS AND METHODS). Analysis of meiotic progeny of the heterozygous *SIZ1*/*siz1*-Δ*::HIS3* diploid, however, did not reveal any detectable phenotypes associated with *SIZ1* deletion. We addressed the possibility that the functions of *SIZ1* and its paralog, *SIZ2*, are redundant, which could have led to our failure to detect any *siz1*-Δ*1::HIS3* phenotype. Two strains containing the complete ORF deletion of *SIZ1* and *SIZ2* marked with *kanMX* were crossed and meiotic progeny were analyzed. Germination of spores was 100% (30 tetrads were analyzed). Two-thirds of the tetrads gave rise to four normally growing spores and one spore that formed a microcolony of $\sim 10^4$ cells. These tetrads in all cases contained only two G418-resistant colonies among the healthy spore progeny, suggesting that a spore with inhibited growth could contain both disruption alleles. This was confirmed by PCR analysis (MATERIALS AND METHODS) of all normal-sized G418-resistant colonies, which showed that all colonies contained only one of the two disruption alleles. Surprisingly, when the *siz1*-Δ*0 siz2*-Δ*0* microcolonies were passaged to fresh media, they failed to grow further, showing zero viability by a plating assay. These findings demonstrate that *siz1*-Δ*0* and *siz2*-Δ*0* are synthetic lethal mutations. The unusual delay of lethality in *siz1*-Δ*0 siz2*-Δ*0* cells may suggest either that these cells are loaded meiotically with the corresponding proteins or that lethality is due to aging or some other cumulative accumulation of cell damage. The severity of the double deletion phenotype is stronger than that of *SMT4* disruption, but does mimic to some extent the low viability of *smt4*-Δ cells. This opens a possibility that Siz1p and possibly Siz2p could be the authentic *in vivo* targets of Smt4p activity. Analysis of a viable conditional mutant in the *SIZ1* gene is required to address the questions of to what degree Smt4$^-$ and Siz$^-$ phenotypes are related and what mechanism may be responsible for bypass of Smt4p function by Siz1p overexpression.

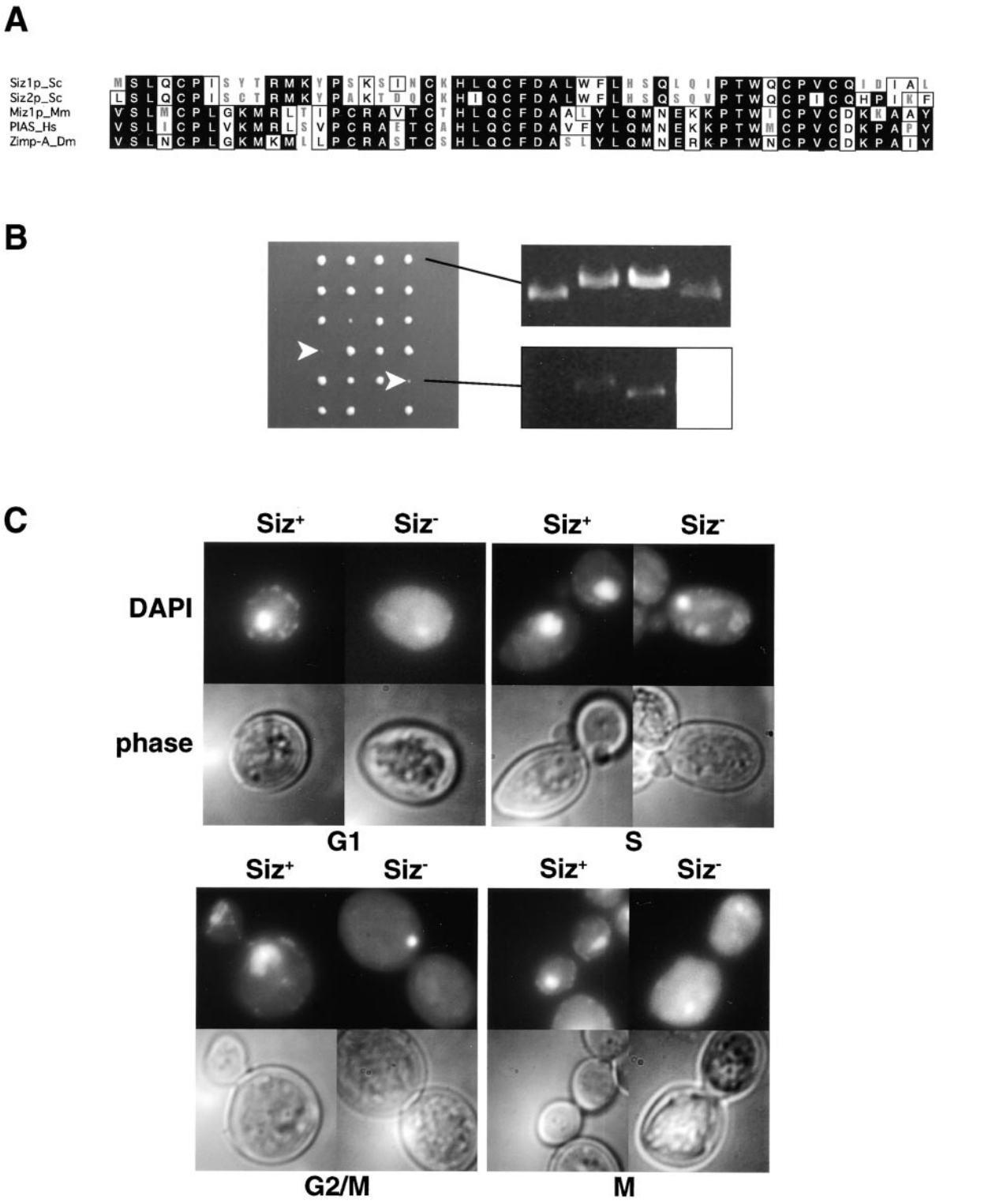

FIGURE 6.—Characterization of *SIZ1*. (A) Alignment of the Miz fingers of Siz1p and Siz2p with homologous sequences from PIAS, Miz1, and Zimp proteins. Conserved residues are shown by reverse shading. (B) A sample of AS399 tetrad analysis. *siz1-Δ0 siz2-Δ0* colonies composed of dead cells are indicated by arrowheads. Two examples of PCR analysis of genomic DNA are shown. The longer product corresponds to *siz1-Δ0*, and the shorter products to *siz2-Δ0*. (C) Cells of inviable *siz1-Δ0 siz2-Δ0* segregant (5dAS399). Double deletion cells (Siz^-) and wild-type cells (YPH499, Siz^+) were stained as described in MATERIALS AND METHODS. Representative samples of four putative stages of cell cycle identified by bud and nuclear DNA morphology are shown.

We analyzed the distribution of nuclear DNA mass and cell morphology in the *siz1*-Δ*0 siz2*-Δ*0* inviable microcolonies compared to an isogenic Siz^+ population (Figure 6C). Distribution of cell types in the *siz1*-Δ*0 siz2*-Δ*0* cell sample was 64% large-budded cells and 36% unbudded, compared to only 19% large-budded cells in the Siz^+ population. *siz1*-Δ*0 siz2*-Δ*0* inviable microcolonies completely lacked cells with small buds, suggesting that lethality is associated with a post-G_1 event. A characteristic feature of the double-mutant cells was apparently very small nuclear DNA mass (Figure 6C). This suggests that the chromatin structure and or content in the *siz1*-Δ*0 siz2*-Δ*0* strain is significantly altered, possibly hypercondensed or underrepresented. It remains to be investigated whether *SIZ1* and *SIZ2* functions are required for proper chromosome structure or for progression through the cell cycle.

DISCUSSION

The role of *SMT4* in chromosome structure maintenance: Isolation of *SMT4* as a dosage suppressor of the *smc2* ts allele and the demonstration that Smt4p is a protease (Stephens *et al.* 1998; Li and Hochstrasser 1999, 2000) expands the list of proteases involved in chromosome metabolism. The most commonly acknowledged protease activities involved in chromosome segregation are the SCF (Willems *et al.* 1999) and anaphase-promoting complex (APC; Fang *et al.* 1999) systems of ubiquitin-dependent protein degradation. These proteosome-dependent pathways are involved in cell-cycle-dependent destruction of a variety of regulatory molecules, only a few of which are chromosomal proteins (Kaplan *et al.* 1997; Weinreich and Stillman 1999; Honda *et al.* 2000; Meimoun *et al.* 2000). It appears likely that a specialized set of proteases is involved in chromatin dynamics. One important proteolytic activity that is crucial for chromosome segregation was described recently. The Esp1 protein is involved in cleavage of the Mcd1/Scc1 protein (Uhlmann *et al.* 1999), one of the key components of cohesin, a complex of four proteins including the Smc1p/Smc3p heterodimer (Losada *et al.* 1998; Toth *et al.* 1999).

The finding that Smt4p is a part of chromatin may indicate the existence of a distinct, chromatin-associated proteolytic system that targets SUMO-modified proteins, in contrast to the ubiquitin-dependent activity of SCF and APC. The enzymatic machinery involved in SUMO modification and maturation has been found to parallel in many aspects the ubiquitination system. However, the biological significance of mono-ubiquitination and mono-SUMO modification in *S. cerevisiae* remains unknown, in part due to the transient nature of these modifications and instability of these moieties in protein extracts.

We utilized a genetic approach to investigate the biology of Smt4p in *S. cerevisiae*. The high-copy suppressor activity of *SMT4* toward the *smc2* mutation suggested a link to chromosome condensation (Strunnikov 1998). Indeed, we demonstrated that the mitotic-specific targeting of the condensin complex to chromatin, in particular rDNA chromatin, is impaired in *smt4* mutant strains. Smt4p, however, is not a stoichiometric part of the condensin complex (A. Strunnikov, unpublished data). This suggests that *SMT4* loss of function either affects the regulation of condensin targeting or impairs the underlying basic chromatin structure, making condensation impossible. There is some evidence in support of the latter model, namely the unusual, intermediate DNA content of $Smt4^-$ cells, which may indicate *SMT4* involvement in DNA replication and/or chromatin maturation. The inability of a significant portion of kinetochores to attach to the mitotic spindle and suppression of *mif2* alleles by *SMT4* increased dosage (Meluh and Koshland 1995) suggest a role of *SMT4* in centromeric chromatin assembly. High chromosome and minichromosome loss (this study; Li and Hochstrasser 2000) and obvious signs of segregation defects in the morphology of *smt4*-Δ cells also suggest that some aspects of chromosome organization are severely impaired. Finally, the phenotype of *smt4*-Δ is reminiscent of some mutants affecting chromatin structure in yeast. One of them is *pds1*-Δ (Yamamoto *et al.* 1996), which is characterized by slow growth, ts lethality, and segregation defects. Other examples include deletions of *ASF1*, a gene for histone chaperone (Tyler *et al.* 1999; Munakata *et al.* 2000), and *CAC1*, a gene encoding chromatin assembly factor subunit (Kaufman *et al.* 1997), which are also characterized by extremely slow growth, ts lethality, and FACS profiles similar to those of *smt4*-Δ. Chromatin association of Smt4p and presence of the DNA-binding SAP module in Siz1p, a bypass suppressor of *SMT4* deletion, also point to abnormal chromatin structure as the primary consequence of Smt4p depletion. It remains to be determined whether other chromosome processes, in addition to chromosome segregation and condensation, are affected by *SMT4* loss, including transcription, DNA repair, and meiotic recombination.

Smt4p functions as an AVP protease: In eukaryotic cells a variety of proteins are shown to be covalently modified by ubiquitin. The *S. cerevisiae* genome encodes a complex network of enzymes involved in this process (Hochstrasser *et al.* 1999). Remarkably, at least a dozen predicted proteases are involved in removal of ubiquitin from these conjugates (Chung and Baek 1999). The recently discovered small ubiquitin-like modifier, SUMO, appears to be a part of an equally complex regulatory and enzymatic network. *SMT3*, the yeast SUMO-encoding gene, is essential for cell viability (Johnson *et al.* 1997). In higher eukaryotes, there are several prominent examples of SUMO modification (Kretz-Remy and Tanguay 1999). It has been recently shown that in *S. cerevisiae*, the SUMO moiety is removed from modified proteins by Ulp1p, an essential protein

that has isopeptidase activity *in vivo* and *in vitro* (Li and Hochstrasser 1999). Ulp1p and Smt4p are members of the AVP family of cysteine proteases (Stephens *et al.* 1998). The AVP family is present only in eukaryotes (with the exception of two bacterial species, *Escherichia coli* and *Chlamydia trachomatis*) and eukaryotic DNA viruses and transposons (Figure 1). The nonviral eukaryotic enzymes of the AVP family, including Ulp1p and Smt4p, show a high level of sequence conservation in the protease domain, which suggests critical, conserved cellular functions. Here we report that the catalytic residues of the AVP protease domain of another yeast member of the family, Smt4, are essential for its *in vivo* function.

Smt4 is likely to be involved in SUMO metabolism *in vivo* as it has been shown to possess a SUMO-cleavage activity *in vitro* with a number of substrates (Li and Hochstrasser 2000). If Smt4p acts as a SUMO peptidase *in vivo* as well as *in vitro* (Li and Hochstrasser 2000), what determines its *in vivo* specificity? The fact that multiple proteins are SUMO modified suggests that a defect in the SUMO-modification pathway may have a catastrophic effect on a variety of cellular processes. *SMT4* disruption has a severe phenotype with a variety of lesions, affecting cell-cycle control, spindle morphology, and chromosome structure (this study; Li and Hochstrasser 2000), which is reminiscent of the phenotypes of the mutants of *SMT3* and genes encoding the SUMO-conjugating machinery components (Johnson and Blobel 1997; Johnson *et al.* 1997). Indeed, in *S. cerevisiae* disruption of *SMT3* is a lethal event.

The specific mechanism of *SMT4* involvement in the control of these processes still remains elusive. Particularly puzzling are the apparent antagonistic roles of Smt4p and Ulp1p in *S. cerevisiae* (Li and Hochstrasser 2000). A key to this antagonism may be provided by the observation that Smt4p and Ulp1p are compartmentalized—as we showed here, Smt4p is a chromatin protein, whereas Ulp1p is concentrated at the nuclear envelope (Li and Hochstrasser 2000). Thus, there is a distinct possibility that Ulp1p is preferentially involved in nuclear transport while Smt4p is a chromatin SUMO hydrolase. Suppression of *smc2* and *mif2* mutants by *SMT4* overexpression provides an additional genetic argument in favor of this hypothesis. The finding that some yeast proteins are modified by Smt3p in a *SMT4*-dependent manner (Li and Hochstrasser 2000) may suggest that Smt4p is a highly specialized SUMO protease that triggers a chain of cell-cycle events resulting in a complex pattern of SUMO modification. This makes identification of the primary substrates of Smt4p a high priority.

Is Siz1p a bridge between chromosome condensation and Smt4p activity? Siz1p has the same organization of conserved domains as PIAS proteins, inhibitors of STATs, which are transcription factors involved in a variety of cellular processes (Starr and Hilton 1999). The study of Zimp in Drosophila demonstrated that *P*-element insertion into the 5′-noncoding region and some excision alleles results in lethality of homozygous embryos but does not affect embryo patterning (Mohr and Boswell 1999). It is not, however, clear whether any of the *P*-element excision alleles are null alleles. It was also reported that mouse Miz1 activates transcription and binds DNA *in vitro* (Wu *et al.* 1997). Yet, biological functions of Zimp and Miz1 are not understood. The functional link of Siz1p-like proteins to transcription is not characterized in sufficient detail, raising the possibility that their interaction with the transcription machinery is fortuitous. Given the presence of the DNA-binding SAP module and the involvement of Miz fingers in protein-protein interactions, it appears likely that all these proteins function by sequence-specific DNA-binding coupled to interaction with other chromatin components.

An attractive hypothesis is that Siz1p, and possibly Siz2p, are targets of the Smt4p activity *in vivo*. It remains to be investigated biochemically whether these proteins are modified by SUMO (Smt3p) *in vivo* in an *SMT4*-dependent fashion. These experiments might also answer the question of whether the suppression of *smt4-Δ2* by *SIZ1* is due to Siz1p being one of the key substrates of Smt4p or is an indirect effect mediated by the excess of Siz1p at the level of its chromatin-associated function.

Indeed, as a DNA-binding protein Siz1p may be a *bona fide* player in condensin targeting to chromatin and SUMO-mediated regulation may provide a mitosis-specific control of this activity. Thus Siz1p may serve as a functional link between the Smt4p-specific branch of SUMO-modification machinery and higher order chromatin structure machinery, represented by condensin. The apparent chromatin hypercondensation and/or diminution phenotype of the *SIZ1/SIZ2* double deletion supports existence of such a link that involves the condensin as well as *SMT4*, *SIZ1*, and *SIZ2* genes. Additional screening for bypass suppressors of the double deletion of *SIZ1* and *SIZ2* and investigation of *SIZ1* and/or *SIZ2* mutants may allow identification of other components of this complex pathway.

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