Supporting Information

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SI Methods

Yeast. Yeast used in this study are described in Table S1. BY4741 and BY4742 were purchased from Open Biosystems. Tandem affinity purification (TAP)-tagged strains were either purchased from Open Biosystems or obtained by genomic integration of the TAP tag at the 3′ end of the gene (1). TAP tagging was verified by PCR and Western blot (WB) analysis. Rpb7 and Asr1 were HA-tagged by a PCR-mediated strategy (2). Oligonucleotide sequences are available upon request.

Plasmids. Plasmids for expressing GST-CTD fusion proteins in *Escherichia coli* were a gift from J. Corden (Johns Hopkins Medical School, Baltimore, MD) (3).

For expression of recombinant Asr1 as an MBP fusion, the plasmid pMAL-Asr1 was constructed. Asr1-coding sequences were amplified by PCR and cloned into the expression vector pMAL-c2x (New England Biolabs). Site-directed mutagenesis (QuikChange XL; Stratagene) was used to introduce mutations within the RING domain (C29A and C50A) and/or the PHD domain (C143A and C146A) of Asr1. The resulting vectors are called pMAL-Ars1-PHDmutant, pMAL-Asr1-RINGmutant, and pMAL-Asr1-RING/PHDmutant.

Plasmid p425-pCUP1-UbG76A (F.G., unpublished data) was used to express polyhistidine-tagged, G76A-mutant, Ub under the control of the copper-inducible *CUP1* promoter.

For galactose-inducible expression of Asr1, we PCR-amplified sequences corresponding to untagged Asr1 (control), or HA-tagged full-length Asr1 or the CBD, and we cloned these fragments into pYES2 (Invitrogen), creating plasmids pYES2-Asr1, pYES2-HA-Asr1, and pYES2-HA-Asr1-CBD, respectively. Site-directed mutagenesis was used to introduce the RING mutations (C26A, C29A, C66A, C69A) into the pYES2-HA-Asr1 vector, creating plasmid pYES2-HA-Asr1-RING. Primer sequences are available upon request. Constructs were sequence-verified.

Antibodies. HA-tagged proteins were detected by 12CA5 (CSHL Monoclonal Antibody Shared Resource) or by a horseradish peroxidase (HRP)-conjugated anti-HA monoclonal antibody 3F10 (Roche). Anti-Rpb1 antibodies 8WG16, H5, H14, y-80, and CTD4H8 were purchased from Covance. Anti-Rpb4 and anti-Rpb7 antibodies were a gift from A. Sentenac (Commissariat à l'Énergie Atomique, Saclay, France) (4). Anti-Rpb3 and anti-Rpb4 antibodies were from NeoClone, whereas anti-Rpb7 antibody (yC-19) was purchased from Santa Cruz Biotechnology. HRP-conjugated anti-GST antibody was from Abcam.

Rabbit anti-Asr1 polyclonal antibodies were raised against a peptide corresponding to carboxyl-terminal amino acids 280–295 of Asr1.

TAP. Asr1–TAP and Rpb1–TAP complexes were isolated according to the published method (1) with the following modifications. Twelve-liter cultures of TAP-tagged and untagged (control) strains of *Saccharomyces cerevisiae* were grown at 30 °C to an A_{600} of ≈5 in YPD containing both glucose and galactose. Cells were collected, snap frozen, and ground in a blender in the presence of (per 2 L of culture) 20 mL of frozen lysis buffer [50 mM Hepes (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 50 mM β-glycerophosphate, 20 mM NaF, 0.1 mM $ZnSO_4$, 0.5 mM DTT, 4 μg/ml Pefabloc (Roche), Complete inhibitor without EDTA (Roche)], 5 mL of glass beads (G8772; Sigma), and dry ice. Samples were processed as described in ref. 1; TEV protease (Invitrogen) cleavage was performed overnight at 4 °C with rotation. For mass spectrometry analysis, proteins in the final eluate from the calmodulin column were trichloroacetic acid-precipitated and resuspended in 8 M urea. For Far-Western and Western blotting, eluates were resuspended in 20 μL of 8 M urea and 40 μL of 2× SDS/PAGE loading buffer. For in vitro transcription or ubiquitylation assays, eluted complexes were kept in the calmodulin column elution buffer [10 mM Tris·HCl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 1 mM magnesium acetate, 1 mM imidazole, 2 mM EGTA, 12% glycerol] and stored frozen in aliquots.

Mass Spectrometry. Asr1–TAP purifications were assayed by mass spectrometry (CSHL Proteomics Shared Resource) by analyzing both gel slices and by solution (shotgun) analysis. For analysis from gel slices, isolated Asr1–TAP components were resolved by SDS/PAGE (4–12% gradient; Invitrogen) and visualized by zinc staining. Bands were excised and processed for in-gel trypsin digestion following standard protocols. The resulting peptides were extracted, purified on C18-Ziptips (Millipore), and resuspended in 5 μL of 1% methanol, 0.1% acetic acid. A fraction of the purified peptides was analyzed by MALDI-TOF mass spectrometry, and the data were analyzed by using the software programs *m/z* (Proteometrics) and the PROFOUND search engine (5). The remaining sample was analyzed by LC-MS/MS on an LCQDeca mass spectrometer (Thermo-Finnigan) by using an in-house nanospray interface. Chromatography was performed by using 0.075-mm × 50-mm Aquasil-C18 PicoFrit columns (Newobjective) developed at 0.5 μL/min. The resulting spectra were analyzed with the SONARS software package (Proteometrics).

For shotgun analysis, trichloroacetic acid-precipitated proteins were resuspended in 8 M urea and then subjected to reduction and alkylation by standard procedures. The urea was diluted to 2 M with 50 mM ammonium bicarbonate, and trypsin digestion was performed. Digestions were stopped by the addition of formic acid to 0.5%. Peptides were purified via C18-Ziptips and analyzed as described above.

In Vitro Transcription/Translation of Asr1-HA, Rpb1-HA, and Rpb1-HA Mutants. Asr1-coding sequences (full-length and the indicated truncation mutants) were PCR-amplified from yeast genomic DNA with a T7-promoter-adapter primer (5′) and used as templates for coupled in vitro transcription/translation by using the TNT kit (Promega). Proteins were radioactively labeled by [^{35}S]methionine during synthesis.

Rpb1 coding sequences were first cloned into pCR4-TOPO (Invitrogen). The resulting plasmid, pCR4-TOPO-Rpb1, was used as a template to introduce alanine substitutions at K1452, K1458, and K1487 (QuikChange; Stratagene), creating pCR4-TOPO-Rpb1TM. These coding sequences (full-length and the indicated truncation mutants) were PCR-amplified from the pCR4-TOPO vectors with a T7-promoter-adapter primer (5′) and an HA-tagging primer (3′) and used as templates for coupled in vitro transcription/translation by using the TNT kit (Promega). Rpb1 proteins produced in this way were not radiolabeled. Where indicated, in vitro synthesized Rpb1 was phosphorylated in vitro by recombinant Cdc2 (New England Biolabs) according to the manufacturer's instructions. Primer sequences are available upon request.

Far-Western Analyses. Fifty-milliliter cultures of DH5α bacterial cells harboring the appropriate GST-CTD expression plasmids were grown to an A_{600} of 0.2 at 37 °C. Protein expression was induced by the addition of 0.1 mM IPTG for 4 h. Cells were collected, resuspended in 1.5 mL of Cdc2 buffer [50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EDTA, 0.01% Brij 35, 2 mM DTT, 0.2 mM ATP], and lysed by sonication. Lysates were then cleared by centrifugation, and GST-fusion proteins were recovered by incubation with glutathione–Sepharose (Amersham) according to the manufacturer's instructions. After washing, resin-bound GST-CTD fusions were phosphorylated by recombinant Cdc2, washed again, and phosphorylation was scored by mobility shift on SDS/PAGE.

For the Far-Western analyses, phosphorylated GST-CTD proteins or purified Asr1–TAP purifications were resolved by SDS/PAGE (4–12% gradient) and transferred to nitrocellulose membranes. The membranes were incubated in Far-Western buffer [20 mM Hepes (pH 7.9), 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 1 mM DTT, 2.5 mM $ZnSO_4$, Complete inhibitor without EDTA] with 5% nonfat milk powder for 4 h at 4 °C. After blocking, the membrane was incubated overnight in Far-Western buffer with 1% nonfat milk powder and radiolabeled Asr1 proteins (produced in vitro as described above), after which time the membrane was washed in Far-Western buffer, and radiolabeled proteins were revealed by PhosphorImaging.

Production of Recombinant Asr1. MBP and MBP-Asr1 proteins were purified from *Escherichia coli* BL21(RIL) by amylose resin chromatography, according to the manufacturer's protocol (New England Biolabs). Protein quality and purification were assessed by SDS/PAGE with Coomassie blue staining.

Ubiquitylation Assays. In vitro ubiquitylation assays were carried out by using a ubiquitylation reaction mixture with the following final concentrations: 0.1 mg/mL MBP-Asr1 or MBP or 50 μg/mL RSP5 (gift from J. Huibregtse, University of Texas Health Science Center, Austin, TX); 6.7 μg/mL human E2 (UbcH5a; Boston Biochem); 5.3 μg/mL yeast E1 (Boston Biochem); 0.17 mg/mL mammalian ubiquitin, methylated ubiquitin, or GST-ubiquitin (Boston Biochem); 2 mM Mg-ATP (Boston Biochem); 50 mM Tris·HCl (pH 7.5); 2.5 mM $MgCl_2$; 0.2 mM $ZnSO_4$; and 0.5 mM DTT. Substrates were either recombinant GST-CTD (phosphorylated by Cdc2), in vitro synthesized Rpb1–HA protein, or purified Rpb1–TAP complexes. In all cases, reactions proceeded at 30 °C for 90 min, after which time the reaction was stopped by the addition of SDS/PAGE loading buffer. Reaction products were resolved by SDS/PAGE and visualized by Western blotting.

Analysis of the in vivo ubiquitylation status of Rpb1 was performed by using the His-tagged ubiquitin (Ub) method, essentially as described in ref. 6. In this case, however, the vector p425-pCUP1-UbG76A was used to express polyhistidine-tagged G76A mutant Ub.

Purification of Pol II Subunits by MBP-Asr1 Resin. Cell extracts were prepared from Δ*asr1* yeast (BY4742) by bead beating in amylose column buffer [20 mM Tris·HCl (pH 7.5), 200 mM NaCl, 0.1 mM $ZnSO_4$, 1 mM DTT, 0.4 mg/mL Pefabloc, Complete inhibitor without EDTA]. Extracts were depleted of ATP by incubation with apyrase (Sigma) and passed over amylose resin bound with either MBP or MBP–Asr1. After washing extensively in column buffer, MBP proteins (and interacting partners) were eluted from the resin with column buffer containing 10 mM maltose and 12% glycerol. Fractions were collected and resolved by SDS/PAGE before analysis by WB.

Effect of Asr1 E3 Activity on the Rpb1–Rpb4/7 Interaction. For analysis of the effects of Asr1 on the Rpb1–Rpb7 interaction in vitro, Rpb1–TAP complexes were purified from yeast expressing both Rpb1–TAP and Rpb7–HA and subjected to ubiquitylation by MBP–Asr1 as described above. After ubiquitylation, Rpb7–HA was recovered by nondenaturing immunoprecipitation using the 12CA5 antibody (or BSA control). Coprecipitating Rpb1 was detected by SDS/PAGE and WB.

To analyze the role of the Asr1–RING finger on the interaction of Rpb1 with Rpb4/7 in vivo, inducible forms of Asr1–HA (in the pYES2 vector, as described above) were expressed in Δ*asr1*-yeast cells (BY4742) for 4 h by galactose induction. Cells were harvested, and lysates were prepared by bead beating in lysis buffer [50 mM Hepes (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 50 mM β-glycerophosphate, 20 mM NaF, 0.1 mM $ZnSO_4$, 0.5 mM DTT, 4 μg/mL Pefabloc (Roche), Complete Inhibitor without EDTA (Roche)]. HA-tagged Asr1 was recovered by immunoprecipitation with the 12CA5 antibody. Coprecipitating Rpb1, Rpb3, Rpb4, and Rpb7 were visualized by SDS/PAGE and WB analysis.

In Vitro Transcription. In vitro transcription assays of Asr1–TAP and Rpb1–TAP complexes were performed as described in ref. 7, using denatured salmon sperm DNA as a template. Before analysis, the relative levels of Rpb1 in both Asr1– and Rpb1–TAP preparations were determined by WB; assays were performed by using TAP eluates that had been normalized to contain comparable levels of Rpb1. Where indicated, α-amanitin was added to a final concentration of 5 μg/mL.

Chromatin Immunoprecipitations (ChIPs). ChIP analyses were performed by using the method described by Kuo and Allis (8). For detection of endogenous, HA-tagged, Asr1 at the *PMA1* gene, parallel ChIP assays were performed by using the 12CA5 monoclonal antibody from congenic yeast at which the *ASR1* locus was either untagged (BY4742), or HA-tagged (BY4742 ASR1-HA). Specific signals from ChIP DNA from the Asr1-HA strain were normalized to the corresponding signals (per primer set) from the untagged yeast. For detection of overexpressed Asr1, cultures (BY4742 Δ*asr1*) carrying galactose-inducible expression vectors for Asr1 or Asr1-HA were grown overnight in raffinose, induced for 2 h by the addition of 2% galactose, and ChIP was performed as above.

ChIP DNA was quantified by real-time PCR. For the *PMA1* gene, amplicons were selected to span the *PMA1* gene, as well as the 5′ end of the inactive *LEU1* gene. Relative to the ATG of *PMA1*, the central point for each amplicon was: −22, +633, +1155, +1565, +2077, +2674, +3048, +3412, +3883, +4099. The *PMA1*-coding sequence extends from +1 to +2757. *PMA1* polyadenylation signals are located at +2823 and +3277. *LEU1* coding sequences begin at +4015. Primer are sequences available upon request.

Total (Y-80) and pSer5 (H14) Rpb1 levels across *PMA1* were quantified by ChIP with the indicated antibodies. Signal was normalized to a noncoding sequence from an intergenic region on chromosome V (9).

Association of endogenous Asr1-HA with the *RPL33a* and *HSP104* genes was determined by ChIP as described above. Cultures were either untreated or treated with 8% ethanol for 5 min before ChIP. Levels of pol II associating with both genes was determined by using antibodies against Rpb3. Signals are presented as percentage of immunoprecipitation efficiency (ChIP/input DNA). Data from ChIP experiments is the average (±SEM) of 4 distinct ChIP assays.

RPC and RC Proteins. We searched the National Center for Biotechnology Information Protein Database for predicted proteins that contain either a RING or RING/PHD combination, together with a domain homologous to the rA9 carboxyl-terminal domain-binding domain (CBD) (Fig. 1*A*). This analysis

led to the identification of 2 distinct sets of proteins: (*i*) RPC proteins, carrying the RING, PHD, and CBD domains, and (*ii*) RC proteins, carrying a RING and PHD domains. Representative RPC proteins are depicted in Fig. 1*B*. The RPC proteins are present in a variety of species, including mammals (e.g., *Homo sapiens* rA9: NP_065925), birds (e.g., *Gallus gallus*: XP_423776), fish (e.g., *Danio rerio*: XP_693020; *Tetraodon nigroviridis*: CAG02388), sea urchins (e.g., *Strongylocentrotus purpuratus*: XP_968013), insects (e.g., *Tribolium castaneum*: XP_968013; *Drosophila melanogaster*: NP_649554), and fungi (e.g., *Neurospora crassa*: XP_958606; *Gibberella zeae*: XP_386932; *Schizosaccharomyces pombe*: NP_588450; *Kluyveromyces lactis*: XP_588450; *S. cerevisiae* Asr1: NP_015418). Only the mammalian (rA9) and *S. cerevisiae* (Asr1) proteins have been named thus far. In all cases examined, there is only 1 predicted RPC protein per species. The mammalian RPC proteins share extensive homology throughout their entire length; RPC members from other species show similarities primarily in the RING, PHD, and CBDs. RPC proteins often contain RS domains, as indicated in the figure. In all cases examined, the CBD is located at the carboxyl terminus of the protein.

The human protein rA9 has been identified in mass spectrometric analyses of the HeLa cell phosphoproteome (10), revealing that it is indeed expressed in human cells.

Proteins belonging to the RC class carry RING and CBDs in a configuration similar to the RPC proteins, but they lack a detectable PHD domain. The prototype of this class of proteins is human SFRS2ip (NP_004710). A single RC protein is found in the genome of most species (although multiple splice variants exist for several of these), including humans (NP_004710), chimpanzees (XP_001165059), monkeys (XP_001093213), dogs (XP_534828), opossums (XP_00137495), fowl (XP_416047), zebrafish (XP_694845), and yeast (*Candida glabrata*: XP_448571). RS domains are also frequently found in this class of proteins.

Phenotype of *asr1*-Null Yeast. Yeast deleted for *asr1* have been reported to be sensitive to detergents and high concentrations of alcohol (11), although this conclusion has been disputed by other groups (11–13). We have compared Δ*asr1* and congenic wild-type yeast (BY4742) for viability in response to treatment with detergent (0.02% SDS), alcohol (8% ethanol or 1% butanol), 6-azauracil, rapamycin, or UV irradiation. We have also examined UV sensitivity in yeast deleted for both *rad16* and *asr1*. In all cases (data not shown), we find no significant differences between Δ*asr1* and control yeast. We also find that *asr1*-null yeast can grow on medium containing galactose as a carbon source (data not shown).

We have probed for genetic interaction between loss of *asr1* and truncation of the carboxyl-terminal domain (CTD) of Rpb1 using a plasmid shuffle assay described by West and Corden (14). Deletion of *asr1* did not influence the growth characteristics of yeast carrying 7 copies of the CTD repeat (gift from J. Corden; data not shown).

Microarray analysis, comparing congenic WT and *asr1*-null yeast at both midlog and stationary phase, failed to detect any consistent differences in patterns of gene expression (data not shown).

Characterization of Asr1–TAP Complexes. Asr1–TAP purifications (and control purifications from untagged yeast strains) were resolved by SDS/PAGE, zinc-stained, and unique bands in the Asr1–TAP purification were excised and identified as described above. This analysis led to the identification of bands containing Asr1, Rpb1, Rpb2, Rpb3, Rpb5, Rpb6, Rpb8, and Rpb9.

We also used solution (shotgun) mass spectrometry to characterize 4 independent Asr1–TAP purifications (and control purifications from untagged yeast strains). We considered proteins to be valid Asr1 interactors if they were identified in at least 3 of the 4 Asr1–TAP purifications and not in the relevant control purifications. This analysis established the presence of Asr1, Rpb1, Rpb2, Rpb3, Rpb5, Rpb6, Rpb8, Rpb10, and Rpb12. We have confirmed the presence of Rpb1, Rpb2, Rpb3, Rpb5, Rpb6, Rpb8, and Rpb11 by Western blotting, using antibodies kindly provided by A. Sentenac (data not shown).

High-throughput proteomic analyses (15–17) have reported that, in addition to Rpb1, Rpb2, Rpb3, Rpb9, and Rpb11, Asr1 also copurifies with Rvs167, Arc15, Crn1, Cap1, Hsc82, Eno2, Fba1, Pgk1, Fpr1, and Ydr131cp. With the exception of the 10 RNA polymerase II subunits reported above, we did not detect any of these proteins in any of our Asr1–TAP preparations. Western blot analysis, using an anti–Arc15 antibody provided by R. Li (Stowers Institute for Medical Research, Kansas City, MO), showed that Arc15 was undetectable in the Asr1–TAP purification (data not shown).

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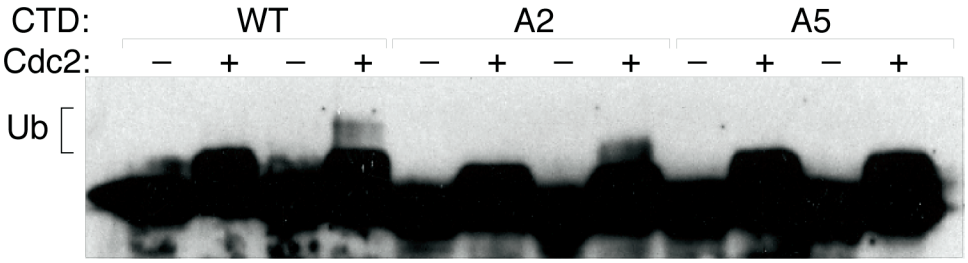

Fig. S1. In vitro ubiquitylation of GST-CTD by Asr1. Recombinant GST-CTD fusion proteins (14) carrying WT or mutant (A2, A5) copies of the CTD repeat were phosphorylated by recombinant Cdc2 (+) and used as substrates for ubiquitylation by recombinant MBP-Asr1. Reaction products were then resolved by SDS/PAGE and detected by WB, using antibodies against GST. WT and the A2 mutant GST-CTD proteins show the characteristic shift in molecular weight that is indicative of ubiquitylation. The A5 mutant is not detectably ubiquitylated.

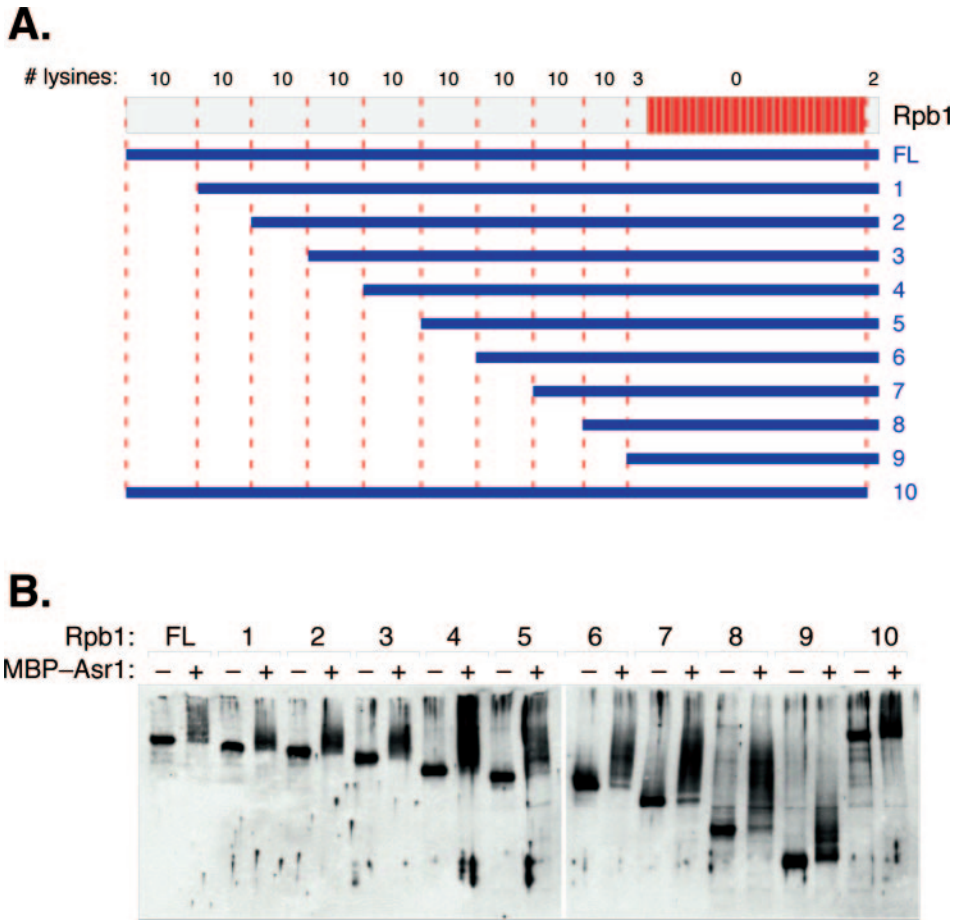

Fig. S2. In vitro ubiquitylation of amino-terminal Rpb1 deletion mutants by Asr1. (*A*) Diagram of Rpb1 and the deletion mutants 1–10. The numbers above the diagram indicate the number of lysine residues in each indicated segment. CTD repeats are shown in red. (*B*) The Rpb1 deletion mutants depicted in *A* were synthesized in vitro and used as substrates for ubiquitylation by recombinant MBP-Asr1. Reaction products were then resolved by SDS/PAGE and detected by WB, using antibody H14. Note that mutant 9, which contains 5 lysine residues surrounding the CBD, is ubiquitylated efficiently by Asr1.

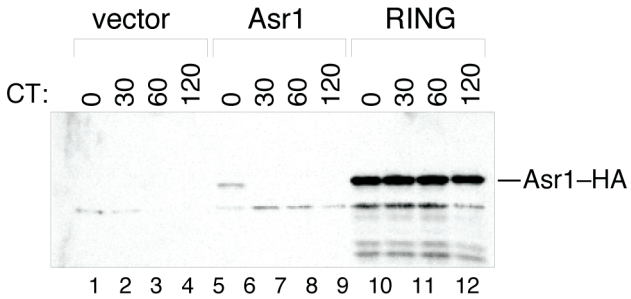

Fig. S3. Asr1 is an unstable protein that is destroyed in a RING finger-dependent manner. Yeast expressing galactose-inducible, HA-tagged versions Asr1, or the Asr1 RING mutant were grown in galactose for 2 h, after which time cells were transferred to glucose-supplemented medium containing cyclohexamide. Protein samples were collected at the indicated chase times (CT; in minutes), and Asr1 was detected by WB. Asr1 is very unstable (compare lanes 6 with 7–9), whereas the RING mutant protein is stable. This result is consistent with Asr1 undergoing autoubiquitylation-dependent proteolysis.

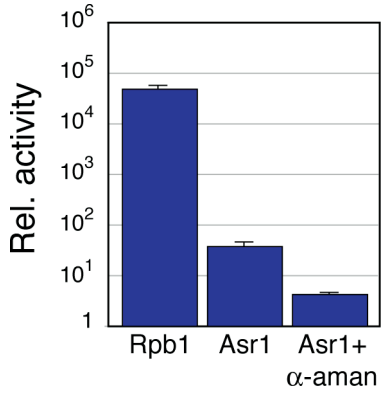

Fig. S4. Analysis of the polymerase activity in the Asr1–TAP complex. Rpb1–TAP (pol II) or Asr1–TAP complexes were analyzed for polymerase activity, with denatured salmon sperm DNA as a template. The amount of each complex used for analysis was normalized to the relative levels of Rpb1. Inclusion of α-amanitin in the Asr1 analysis showed that some, but not all, of the residual polymerase activity was caused by RNA polymerase II.

Table S1. Yeast used in this work

Name	Genotype	Source
BY4742	MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0	Open Biosystems
BY4741	*MATa*, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*	Open Biosystems
W303–1a	*MATa*, *ura3-1*, *ade2-1*, *his3-11,15*, *trp1-1*, *leu2–3,112*, *can1–100*	B. Futcher
W303–1a Asr1–TAP	W303-1a but *ASR1–TAP*::*KlURA3*	This work
BY4741 Rpb1–TAP	By4741 but *RPB1–TAP*::*His3MX*	Open Biosystems
BY4742 ΔAsr1	BY4742 but Δ*asr1*::*KanMX*	Open Biosystems
BY4742 Asr1–TAP	BY4742 but *ASR1–TAP*::*KlURA3*	This work
BY4742 Asr1–HA	BY4742 but *ASR1–HA3*::*KanMX*	This work
BY4741 Rpb1–TAP Rpb7–HA	BY4741 but *RPB1–TAP*::*His3MXl RPB7–HA*::*KanMX6*	This work
BY4742 ΔRrp6	BY4742 but Δ*rrp6*::*KanMX*	Open Biosystems
BY4742 ΔRrp6 ΔAsr1	BY4742 but Δ*rrp6*::*KanMX;* Δ*asr1*::*His3MX6*	This work