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Sir Antagonist 1 (San1) Is a Ubiquitin Ligase*

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Mutations in *Sir Antagonist 1* (*SAN1*) suppress defects in *SIR4* and *SPT16* in *Saccharomyces cerevisiae*. San1 contains a RING domain, suggesting that it functions by targeting mutant sir4 and spt16 proteins for degradation by a ubiquitin-mediated pathway. Consistent with this idea, mutant sir4 and spt16 proteins are unstable in *SAN1* cells but are stabilized in *san1*Δ cells. We demonstrate that San1 possesses ubiquitin-protein isopeptide ligase activity *in vitro*, and the ubiquitin-protein isopeptide ligase activity of San1 is required for its function *in vivo*. Wild-type Sir4 has a half-life of about 21 min, and *san1*Δ increased Sir4 half-life to >90 min. In contrast, *san1*Δ did not affect the stability of wild-type Spt16, Sir3, Sir2, or the Spt16-associated proteins Pob3 and Nhp6. Loss of *SAN1* also did not affect the stability of Ste6-166, a highly unstable protein in yeast. These results support the idea that San1 controls the turnover of a specific class of unstable nuclear proteins. Sir4 nucleates the assembly of silent chromatin at telomeres and the silent mating-type loci (*HM*) in *S. cerevisiae*. Sir4 can also affect silencing in the rDNA indirectly by sequestering limiting Sir2. Increasing the stability of wild-type Sir4 by deleting *SAN1* had only subtle effects on silencing, suggesting that silent chromatin in yeast is robustly buffered against changes in Sir4 stability. Consistent with the idea that San1 participates as an accessory factor to regulate silent chromatin, including the silent mating-type loci, microarray analysis defined a small but statistically significant role for San1 in transcription of several mating pheromone-responsive genes.

Transcriptional silencing of specific chromosomal domains results from the assembly of repressive chromatin structures composed of histones, Sir proteins, and specific DNA-binding proteins that are required for Sir protein recruitment (1). Silent chromatin is also characterized by histone hypoacetylation and methylation in some organisms (2). There are three silenced chromatin domains in the yeast *Saccharomyces cerevisiae*. Silencing at *HML* and *HMR* requires Sir1–4, whereas telomeric silencing requires Sir2, Sir3, and Sir4 (3–7). Silencing of RNA polymerase II transcription at rDNA requires Sir2 but not the other Sir proteins (8, 9). The strength of rDNA silencing is determined by Sir2 protein levels, which are limiting in yeast (10). Consistent with this idea, although Sir4 is not required for rDNA silencing, elevated levels of Sir4 impair rDNA silencing by titrating limiting Sir2 away from rDNA (10). The competition for Sir2 among various silenced chromatin sites suggests that despite being stably maintained in a quiescent state, the constituents of silent chromatin are nonetheless in equilibrium.

Silencing involves distinct establishment and maintenance phases. Establishment of silencing at *HM* loci is under cell cycle control and requires the Sir1 protein (11, 12). Sir2, Sir3, and Sir4 are required for both the establishment and the maintenance of the silenced state (13). Telomeric silencing is also influenced by the cell cycle such that it is stronger during G_1/S and weaker during G_2/M (14). Interestingly, Sir3 and Sir4 are partially released from telomeres during G_2/M (15), and robust establishment of silencing at HMR is not achieved until cells fully traverse the cell cycle and enter the next G_1 phase (16). Transcriptional silencing is thought to result from the limited access of the transcriptional machinery to Sir-containing chromatin, which is generally inaccessible to DNA-modifying enzymes (17–20). Nonetheless, DNA packaged into silent chromatin can be accessed by replication, repair, and recombination machineries (see Ref. 1 for review). Additionally, a transcriptional activator and the RNA polymerase II general transcription machinery can bind to silenced chromatin along with Sir proteins (21). The mechanisms regulating access of silenced chromatin are poorly understood. However, two recent reports (22, 23) demonstrate that HP1, a major component of heterochromatin in vertebrate cells, is dynamically associated with chromatin *in vivo*, indicating that the repressive chromatin state in these cells does not result from generation of static, condensed DNA.

Mutations in *SAN1* can suppress mating defects in *sir4* cells (24). Although the suppression caused by loss of *SAN1* is not allele-specific, *san1*Δ cannot by-pass the requirement for Sir4 (24). Deletion of *SAN1* does not affect *SIR4* mRNA levels, suggesting that *SAN1* functions by a post-transcriptional mechanism (24). Mutations in *SAN1* also suppress a defect in *CDC68/SPT16* (25). Here we report that San1 is a RING domain-containing E3[1] ubiquitin ligase. Sir4 (wild-type and mutant protein) and mutant spt16 are turned over by a San1-dependent mechanism. These results describe a novel mechanism for turnover of unstable nuclear proteins in yeast and suggest that silent chromatin may be more dynamic than described previously.

EXPERIMENTAL PROCEDURES

Plasmids and Strains—Plasmids carrying the wild-type *SAN1* gene (pAD27), *san1-W269A* (pAD31), and *san1-C165A* (pAD32) were con-

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[1] The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; HA, hemagglutinin; 5-FOA, 5-fluoroorotic acid.

This paper is available on line at http://www.jbc.org

TABLE I
Yeast strains used in this study

Strain name	Genotype	Ref. or source
JS222	*MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-167 RDN1 (NTS1)::mURA3-HIS3*	8
YAD144	*MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-167 RDN1 (NTS1)::mURA3-HIS3 san1Δ::KAN*	This study
JS237	*MATα his3Δ200 leu2Δmet15Δ0 trp1Δ63 RDN1 (NTS2)::Ty1-MET15*	33
YAD145	*MATα his3Δ200 leu2Δmet15Δ0 trp1Δ63 RDN1 (NTS2)::Ty1-MET15 san1Δ::KAN*	This study
YCB647	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11*	J. Boeke
YCB652	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 ADH4::URA3-TEL sir2Δ::TRP1*	J. Boeke
YLS59	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hmrΔA::TRP1*	D. Shore
MC119	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hmrΔA::TRP1 sir2::HIS3*	D. Shore
YAD96	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hmrΔA::TRP1 san1Δ::KAN*	This study
YJB958	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hmrΔA::TRP1 cac1-Δ1*	J. Berman
YAD92	*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hmrΔA::TRP1 cac1-Δ1 san1Δ::KAN*	This study
YAD125	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 san1Δ::HIS3*	This study
YAD126	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hmr ΔA::TRP1 san1Δ::HIS3*	This study
YAD121	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 cac1Δ::KAN*	This study
YAD122	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 san1Δ::HIS3 cac1Δ::KAN*	This study
YAD123	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 cac2Δ::KAN*	This study
YAD124	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 san1Δ::HIS3 cac2Δ::KAN*	This study
PKY 090	*MAT***a** *leu2-3, 112 ura3-1 his3-11, 15 trp1-1 ade2-1 can1-100 adh4::URA3-TEL VIIL*	P. Kaufman
YAD102	*MAT***a** *leu2-3, 112 ura3-1 his3-11, 15 trp1-1 ade2-1 can1-100 adh4::URA3-TEL VIIL san1Δ::KAN*	This study
PKY993	*MAT***a** *leu2-3, 112 ura3-1 his3-11, 15 trp1-1 ade2-1 can1-100 adh4::URA3-TEL VIIL asf1Δ::TRP1*	P. Kaufman
YAD105	*MAT***a** *leu2-3, 112 ura3-1 his3-11, 15 trp1-1 ade2-1 can1-100 adh4::URA3-TEL VIIL asf1Δ::TRP1 san1Δ::KAN*	This study
YPH499	*MAT***a** *ura3-52 lys3-52 lys2-801*^{a} *ade 2-101*^{o} *trp1-Δ63 his 3-Δ200 leu2-Δ1*	P. Hieter
YAD128	*MAT***a** *ura3-52 lys3-52 lys2-801*^{a} *ade 2-101*^{o} *trp1-Δ63 his 3-Δ200 leu2-Δ1 san1Δ::KAN*	This study
YPH500	*MATα ura3-52 lys3-52 lys2-801*^{a} *ade 2-101*^{o} *trp1-Δ63 his 3-Δ200 leu2-Δ1*	P. Hieter
YAD130	*MATα ura3-52 lys3-52 lys2-801*^{a} *ade 2-101*^{o} *trp1-Δ63 his 3-Δ200 leu2-Δ1 san1Δ::KAN*	This study
YAD140	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 dot4Δ::KAN*	This study
YAD141	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 san1Δ::HIS3 dot4Δ::KAN*	This study
JRY50	*MAT***a** *sir4-9 his3-532 trp1-289*^{a} *ura3-52*	J. Rine
YAD50	*MAT***a** *sir4-9 his3-532 trp1-289*^{a} *ura3-52 san1Δ::KAN*	This study
YAD132	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 san1Δ::HIS3 ris1Δ::KAN*	This study
YAD131	*MAT***a** *his3Δ200 leu2Δ1 lys2Δ202 ura3-52 leu2Δ::TRP1 ADH4::URA3-TEL-V11 ris1Δ::KAN*	This study
YAD134	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hmrΔA::TRP1 san1Δ::HIS3 ris1Δ::KAN*	This study
YAD133	*MATα ade2-1 can1-100 his3-11,15 leu2-3, 112 trp1-1 ura3-1 hmrΔA::TRP1 ris1Δ::KAN*	This study
FY56	*MATα lys2-138δ ura3-52 his4-912δ*	F. Winston
L577	*MATα lys2-138δ spt16-197 ura3-52 his4-912δ*	F. Winston
FY56	*MATα lys2-138δ ura3-52 his4-912*	F. Winston
YAD142	*MATα lys2-138δ spt16-197 ura3-52 his4-912δ san1Δ::KAN*	This study
YAD143	*MATα lys2-138δ ura3-52 his4-912δ san1Δ::KAN*	This study
YAD118	*MATα leu2-2, ura3-1 his3-11, 15 trp1-1 ade2-1 can1-100 SAN1-TAP(URA3)CDC54::13XMYC (HIS3)*	This study

structed by insertion of PCR-generated alleles of *SAN1* into the HindIII and NotI sites of the *CEN ARS URA3* plasmid pRS316 (26). The fragments contain the entire *SAN1* open reading frame and 335 bp of 5′-flanking DNA. Plasmid pSIR4-URA carrying *SIR4* in pRS316 was provided by Dan Gottschling. pLP305 (2 μm *SIR4*) and pLP754 (2 μm *SIR4-42*) were described previously (10). A plasmid for bacterial expression of glutathione *S*-transferase-ubiquitin was provided by Jon Huibregtse, and plasmids expressing wheat E1 and human Ubc-H5B were provided by Allan Weissman. PCR was used to append five copies of the Myc epitope to the 3′ end of the full-length *SAN1* gene, which was carried on pRS316. The San1-myc fusion protein was detected using the Myc-specific 9E10 monoclonal antibody (1:5000 dilution). Yeast strains used in the study are listed in Table I. Strains were constructed by disruption of the respective genes by single-step gene replacement in the indicated strains using PCR-generated DNA disruption cassettes. Appropriate targeting of the disruption cassettes was confirmed by PCR or by standard genetic crosses and tetrad analysis. A strain bearing the temperature-sensitive allele *spt16-197* and the congenic wild-type *SPT16* strain were provided by Fred Winston (27). The *sir4-9* strain JRY50 (24) was provided by Rohinton Kamakaka.

Expression of Recombinant Proteins and Ubiquitination Assays—BL21 DE3 bacterial cells harboring plasmids for recombinant wheat E1 (28), Ubc-H5B (28), and full-length San1 were induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside at an A_{600} of 0.6-1.0 for 3 h at 37 °C. The cells were sonicated in 50 mM Tris-Cl (pH 8.0), 1 mM EDTA, 1% Triton X-100 using 1 ml of sonication buffer per 50 ml of induced cell culture. After sonication, the extract was clarified by centrifugation at 14,000 rpm for 30 min in a Sorvall SS34 rotor, and the supernatant was stored in small aliquots at −80 °C.

Ubiquitination assays were carried out using 20 ng of E1, E2, and/or E3 lysate in 50 mM Tris-Cl (pH 7.5), 0.2 mM ATP, 0.5 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 mM creatine phosphate, 15 units of creatine phosphokinase, and 1 μg of FLAG-tagged ubiquitin (Sigma) in a reaction volume of 30 μl (28, 29). The reactions were incubated for 1.5 h at 30 °C. Samples were boiled in protein gel loading buffer with dithiothreitol, resolved on 10% SDS-polyacrylamide gels, and analyzed by Western blotting using anti-FLAG M2 monoclonal antibodies (Sigma) at a dilution of 1:2000. Similar results were obtained when the assay was performed using radiolabeled glutathione *S*-transferase-ubiquitin and detection of reaction products by autoradiography (not shown; see Refs. 28 and 29).

For the experiment in Fig. 1*C*, the ubiquitination assays were carried out as above except that 2 μg of wild-type ubiquitin (FLAG-tagged), ubiquitin mutants having only Lys-6, Lys-48, or Lys-63 (Boston Biochem Inc.), or ubiquitin with all lysines mutated to arginine (Boston Biochem Inc.) were added to the reaction. The reactions were resolved on 8% SDS-polyacrylamide gels, and the conjugated products were detected by Western blotting using the Py (polyoma virus medium T) antibody (68) to detect the N-terminal epitope tag on San1.

Western Blotting and Analysis of Protein Turnover—To estimate the stability of specific proteins in the absence of new protein synthesis, wild-type and *san1*Δ cells were grown in YPD at 30 °C to an A_{600} of about 1.0. Cycloheximide was then added to a concentration of 0.5 mg/ml, and aliquots of the cultures were removed at the indicated times. Harvested cells were washed with 200 mM Tris-Cl (pH 8.0), 400 mM ammonium sulfate, 10 mM magnesium chloride, 1 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol, 0.3 μg/ml leupeptin, 2 μg/ml chymostatin, 1.4 μg/ml pepstatin, 1.25 mg/ml *N*-ethylmaleimide, and 10% glycerol. Cells were lysed in the same buffer using glass beads and vortexing with intermittent cooling on ice. Extract proteins were resolved on 8 or 10% polyacrylamide protein gels except for detection of Nhp6 (15% gels). Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes and probed with antibodies to Sir2 or Sir4 (Santa Cruz Biotechnology) or Sir3 (kindly provided by Lorraine Pillus). Sir2 antisera was used at a dilution of 1:1000, and Sir4 antisera were used at 1:100 in TBST (50 mM Tris-Cl (pH 7.5), 154 mM NaCl, 0.05% Tween 20, and 50 μl of antifoam A per liter) containing 5% nonfat dry milk, followed by incubation with anti-goat

secondary antibody (1:10,000; Santa Cruz Biotechnology). Santa Cruz Biotechnology Sir4 antisera directed against either the N or C terminus of Sir4 worked indistinguishably. Sir3 antisera were used as described previously (30). Spt16, Pob3, and Nhp6 were detected using antisera kindly provided by Tim Formosa and David Stillman. Spt16 antisera were used at a dilution of 1:10,000, Pob3 antisera at a dilution of 1:5000, and Nhp6 antisera at a dilution of 1:4000 in TBST containing 0.01% (α-Spt16) or 0.1% Tween 20 (α-Pob3, α-Nhp6) followed by incubation with anti-rabbit secondary antibody (1:5000, Amersham Biosciences). Plasmid-expressed HA-tagged Ste6-166 (31) was detected by using 12CA5 HA antisera at a dilution of 1:3000. Detection of all proteins utilized the ECL+ kit (Amersham Biosciences) as recommended by the manufacturer.

The amount of protein present at various times following cycloheximide addition was determined by densitometry, and kinetic analysis of protein stability was performed by fitting the data to exponential equations of the form $Y = \mathrm{M0} \times e^{\mathrm{M1} \times X}$ using Kaleidagraph software. First order rate constants for the decay were determined directly from the resulting equations and were used to determine the $t_{1/2}$ values. The quality of the fits was described by R values; the $t_{1/2}$ and R values for the Sir4 data are reported in Fig. 3 legend.

Metabolic Labeling—Metabolic labeling of yeast proteins was performed as described (31). Cells were grown in synthetic dextrose media (supplemented with appropriate amino acids) to an A_{600} of about 1.0. Ten A_{600} units of cells (total) were resuspended in 2 ml of synthetic dextrose media and incubated at 30 °C for 10 min with shaking; cells were then pulse-labeled using 300 μCi of Express ^{35}S-labeling mix (PerkinElmer Life Sciences) for 10 min at 30 °C. Specifically labeled proteins were detected by autoradiography of gel-fractionated immunoprecipitates obtained by using polyclonal antisera raised against the protein of interest or antibodies directed against particular epitope tags as indicated. Under these conditions, radiolabeled HA-tagged Ste6 expressed from a gene carried on a 2-μm plasmid (31) was readily detected (not shown). By using this protocol to detect Sir proteins (whose expression was driven by chromosomal copies of their genes), radiolabeled Sir2 and Sir3 were barely detectable, and radiolabeled Sir4 could not be detected by using either polyclonal antisera directed against untagged Sir4 (Santa Cruz Biotechnology) or by immunoprecipitation of TAP-tagged Sir4 (32) by using calmodulin beads. Metabolic labeling was used to determine the half-life of Ste6; the turnover of Ste6 was in good agreement with previously published data (31).

Yeast Methods—Mating assays were performed by growing cells in patches overnight on YPD plates at 30 °C. The patches were then replica-plated to either YPD or lawns of mating tester strains on synthetic dextrose plates. Plates were incubated for 2 days at 30 °C prior to being photographed. For testing telomeric silencing, a tester strain was used that has *URA3* located in the telomere on the left arm of chromosome VII (33, 34). Overnight cultures of cells were grown on YPD or selective medium, and serial 10-fold dilutions of each strain were spotted onto synthetic complete medium or medium containing 1 mg/ml 5-FOA. For testing *HM* silencing, strains were used in which *TRP1* is integrated at *HMR*, and cells were grown and serially spotted onto medium with or without tryptophan. Plates were photographed after incubation for 2 days at 30 °C. The *HM* tester strains used in Fig. 4 were derived from a strain missing the Rap1-binding site in *HMR* (*hmrΔE*::*TRP1*, see Ref. 35). Similar results were obtained by using *HM* tester strains with crippled *HMR* silencers because of deletion of either the origin recognition complex or Abf1-binding sites in *HMR* (not shown; see Refs. 35 and 36). Silencing tester strains with deletions of *CAC1*, *CAC2,* or *ASF1,* which have been described previously (37), were used to delete *SAN1* in them for the experiments shown in Fig. 4, *A* and *B*. For testing rDNA silencing, strains were used with *URA3* or *MET15* reporter genes integrated in rDNA (10). Where indicated, the *SAN1* gene was deleted by single step gene replacement, and silencing was assayed in Fig. 4*C* by spotting cells on synthetic media with or without uracil. To determine whether mutations in the San1 RING domain suppress a defect in *SPT16*, cultures of wild-type and mutant cells were grown in YPD or selective medium at 30 °C. 10-fold serial dilutions of cells were spotted on YPD or synthetic medium without uracil (to select for the *SAN1*-containing plasmid as indicated in the figure). Plates were incubated at 30 or 34 °C (as indicated in Fig. 2) for 2 days.

Microarray Analysis—Poly(A)$^+$ RNA was isolated from wild-type *SAN1* cells (YPH499) (26) and congenic *san1Δ* cells exactly as described previously (38), with the exception that cells were harvested at A_{600} ~1.0 in YPD media at 30 °C. Hybridizations to glass slide microarrays and data analyses were performed by the NIEHS Microarray Facility exactly as described previously (38); hybridizations were performed in triplicate, and the genes listed in Table II represent the total set of genes whose message levels were affected in a statistically significant manner when the data were analyzed at the 95% confidence level. The microarray data are available at dir.niehs.nih.gov/microarray/datasets/auble851.txt.

Isolation and Characterization of San1 in Yeast Whole Cell Extracts—For purification of San1, whole cell extracts were made from 4-liter cultures of yeast cells expressing TAP-tagged San1 (and in some cases Myc-tagged Mcm4) or the congenic strain with untagged San1. Cells were grown to mid log phase in yeast extract peptone media (YPD), and whole cell extracts were made in Buffer A (40 mM HEPES-KOH (pH 7.5), 350 mM NaCl, 10% glycerol, 0.1% Tween 20, 2 mM $CaCl_2$, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The extracts were clarified by centrifugation at 24,000 rpm for 2 h in an SW28 rotor in a Beckman ultracentrifuge. The extract was then incubated with 200 μl of pre-equilibrated calmodulin beads (Stratagene) for 2 h. The beads were then washed with 30 ml of extraction buffer, and five 200-μl elutions were performed with calmodulin elution buffer (10 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM magnesium acetate, 10 mM β-mercaptoethanol, 1 mM imidazole, 2 mM EGTA, 0.1% Nonidet P-40, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The peak fractions were identified by Western blotting with anti-protein A antibodies (Sigma) at a dilution of 1:50,000. The pooled material was then fractionated on a Superose-6 gel filtration column in Buffer A. Fractions were analyzed by Western blotting to identify the proteins indicated in Fig. 5. For the immunoprecipitation experiment shown in Fig. 5, whole cell extracts prepared as above from the tagged and untagged strains were incubated with calmodulin beads. The beads were washed with Buffer A as mentioned above and boiled in SDS-PAGE sample buffer, and TAP-tagged San1 or Myc-tagged Mcm4 was detected by Western blotting with anti-protein A (1:50,000 dilution) or anti-Myc 9E10 monoclonal antibodies (1:5000 dilution). Sir4 was detected as described above by using Sir4 antibodies (Santa Cruz Biotechnology).

RESULTS

San1 was reported to contain a RING domain (39) and has a core that is similar to other RING domain-containing proteins, although a glycine residue has replaced a cysteine at one of the putative zinc coordinating positions (Fig. 1*A*). To determine whether San1 can function as an E3 *in vitro*, recombinant San1 was added to reactions containing recombinant E1, E2, and ubiquitin. Under these conditions, many RING-containing E3s can catalyze covalent transfer of ubiquitin to proteins in the reaction even though the physiologically relevant substrate may not be present (29). As shown in Fig. 1*B*, San1 demonstrated robust E3 activity *in vitro* by using this assay. The activity of recombinant San1 was similar to that of the E3 AO7T described previously (29), although each RING domain protein catalyzes the formation of a distinct pattern of ubiquitinated species (Fig. 1*B, lane 4 versus 13*). An E2-RING domain co-crystal structure shows that an appropriately positioned hydrophobic residue is critical for the interaction (40). The analogous residue in San1 is Trp-269, and mutation of Trp-269 to alanine destroyed E3 activity *in vitro* for San1 (Fig. 1, *lane 4 versus 7*). Mutation of the putative zinc-coordinating residue Cys-165 to alanine also diminished the E3 activity of San1 *in vitro* (Fig. 1*B, lane 4 versus 10*). To characterize further the E3 activity of San1 and to determine whether San1 itself becomes ubiquitinated, the fate of San1 was monitored in reactions that were carried out in the presence of wild-type or mutant ubiquitins (Fig. 1*C*). San1 was ubiquitinated in reactions containing wild-type ubiquitin (Fig. 1*C, lane 8*), and it was also ubiquitinated by ubiquitin molecules that contained only lysine 6, lysine 48, or lysine 63 (Fig. 1*C, lanes 4–6*), although the distribution of ubiquitinated species was different using each of these ubiquitins. As expected, ubiquitin in which all of the lysines were mutated to arginine was not conjugated to San1, and the ubiquitination of San1 depended on addition of E1 and E2 enzymes. Ubiquitination of the RING domain containing E3s has been reported and may be a general property of these proteins in this assay (29).

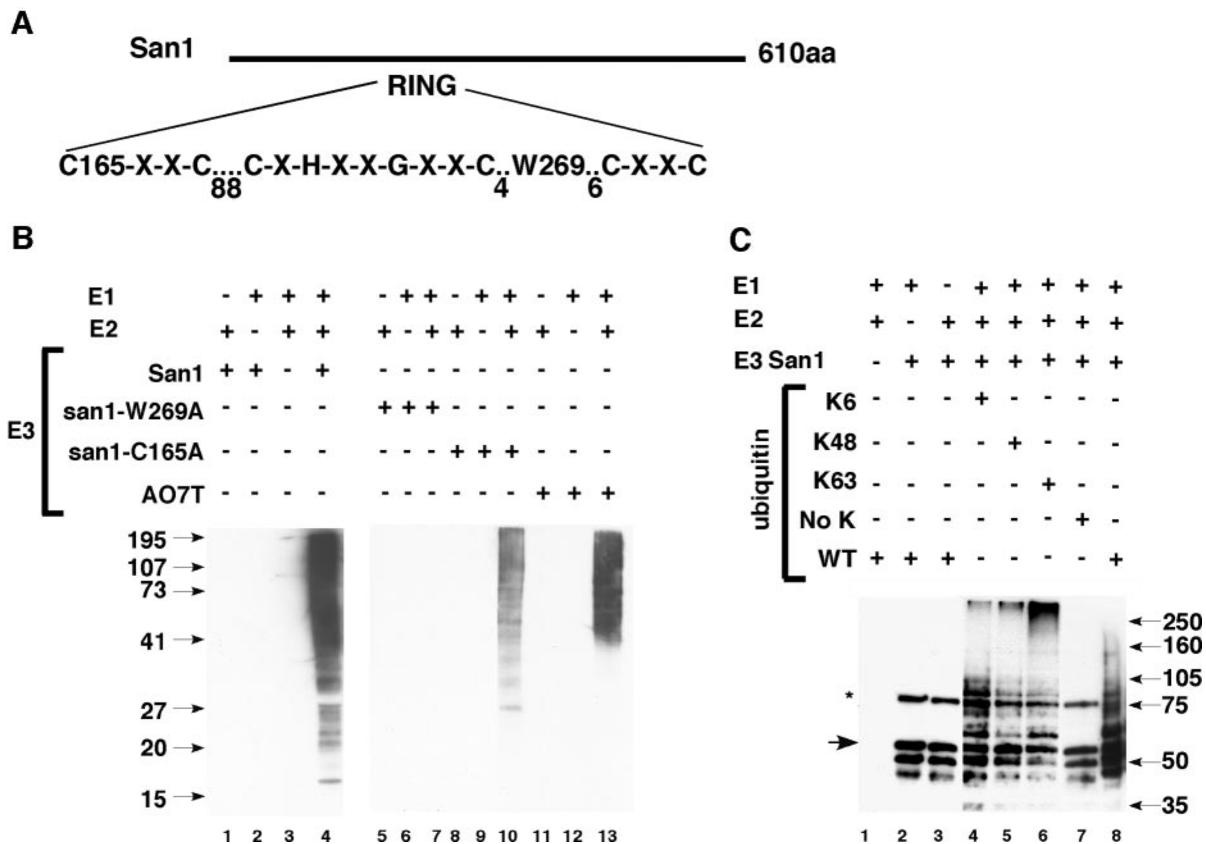

FIG. 1. **Recombinant San1 possesses E3 activity *in vitro*.** *A*, *top schematic* shows the residues that comprise the RING domain. *B*, bacterial lysates containing recombinant E1, E2, and/or recombinant San1 were added to reactions containing wild-type ubiquitin (FLAG-tagged). Reactions in *lanes 1*, *2*, and *4–10* contained equivalent amounts of wild-type or mutant San1 as judged by immunoblotting. For comparison, the reactions in *lanes 11–13* contained bacterial extract from cells expressing the previously characterized ubiquitin ligase AO7T (29). The reactions were resolved by SDS-PAGE under reducing conditions, and ubiquitin conjugates were detected by Western blotting with the FLAG antibody. *Lanes 5–13* were exposed five times longer than *lanes 1–4*. *C*, bacterial lysates containing recombinant E1, E2, and/or recombinant San1 were added to reactions containing wild-type ubiquitin, ubiquitin with only a single lysine (Lys-6, Lys-48, or Lys-63 as indicated), or ubiquitin with lysines mutated to arginine (*No K*). The reactions were resolved by SDS-PAGE as in *B,* and the ubiquitin-conjugated San1 species were detected by Western blotting by using the Py (polyoma virus medium T) monoclonal antibody (68), which recognizes the N-terminal epitope tag on San1. The *arrow* indicates the size of full-length unmodified San1. The *asterisk* denotes a nonspecific band.

There are no known phenotypes associated with *san1*Δ in otherwise wild-type cells (24, 25). To determine whether the E3 activity of San1 correlated with its *in vivo* function, wild-type and mutant alleles of *SAN1* were introduced into *sir4-9* and *spt16-197* strains to determine whether alleles of *SAN1*-encoding RING mutations can suppress the mating or growth defects caused by *sir4* or *spt16*, respectively, as has been described for other *san1* strains (24, 25). *san1-W269A* and *san1-C165A* behaved indistinguishably from *san1*Δ in *sir4-9* cells (Fig. 2*A*) and *spt16-197* cells (Fig. 2, *B* and *C*). Most important, wild-type San1, san1-C165A, and san1-W269A were all comparably expressed in yeast (Fig. 2*D*). These results indicate that the E3 activity of San1 is required to antagonize Sir4 and Spt16 function *in vivo*.

To determine whether San1 antagonizes Sir4 and Spt16 function by targeting them for degradation, extracts were prepared from wild-type and *san1*Δ cells, and protein levels were determined by Western blotting. Deletion of *SAN1* caused less than a 2-fold change in steady-state levels of Sir4 and Spt16 (Fig. 3 and data not shown). There was also little or no detectable change in steady-state levels of Sir4-associated proteins Sir2 and Sir3 or Spt16-associated proteins Pob3 and Nhp6. To determine whether protein turnover was affected by San1, cycloheximide was added to cells to inhibit protein synthesis, and whole cell extracts were prepared at different times following cycloheximide addition. As shown in Fig. 3, *A* and *B*, Sir2 and Sir3 are very stable proteins with no detectable change in steady-state protein levels 2 h after cycloheximide addition. Remarkably, and in sharp contrast to Sir2 and Sir3, Sir4 was rapidly degraded in wild-type cells following inhibition of protein synthesis (Fig. 3*C*; half-life of ~21 min). Deletion of *SAN1* stabilized Sir4 (Fig. 3*C*; half-life >90 min), indicating that San1 participates in Sir4 turnover *in vivo*. Spt16, Pob3, and Nhp6 protein levels were monitored in the same way (Fig. 3*E*); the results indicate that all three of these wild-type proteins are very stable proteins, and *SAN1* deletion did not affect their stability. Similarly, deletion of *SAN1* did not affect the stability of Ste6-166, a very unstable protein (Fig. 3*F*) (31). In *sir4-9* cells, sir4 protein was nearly undetectable, but deletion of *SAN1* increased the levels of mutant Sir4 (Fig. 3*D*). Stabilization of sir4-9 by deletion of *SAN1* can explain the suppression of the *sir4-9* mating defect (Fig. 2). Similarly, spt16-197 was rapidly degraded following cycloheximide addition, but deletion of *SAN1* stabilized the protein. The Spt16-associated Pob3 protein was also destabilized in *spt16-197* cells, and Pob3 levels were stabilized in *spt16-197 san1*Δ cells (Fig. 3*E*). Stabilization of spt16-197 in the *san1*Δ strain is consistent with results published previously (41) and can explain how loss of San1 suppresses the conditional growth defect in the *spt16-197* strain; the effects on Pob3 could result from the fact that Spt16 and Pob3 form a tight complex (42). Thus, San1 controls the turnover of a subset of yeast nuclear proteins, including wild-type and mutant Sir4 and mutant spt16.

To determine whether changes in Sir4 half-life affect silenc-

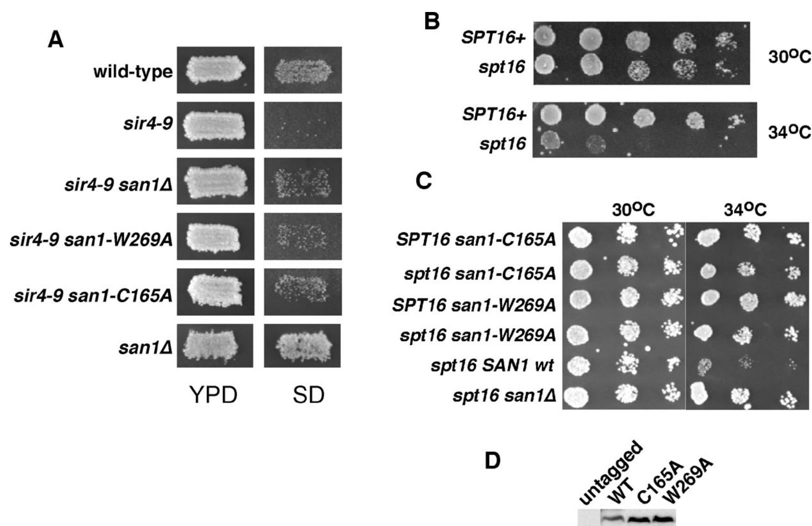

FIG. 2. **San1 RING domain is required for function *in vivo*.** *A*, mutations in the San1 RING domain suppress *sir4-9* mating defects. *MAT**a** sir4-9 san1*Δ cells carrying wild-type *SAN1* or mutant *san1* alleles on low copy *URA3*-marked plasmids were grown as patches on synthetic complete media lacking uracil. The patches were then replica-plated onto YPD (*left column*) and onto a lawn of *MAT*α mating type tester cells on a synthetic dextrose plate (*right column*). The wild-type strain was made by introduction of plasmid-borne wild-type *SIR4* into the *sir4-9* strain. Wild-type congenic (*SIR4 SAN1*) and *san1*Δ strains were included as controls. *B*, temperature-sensitive growth defect in the *spt16* strain. *SPT16* or *spt16-197* strains were spotted in 10-fold serial dilutions on YPD plates and incubated at 30 or 34 °C. *C,* mutations in the San1 RING domain suppress a defect in *SPT16*. Cultures of *SPT16* or *spt16-197* strains harboring the indicated plasmid-borne *SAN1* alleles were spotted on synthetic media minus uracil and incubated at 30 or 34 °C. Growth of the congenic *spt16-197 san1*Δ strain is shown for comparison. *D,* wild-type and mutant San1 proteins were expressed at equivalent levels in yeast. Western analysis was performed using whole cell extracts from cells harboring each of the indicated Myc-tagged San1 alleles expressed under control of the *SAN1* promoter.

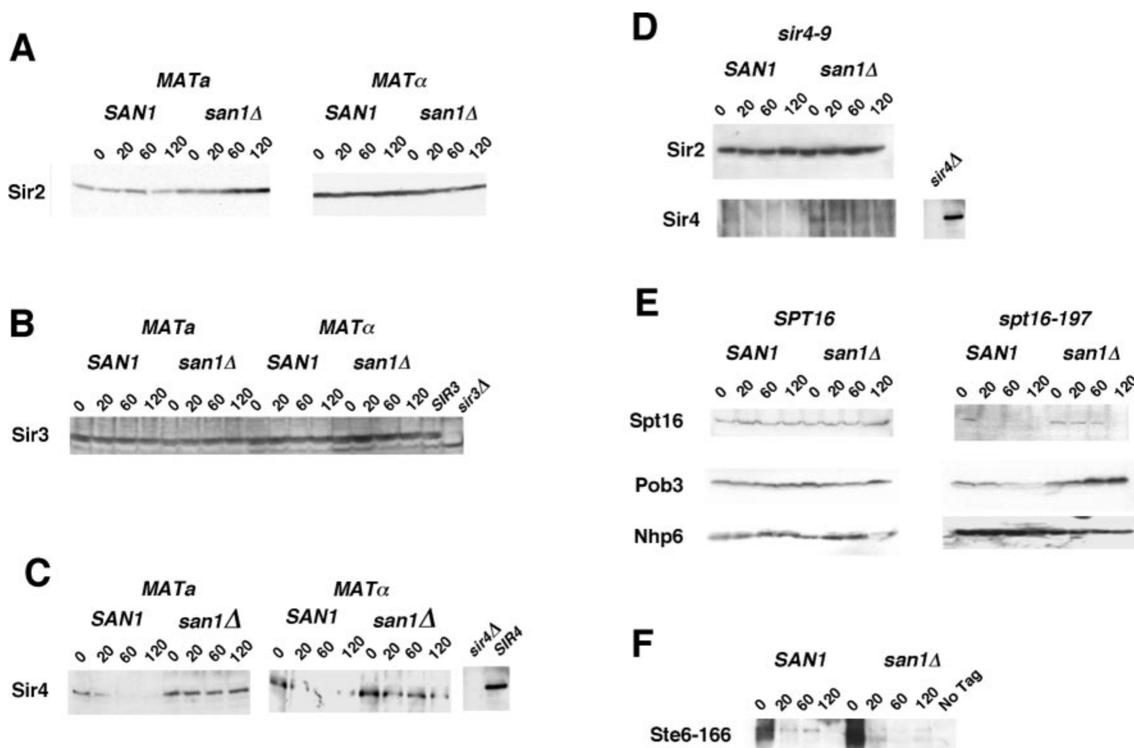

FIG. 3. **Effect of San1 on protein stability.** In all panels, cycloheximide (0.5 mg/ml) was added to logarithmically growing cultures of the indicated strains at time 0, and cells were harvested subsequently at the times indicated in minutes *above* each lane. Whole cell extracts were prepared, and the levels of the indicated proteins were detected by Western blotting (see "Experimental Procedures"). The blots in *A–C* and the *left-hand panel* of *E* were performed with the same batches of extracts. *A*, Sir2 stability in *MAT**a*** or *MAT*α *SAN1* and *san1*Δ strains. In the experiment shown, *MAT**a** san1*Δ cells appear to have more Sir2 at steady state than *MAT**a** SAN1* cells, but this was not reproducible. *B*, Sir3 levels in cells as performed in *A*. *C*, Sir4 stability in cells as performed in *A*. Note the disappearance of Sir4 in wild-type cells treated with cycloheximide and the marked stabilization of Sir4 in *san1*Δ cells of either mating type. Fitting the data to a single exponential (see "Experimental Procedures") yielded a $t_{1/2}$ for Sir4 in *MAT**a** SAN1* cells of 23 min (R = 0.99), and the $t_{1/2}$ for Sir4 in *MAT*α *SAN1* cells was 18.2 min (R = 0.999). The $t_{1/2}$ for Sir4 in *MAT**a** san1*Δ cells was 95 min (R = 0.88), and the $t_{1/2}$ for Sir4 in *MAT*α *san1*Δ cells was 182 min (R = 0.97). *D*, stability of Sir2 (*top panel*) and sir4-9 (*bottom panel*). sir4-9 was nearly undetectable in *SAN1* cells but was detected in the *san1*Δ strain. *E*, stability of Spt16, spt16-197, Pob3, and Nhp6 in *SPT16* or *spt16-197* cells. *F*, levels of Ste6-166 were monitored in *SAN1* and *san1*Δ cells by using anti-HA antibodies that recognize the HA-tagged Ste6-166 protein (31).

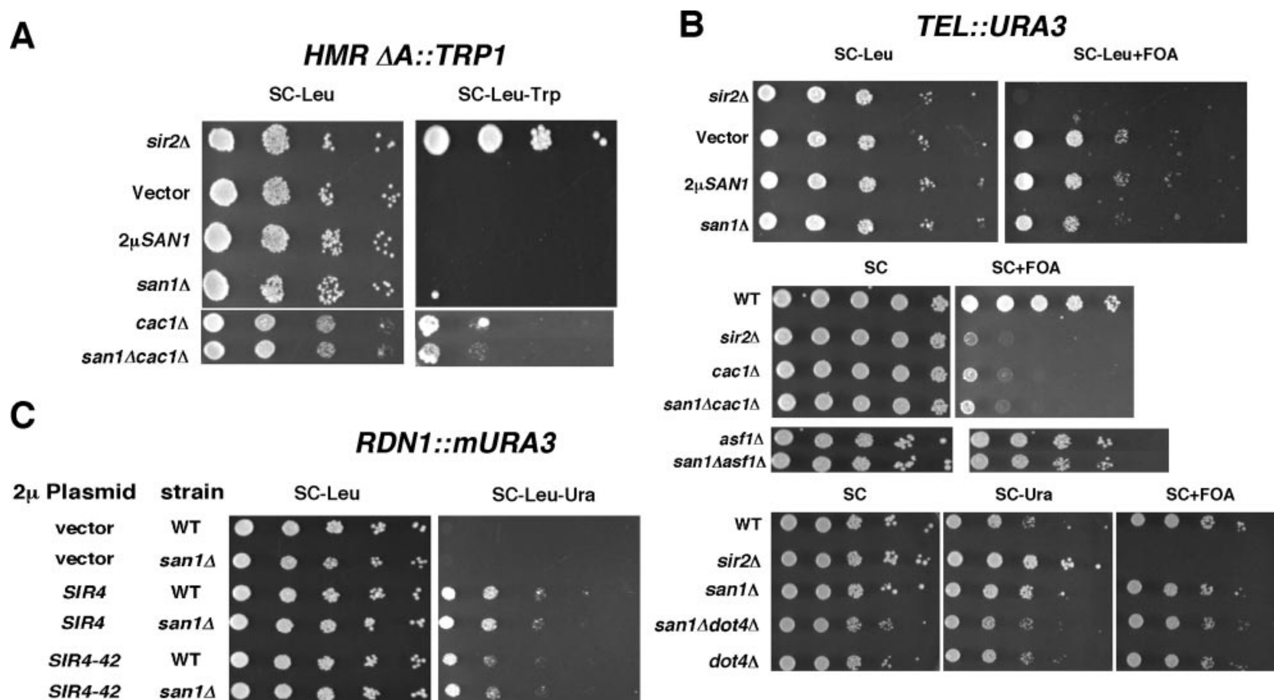

FIG. 4. **Little or no effect of high copy *SAN1* or *san1*Δ on silencing at *HMR*, telomeres, or rDNA.** *A*, growth of *HMR*::*TRP1* strains with the indicated gene deletions or high copy *SAN1* were compared by spotting serial dilutions of cells onto synthetic complete (*SC*) media or SC media without tryptophan. Loss of silencing at *HMR* results in expression of the *TRP1* reporter and allows cells to grow in media without tryptophan; strengthened silencing would give rise to poorer growth. Leucine was omitted from the media to select for the appropriate plasmid (2 μm *SAN1* or vector). *B*, growth of *TEL*::*URA3* strains with the indicated gene deletions or high copy *SAN1* plasmid marked with *LEU2* were compared by spotting serial dilutions of cells onto SC media with or without FOA. All strains grew equivalently on SC media, but loss of telomeric silencing resulted in de-repression of the telomeric *URA3* gene and growth inhibition on FOA. Enhanced silencing would give rise to better growth on FOA-containing media. *C*, growth of *RDN1*::*URA3* strains with wild-type *SAN1* or *san1*Δ. The strains were transformed with *LEU2* plasmid vector pRS426 (67) or high copy plasmids expressing full-length *SIR4* or the dominant *SIR4* allele, *SIR4-42* (10, 46). Strains grew equivalently on SC media without leucine, whereas impairment of rDNA silencing allowed better growth on SC media without leucine and uracil. Similar results were seen using strains with a *MET15* reporter integrated in the rDNA (10) (not shown).

ing, *SAN1* gene dosage was altered in yeast silencing tester strains. There was no detectable effect of *SAN1* deletion or high copy *SAN1* (2 μm plasmid) on *HM* silencing (Fig. 4*A*). This is largely consistent with previous results demonstrating no effect of *san1*Δ on *HM* silencing and a small effect of high copy *SAN1* only when using one particular *HM* silencing reporter strain (24). There was also no detectable effect of high copy *SAN1* on telomeric silencing (Fig. 4*B*). Deletion of *SAN1*, however, resulted in a subtle but reproducible loss (~5-fold) in telomeric silencing (Fig. 4*B*). The *TEL*::*URA3 san1*Δ cells also formed larger colonies than the *TEL*::*URA3 SAN1* cells on media without uracil (Fig. 4*B*). Loss of *SAN1* did not detectably affect the partial loss of silencing observed in *cac1*Δ or *cac2*Δ cells nor was silencing affected when *san1*Δ was combined with *asf1*Δ (37) (Fig. 4, *A* and *B,* and data not shown). These results indicate that stabilization of Sir4 does not strengthen silencing even when silencing is partially defective. Dot4 is a ubiquitin-processing protease (43) that was identified as a high copy disrupter of silencing at telomeres, *HM* loci, and rDNA (44). Dot4 interacts with Sir4, and *dot4*Δ cells have a reduced level of Sir4 (43), suggesting that Dot4 could antagonize the E3 activity of San1 by cleaving ubiquitin from Sir4. Deletion of *DOT4* caused a slight growth defect but no detectable effect on silencing. Deletion of *SAN1* did not suppress the *dot4*Δ growth defect, and the *san1*Δ *dot4*Δ strain had no detectable silencing defect (Fig. 4B).

Although Sir4 is not required for rDNA silencing, increased levels of Sir4 partially inhibit rDNA silencing by sequestering limiting Sir2 (10). Loss of Sir4 also results in redistribution of Sir3 to the nucleolus (45). We therefore considered the possibility that changing the rate of Sir4 turnover might affect rDNA silencing by altering the steady-state amount of Sir2 available for silencing at rDNA. Deletion of *SAN1* did not affect rDNA silencing even in cells with a partial loss of rDNA silencing resulting from high copy *SIR4* or the dominant allele of *SIR4*, *SIR4-42* (10, 46) (Fig. 4*C*). There was also no effect of high copy *SAN1* on rDNA silencing in this system (not shown). The high rate of Sir4 protein turnover suggests that Sir4-containing chromatin is surprisingly dynamic, but these silencing results indicate that the silenced state is strongly buffered against changes in the stability of one of its essential components.

As *san1*Δ stabilizes at least two proteins involved in regulating chromatin structure, it was possible that insight into the global role of San1 could be obtained by comparing the gene expression profiles in wild-type *SAN1* and *san1*Δ cells. Genes whose expression was affected by deletion of *SAN1* were identified by co-hybridization of poly(A)$^+$ RNA from wild-type and *san1*Δ cells exactly as we reported previously (38) (see "Experimental Procedures"). Affected genes were defined based on statistical treatment of the data obtained from three independent hybridization experiments; the genes identified are shown in Table II along with the magnitudes of the transcriptional defects (±S.D.) for data analyzed at the 95% confidence level. Transcription of only seven genes was found to be affected in a statistically significant manner when *SAN1* was deleted, and the transcriptional effects for all seven genes were rather modest. When data were analyzed at the more stringent 99% confidence level, only three genes were found to be affected by loss of *SAN1*: *YLL034C*, *PRM7,* and *YLL053C.* Thus, *SAN1* does not play a large role in transcriptional control. Interestingly, most of the genes affected by *san1*Δ are affected transcription-

TABLE II
Gene expression affected by san1D

Gene	-Fold change[a] (*san1Δ[ρ] versus SAN1*)	S.D.
YLL034C	3.0	±0.2
HSP12	1.5	±0.2
PEX7	1.4	±0.1
YDL037C	0.7	±0.1
PRM7	0.7	±0.1
YDL038C	0.7	±0.1
YLL053C	0.6	±0.1

[a] Data were analyzed at the 95% confidence level.

ally by mating pheromone (*YLL034C, HSP12, YDL037C,* and *YLL053C*; see Ref. 47) or are involved in the pheromone response pathway (*PRM7*). These transcriptional effects may be related in some way to the effect of San1 on Sir4 turnover. *PRM7, YDL038C,* and *YDL037C* are adjacent to one another on chromosome IV; perhaps they are coordinately regulated by virtue of their physical location.

To determine whether San1 functions alone or in combination with other proteins, native TAP-tagged San1 was isolated from yeast whole cell extracts. As shown in Fig. 5*A*, San1 present in yeast whole cell extracts (*upper panel*) or after affinity purification using the TAP tag (*middle panel*) migrated similarly when fractionated on a Superose 6 gel filtration column. The peak of immunoreactive San1 eluted in fractions ~25–27, corresponding to an apparent native molecular mass of 400–500 kDa. This suggests either that San1 multimerizes in a discrete fashion or that it is stably associated with other polypeptides. As shown in the *lower panel* of Fig. 5*A*, there was no detectable Sir4 in the affinity-purified San1. In the high throughput study of Ho *et al.* (48), Cdc54/Mcm4 was identified as a San1-associated protein. To determine whether Cdc54/Mcm4 is present in the purified San1 complex, the affinity purification of TAP-tagged San1 was repeated by using a strain that also contained Myc-tagged Cdc54/Mcm4. As shown in Fig. 5*B*, whereas Cdc54/Mcm4 in whole cell extracts eluted in fractions overlapping the profile for San1, there was no detectable Cdc54/Mcm4 in the Superose 6 fractions that contained the affinity-purified San1. Additionally, there was no detectable Cdc54/Mcm4 in San1-TAP immunoprecipitates performed using yeast whole cell extracts (Fig. 5*C*). Thus, Cdc54/Mcm4 is not stably associated with San1 under these conditions. Further characterization of the San1 complex will be the subject of future work.

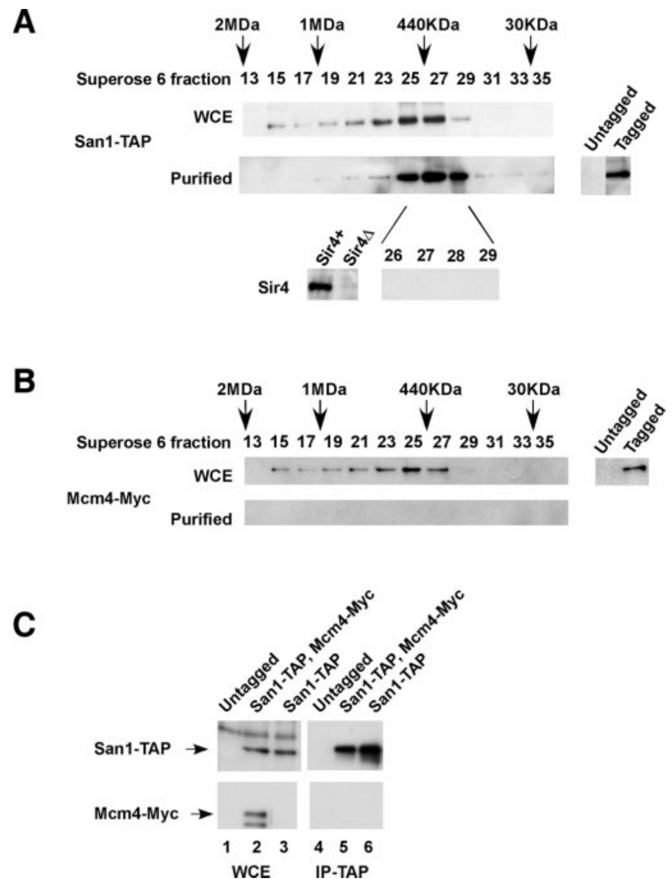

FIG. 5. **Native San1 exists in a discrete 400–500-kDa complex but is not stably associated with Sir4 or Mcm4.** *A*, yeast whole cell extracts (*WCE*) were prepared from a strain expressing TAP-tagged San1 and Myc-tagged Mcm4 or from a congenic wild-type strain with untagged San1, and were purified over calmodulin beads that recognize the TAP tag. As shown by Western blotting, the San1 protein was detected in the affinity-purified material (lane labeled *tagged*), whereas there was no immunoreactivity detected in a mock purification by using extract from cells with untagged San1 (lane labeled *untagged*). Whole cell extract or the affinity-purified protein was subjected to Superose 6 gel filtration chromatography. Western blotting of the Superpose 6 fractions was performed with antibodies against the protein A portion of the TAP tag. The *number above each lane* corresponds to the fraction number. The peak of San1 protein was present in fractions 25–27 in whole cell extracts (*upper panel*), and affinity-purified San1 eluted very similarly (*middle panel*). Western blotting of Superpose 6 fractions was performed with polyclonal antibodies against untagged Sir4; there was no detectable Sir4 in the affinity-purified San1-containing material (*lower panel*). *B*, Western blotting of the Superpose 6 fractionated material with monoclonal antibodies against the Myc tag to detect Mcm4. Western analysis of whole cell extracts containing untagged or tagged Mcm4 is indicated. Note that while Mcm4 in whole cell extracts eluted in fractions 15–27 (*upper panel*), there was no detectable Mcm4 in the affinity-purified San1 preparation (*lower panel*). *C*, extracts from strains expressing untagged or TAP-tagged San1 and untagged or Myc-tagged Mcm4 (as indicated above the panels) were incubated with calmodulin beads to immunoprecipitate TAP-tagged San1 and associated proteins. The immunoprecipitated material was analyzed by Western blotting to detect the proteins indicated by the *arrows.* No Mcm4-myc was detected in the San1-TAP immunoprecipitate (*IP*) (*lane 5*), indicating that the proteins do not stably co-associate under these conditions.

DISCUSSION

San1 was previously identified as a suppressor of a defective allele of *SIR4* (24) and independently as a suppressor of a defect in *SPT16/CDC68* (25). Although it had been argued that San1 functions by a post-transcriptional mechanism (24), the specific function of San1 was unknown. Sequence analysis indicated that San1 contains a RING domain (39), suggesting that it might function as a ubiquitin ligase (49). Biochemical analysis supports this idea as recombinant San1 has robust E3 activity *in vitro*, on par with the activity of the previously characterized E3 A07T (Fig. 1) (29). Additionally, mutations in the San1 RING domain that impair E3 activity *in vitro* cripple its function *in vivo* (Fig. 2). Deletion of *SAN1* results in some stabilization of mutant sir4 and spt16, offering an explanation for why *san1Δ* suppresses these defects. The suppression of the *sir4-9* mating defect by *san1Δ, san1-W269A*, and *san1-C165A* was only partial, probably because sir4-9 has a biochemical defect that cannot be overcome by simply elevating the level of the protein. In addition, there may be other pathways that are also responsible for turnover of sir4-9. Another allele of *SPT16, cdc68-1*, was shown previously (41) to be stabilized by a mutant version of *SAN1, san1-3*. Xu *et al*. (50) reported that most aspects of the *cdc68-1* mutant phenotype could also be reversed by mutations in the proteasomal subunit Sug1, again supporting the role for a ubiquitin-mediated pathway regulating turnover of cdc68-1/spt16. As there are no known mechanistic links between Sir4 and Spt16, one possibility is that San1 functions as a general co-factor for directing turnover of unstable proteins in the nucleus. The fact that *san1Δ* has no effect on the

turnover of the endoplasmic reticulum protein Ste6-166 and that deletion of *SAN1* alone has no known phenotypes suggests that San1 directs the turnover of a subset of short lived proteins *in vivo*, and the San1 pathway is either functionally redundant with another turnover pathway or the cell has the capacity to be buffered against changes in the turnover of San1-targeted substrates.

Remarkably, we find that the half-life of wild-type Sir4 protein is affected by *san1*Δ. This conclusion is based on the determination of protein stability in cycloheximide-treated cells. This approach was employed because it was not possible to detect Sir4 by metabolic labeling by using two different approaches (see "Experimental Procedures"), and previously published work has shown that there is excellent agreement between the results obtained by cycloheximide block and metabolic labeling protocols (51) (see under "Experimental Procedures"). There are wide variations in the mating efficiencies of various wild-type yeast strains; differences in the half-life of Sir4 in different strain backgrounds might account for some of this variability. Other links between silencing and ubiquitin have been described previously. For instance, the ubiquitin-conjugating enzyme Rad6 is required for all three types of silencing in *S. cerevisiae* (9, 52). The ubiquitin-conjugating activity of Rad6 is required for its silencing function (52, 53), which is mediated via ubiquitination of histone H2B in budding yeast (54, 55). A defect in the *Schizosaccharomyces pombe* homolog of *RAD6, rhp6*, causes derepression of the silent donor loci and an increase in chromatin accessibility (56). Consistent with these observations, the deubiquitinating enzyme Ubp3 physically associates with Sir4 and inhibits silencing at telomeres and *HML* (57). These results suggest collectively that ubiquitin modification of H2B (and perhaps other chromatin-associated proteins) contributes to the silenced state by affecting chromatin conformation, and this modification may confer a signaling function rather than leading to protein degradation as in the San1 pathway.

In addition to testing for genetic interactions between *san1*Δ and *cac1*Δ, *cac2*Δ, *asf1*Δ, and *dot4*Δ (Fig. 4*B*), we considered the possibility that San1 function is redundant with the RING domain-containing protein Ris1 which has been implicated in regulation of silent chromatin (58). However, no synthetic effect on silencing was observed in *san1*Δ *ris1*Δ cells (data not shown). Why does altered San1 dosage have little or no detectable effect on silencing? The observation of relatively rapid turnover of Sir4 in wild-type cells suggests that stable, continuous binding of Sir4 to silent chromatin is not required to maintain the silenced state. Loss of San1, and the consequent stabilization of Sir4, would have no effect on silencing if silencing strength were already set at a maximum level by some steps in the assembly/disassembly pathway for silent chromatin that is separate from steps requiring loading or activity of Sir4. Likewise, maintenance of the silenced state may be governed by a step that is mechanistically uncoupled from Sir4. Alternatively, the half-life of a pool of chromatin-associated Sir4 may be different from the half-life of "bulk" Sir4 measured in these experiments. It is remarkable that whereas *san1*Δ effects a large change in Sir4 half-life, the steady-state levels of Sir4 are only modestly affected by deletion of *SAN1*. San1 does not control *SIR4* transcription (Table II, Ref. 24) suggesting that there is a post-transcriptional mechanism for maintaining steady-state Sir4 protein levels despite variations in Sir4 half-life.

One possibility is that the silenced chromatin state is buffered against changes in Sir4 half-life by hypoacetylation of histone tails, which provide a surface for interaction with Sir complexes. Histone hypoacetylation is correlated with silencing, and the NAD-dependent histone deacetylase activity of Sir2 is required for its silencing function (see Refs. 1 and 59 for review). By using a hyperacetylated chromatin template, Sir2 was found to possess potent NAD-dependent transcriptional repression activity and to alter chromatin conformation *in vitro* in the absence of other Sir proteins (60). Similarly, recombinant Sir3 alone can direct the assembly of nucleosomal arrays into conformationally distinct supramolecular assemblies (61). Thus, dynamic association of Sir4 with chromatin need not imply loss of the silenced state. Two studies reported recently (22, 23) that heterochromatin protein 1 (HP1) is highly mobile in mammalian cells and only transiently associated with heterochromatin. The dynamic nature of *S. cerevisiae* silent chromatin suggested by the rapid turnover of Sir4 reported here is consistent with the dynamic nature of HP1 association with heterochromatin in mammalian cells, and the transient association of HP1 or other components of silent chromatin may in turn be regulated by proteolysis. Proper stoichiometry of silent chromatin components is critical for the silenced state (62), with overexpression of Sir proteins or other silencing factors causing reduced silencing (10, 37, 43, 63–65). The function for Sir4 instability is unknown, but the ability to combine changes in Sir4 half-life with changes in the activities of other regulators of silent chromatin could provide a rapid mechanism for controlling the establishment or maintenance of the silenced state. The notion of silent chromatin dynamics dictated by Sir4 instability may also be important for allowing switching between otherwise stable epigenetic states by contributing to nucleosome dynamics resulting from processes including histone modification and replacement (66).

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REFERENCES

1. Moazed, D. (2001) *Mol. Cell* **8,** 489–498
2. Richards, E. J., and Elgin, S. C. R. (2002) *Cell* **108,** 489–500
3. Haber, J. E., and George, J. P. (1979) *Genetics* **93,** 13–35
4. Klar, A. J. S., Fogel, S., and Macleod, K. (1979) *Genetics* **93,** 37–50
5. Rine, J., Strathern, J. N., Hicks, J. B., and Herskowitz, I. (1979) *Genetics* **93,** 877–901
6. Aparicio, O. M., Billington, B. L., and Gottschling, D. E. (1991) *Cell* **66,** 1279–1287
7. Strahl-Bolsinger, S., Hecht, A., Luo, K., and Grunstein, M. (1997) *Genes Dev.* **11,** 83–93
8. Smith, J. S., and Boeke, J. D. (1997) *Genes Dev.* **11,** 241–254
9. Bryk, M., Banerjee, M., Murphy, M., Knudsen, K. E., Garfinkle, D. J., and Curcio, M. J. (1997) *Genes Dev.* **11,** 255–269
10. Smith, J. S., Brachmann, C. B., Pillus, L., and Boeke, J. D. (1998) *Genetics* **149,** 1205–1219
11. Pillus, L., and Rine, J. (1989) *Cell* **59,** 637–647
12. Miller, A. M., and Nasmyth, K. A. (1984) *Nature* **312,** 247–251
13. Ivy, J. M., Klar, A. J., and Hicks, J. B. (1986) *Mol. Cell. Biol.* **6,** 688–702
14. Aparicio, O. M., and Gottschling, D. E. (1994) *Genes Dev.* **8,** 1133–1146
15. Laroche, T., Martin, S. G., Tsai-Pflugfelder, M., and Gasser, S. M. (2000) *J. Struct. Biol.* **129,** 159–174
16. Lau, A., Blitzblau, H., and Bell, S. D. (2002) *Genes Dev.* **16,** 2935–2945
17. Gottschling, D. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 4062–4065
18. Loo, S., and Rine, J. (1994) *Science* **264,** 1768–1771
19. Singh, J., and Klar, A. J. (1992) *Genes Dev.* **6,** 186-196
20. Wallrath, L. L., and Elgin, S. C. R. (1995) *Genes Dev.* **9,** 1263–1277
21. Sekinger, E. A., and Gross, D. S. (2001) *Cell* **105,** 403–414
22. Cheutin, T., McNairn, A. J., Jenuwein, T., Gilbert, D. M., Singh, P. B., and Misteli, T. (2003) *Science* **299,** 721–725
23. Festenstein, R., Pagakis, S. N., Hiragami, K., Lyon, D., Verreault, A., Sekkali, B., and Kioussis, D. (2003) *Science* **299,** 719–721
24. Schnell, R., D'Ari, L., Foss, M., Goodman, D., and Rine, J. (1989) *Genetics* **122,** 29–46

25. Xu, Q., Johnston, G. C., and Singer, R. A. (1993) *Mol. Cell. Biol.* **13,** 7553–7565
26. Sikorski, R. S., and Hieter, P. (1989) *Genetics* **122,** 19–27
27. Malone, E. A., Clark, C. D., Chiang, A., and Winston, F. (1991) *Mol. Cell. Biol.* **11,** 5710–5717
28. Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) *Cell* **75,** 495–505
29. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11364–11369
30. Stone, E. M., and Pillus, L. (1996) *J. Cell Biol.* **135,** 571–583
31. Loayza, D., Tam, A., Schmidt, W. K., and Michaelis, S. (1998) *Mol. Biol. Cell* **9,** 2767–2784
32. Hoppe, G. J., Tanny, J. C., Rudner, A. D., Gerber, S. A., Danaie, S., Gygi, S. P., and Moazed, D. (2002) *Mol. Cell. Biol.* **22,** 4167–4180
33. Smith, J. S., Brachmann, C. B., Celic, I., Kenna, M. A., Muhammad, S., Starai, V. J., Avalos, J. L., Escalante-Semerena, J. C., Grubmeyer, C., Wolberger, C., and Boeke, J. D. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6658–6663
34. Gottschling, D. E., Aparicio, O. M., Billington, B. L., and Zakian, V. A. (1990) *Cell* **63,** 751–762
35. Enomoto, S., and Berman, J. (1998) *Genes Dev.* **12,** 219–232
36. Sussel, L., Vannier, D., and Shore, D. (1993) *Mol. Cell. Biol.* **13,** 3919–3928
37. Sharp, J. A., Fouts, E. T., Krawitz, D. C., and Kaufman, P. D. (2001) *Curr. Biol.* **11,** 463–473
38. Dasgupta, A., Darst, R. P., Martin, K. J., Afshari, C. A., and Auble, D. T. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 2666–2671
39. Aravind, L., and Koonin, E. V. (1998) *Curr. Biol.* **8,** R111–R113
40. Zheng, N., Wang, P., Jeffrey, P. D., and Pavletich, N. P. (2000) *Cell* **102,** 533–539
41. Evans, D. R. H., Brewster, N. K., Xu, Q., Rowley, A., Altheim, B. A., Johnston, G. C., and Singer, R. A. (1998) *Genetics* **150,** 1393–1405
42. Wittmeyer, J., Joss, L., and Formosa, T. (1999) *Biochemistry* **38,** 8961–8971
43. Kahana, A., and Gottschling, D. E. (1999) *Mol. Cell. Biol.* **19,** 6608–6620
44. Singer, M. S., Kahana, A., Wolf, A. J., Meisinger, L. L., Peterson, S. E., Goggin, C., Mahowald, M., and Gottschling, D. E. (1998) *Genetics* **150,** 613–632
45. Gotta, M., Strahl-Bolsinger, S., Renauld, H., Laroche, T., Kennedy, B. K., Grunstein, M., and Gasser, S. M. (1997) *EMBO J.* **16,** 3243–3255
46. Kennedy, B. K., Gotta, M., Sinclair, D. A., Mills, K., McNabb, D. S., Murthy, M., Pak, S. M., Laroche, T., Gasser, S. M., and Guarente, L. (1997) *Cell* **89,** 381–391
47. Roberts, C. J., Nelson, B., Marton, M. J., Stoughton, R., Meyer, M. R., Bennett, H. A., He, Y., Dai, H., Walker, W. L., Hughes, T. R., Tyers, M., Boone, C., and Friend, S. H. (2000) *Science* **287,** 873–880
48. Ho, Y., Gruhler, A., Heilbut, A., Bader, G. D., Moore, L., Adams, S. L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., Yang, L., Wolting, C., Donaldson, I., Schandorff, S., Shewnarane, J., *et al.* (2002) *Nature* **415,** 180–183
49. Joazeiro, C. A. P., and Weissman, A. M. (2000) *Cell* **102,** 549–552
50. Xu, Q., Singer, R. A., and Johnston, G. C. (1995) *Mol. Cell. Biol.* **15,** 6025–6035
51. Lommel, L., Ortolan, T. G., Chen, L., Madura, K., and Sweder, K. S. (2002) *Curr. Genet.* **42,** 9–20
52. Huang, H., Kahana, A., Gottschling, D. E., Prakash, L., and Liebman, S. W. (1997) *Mol. Cell. Biol.* **17,** 6693–6699
53. Freiberg, G., Mesecar, A. D., Huang, H., Hong, J. Y., and Liebman, S. W. (2000) *Curr. Genet.* **37,** 221–233
54. Robzyk, K., Recht, J., and Osley, M. A. (2000) *Science* **287,** 501–504
55. Sun, Z.-W., and Allis, C. D. (2002) *Nature* **418,** 104–108
56. Singh, J., Goel, V., and Klar, A. J. (1998) *Mol. Cell. Biol.* **18,** 5511–5522
57. Moazed, D., and Johnson, A. D. (1996) *Cell* **86,** 667–677
58. Zhang, Z., and Buchman, A. R. (1997) *Mol. Cell. Biol.* **17,** 5461–5472
59. Guarente, L. (2000) *Genes Dev.* **14,** 1021–1026
60. Parsons, X. H., Garcia, S. N., Pillus, L., and Kadonaga, J. T. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 1609–1614
61. Georgel, P. T., Palacios DeBeer, M. A., Pietz, G., Fox, C. A., and Hansen, J. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 8584–8589
62. Buck, S. W., and Shore, D. (1995) *Genes Dev.* **9,** 370–384
63. Cockell, M., Palladino, F., Laroche, T., Kyrion, G., Liu, C., Lustig, A. J., and Gasser, S. M. (1995) *J. Cell Biol.* **129,** 909–924
64. Marshall, M., Mahoney, D., Rose, A., Hicks, J. B., and Broach, J. R. (1987) *Mol. Cell. Biol.* **7,** 4441–4452
65. Renauld, H., Aparicio, O. M., Zierath, P. D., Billington, B. L., Chhablani, S. K., and Gottschling, D. E. (1993) *Genes Dev.* **7,** 1133–1145
66. Ahmad, K., and Henikoff, S. (2002) *Cell* **111,** 281–284
67. Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., and Hieter, P. (1992) *Gene (Amst.)* **110,** 119–122
68. Schneider, K. R., Smith, R. L., and O'Shea, E. K. (1994) *Science* **266,** 122–126