SUPPORTING INFORMATION

Supplementary Materials and Methods

All reagents, unless otherwise stated, were procured from Sigma-Aldrich, USA. [*14C]-labeled uracil was procured from Ogene Systems, Hyderabad, India. [*γ-32P]ATP, [*35S]Met/Cys, and [*α-32P]UTP were procured from JONAKI/BRIT, Hyderabad, India.

Yeast strains: All yeast strains used in this study are listed in Table S1. The DDY1810 *S. cerevisiae* strains were a gift from Dr. Adolfo Saiardi [1], and the *kanMX4* cassette in vip1Δ and *ks1ΔddplΔ* strains was removed by using the Cre-loxP recombination system [2]. The BY4741 *ks1Δ* strain was a gift from Dr. Beverley Wendland [3]. The NOY222 RPA190 shuffle strain carrying a complemented deletion of *RPA190*, and the plasmids encoding WT and Ser to Ala mutant versions of RPA190 were gifted by Dr. Herbert Tschochner [4]. An *rpa190* and *rpa34* double mutant strain was generated by mating BY4741 *rpa34Δ* with NOY222 using standard yeast genetic techniques [5], and the resulting haploid strain was phenotyped for the presence of auxotrophy and drug resistance markers (Table S1). A genomic mutation on the *RPA43* gene was inserted into the NOY222 *rpa34Δ* strain and the BY4741 WT strain. pRS314-*RPA43* (a gift from Dr. Herbert Tschochner, [6]) was used as a template for PCR-based site-directed mutagenesis to create a mutant version of *RPA43* (*RPA43*S322/S323/S325A). Using homologous recombination methods, the indicated Ser codons were substituted with Ala in the C-terminal tail of RPA43, by inserting the nourseothricin N-acetyltransferase (*nat1*) gene [7] between the 3′UTR of *RPA43* and 5′UTR of the downstream gene, *UBC11* (Table S4). Plasmids encoding WT and mutant *RPA190* (*RPA190*S1413/S1415/S1417A) were introduced into the indicated strains (Table S1) by shuffling, as described earlier [4].

Drug sensitivity assay: Analysis of sensitivity to translation inhibitors was conducted in the DDY1810 *S. cerevisiae* strain background, that does not contain the *kan* selection marker (Table S1). Sensitivity to 6-azauracil (6AU) was monitored in the BY4741 or NOY222 strain backgrounds (Table S1). As uracil is a competitive inhibitor of 6AU, the plasmid p416GPD, carrying the *URA3* gene [8] was introduced into BY4741-derived strains to complement the *URA3* deletion in this strain. Overnight cultures grown in YPD or synthetic complete (SC) medium without uracil, were diluted to OD600 0.25, followed by 5 fold serial dilutions, and 3μL of each dilution was spotted on a YPD-agar plate containing the indicated concentrations of translation inhibitors, or an SC-Ura agar plate, containing the indicated concentrations of 6AU. Growth was monitored at 30°C for 2-3 days.

Protein synthesis assay: 1 OD600 unit of cells from mid-log phase yeast cultures grown in YPD were labeled in SC medium without Methionine (SC-Met) containing 25 μCi/mL [*35S]Met/Cys for 5 min. Cells were lysed by bead-beating in Tris-buffered saline (20 mM Tris-HCl pH 7.2, 0.9% NaCl) with protease inhibitor cocktail and centrifuged at 12,000 g for 15 min at 4°C. Sodium deoxycholate (final concentration 0.1 mg/mL) was added to the supernatant and incubated on ice for 30 min. Trichloroacetic acid was added to a final
concentration of 6%, followed by incubation on ice for 1 h and centrifugation at 15,000 g for 15 min at 4°C. The pellet obtained was suspended in Tris-buffered saline and counted in a liquid scintillation counter (Perkin Elmer Tri-carb 2900). The cpm (counts per min) values obtained were plotted using GraphPad Prism.

**Ribosome profiles:** Ribosome profiles were generated as described earlier [9] with some modifications. Yeast were grown in YPD till mid-log phase and were treated with cycloheximide (50 μg/mL), chilled on an ice-salt bath for 2-5 min and centrifuged immediately at 4,000 g. Cells were lysed in 1 mL lysis buffer (10 mM Tris pH 7.4, 100 mM NaCl, 30 mM MgCl₂, 50 μg/mL cycloheximide, 200 μg/mL heparin, in 0.2% diethyl pyrocarbonate (DEPC)-treated water) and centrifuged at 10,000 g for 10 min at 4°C. Cell lysates equivalent to 10 A₂₅₄ units were loaded on top of a 10%-50% sucrose continuous gradient in buffer (50 mM Tris-HCl pH 7.4, 50 mM NH₄Cl, 12 mM MgCl₂, 1 mM DTT, 0.1% DEPC) and centrifuged at 100,000 g at 4°C for 6 h in an SW41 rotor (Beckman). Ribosome levels were measured by gradient analysis on an ISCO UV-6 gradient collector by monitoring absorbance at 254 nm. To analyse individual ribosome subunits, lysates were resolved on a 10%-30% sucrose continuous gradient in buffer lacking MgCl₂.

**Doubling time and viability assessment:** Overnight grown yeast were sub-cultured in SC medium or in YPD at 0.1 OD₆₀₀. Growth was monitored for 72 h by measuring OD₆₀₀ of the culture at regular intervals, and doubling time was calculated from the exponential phase of growth by linear regression analysis on a semi-logarithmic scale, using GraphPad Prism. To determine yeast cell mass, cells equivalent to 5 OD₆₀₀ were harvested from mid-log and stationary phase cultures, and washed with PBS. Cell pellets were dried at 50°C for 20 min and the dry weight of yeast was measured. To assess the cell number, cells in mid-log or stationary phase were counted using a Neubauer chamber and the number of cells present in 1 OD₆₀₀ was calculated. Cell death was monitored by incubating yeast cells in 0.2% trypan blue solution (Sigma-Aldrich) for 10 min, and scoring dead cells that take up the dye. To monitor cell viability, cells equivalent to 10⁻⁵ OD₆₀₀ from mid-log and stationary phase cultures were plated on YPD-agar, incubated at 30°C for 48 h, and colonies were counted to extrapolate viable cell count per OD₆₀₀.

**RNA extraction and analysis:** Total RNA was isolated by hot phenol extraction as described earlier [10] with slight modifications. 1 OD₆₀₀ unit of cells from mid-log phase yeast cultures grown in YPD were lysed in AE solution (50 mM CH₃COONa pH 5.3, 10 mM EDTA), containing 1% SDS and an equal volume of acid-buffered phenol, pH 4.3, followed by incubation at 65°C for 15 min with continuous shaking. Lysates were chilled on ice and centrifuged at 12,000 g for 10 min. The aqueous phase was transferred to a tube containing an equal volume of chloroform, mixed well and centrifuged at high speed. RNA was precipitated by the addition of 50 μL 3M sodium acetate followed by 100% ethanol, and dissolved in DEPC-treated water. RNA was estimated by measuring A₂₆₀ using a spectrophotometer (Thermo Scientific ND-1000). To monitor rRNA levels, 10μg of total RNA from each strain was resolved on a 1.2% formaldehyde-agarose gel.
RNA labelling experiments were performed by harvesting mid-log phase yeast cells grown in YPD. Cells equivalent to 1 OD₆₀₀ unit were incubated in SC-Ura medium containing 3 μCi/mL [¹⁴C]uracil for different lengths of time, and RNA was extracted as described previously. Equal total RNA was resolved on a formaldehyde agarose gel, stained with ethidium bromide and transferred to an N⁺ Hybond membrane (GE Life Sciences). Radiolabeled rRNA was detected using a phosphorimager scanner (Fujifilm FLA-9000). Pulse-chase analysis of rRNA was performed as described earlier [9], with slight changes. Yeast cells were harvested at an OD₆₀₀ of 0.5-0.7. The cells were washed and labeled in 1 mL SC-Ura medium containing 3 μCi/mL [¹⁴C]uracil for 5 min at 30°C. A chase was performed with SC medium containing 240 mg/L unlabeled uracil. Samples were harvested 0, 1, 5, 15 and 20 min after the chase, and centrifuged at 12,000 g for 1 min at 4°C. RNA was extracted, and incorporation of radioactivity was detected as described earlier.

**ChIP**: The assay was performed as described earlier [11] with slight modifications. 45 mL of mid-log phase yeast cultures grown in YPD were subjected to cross linking with 1% formaldehyde for 15 min at room temperature. Cross linking was quenched by adding glycine to a final concentration of 0.1 M. Cells were washed in ice cold Tris-buffered saline and were lysed in 500 μL of ice cold lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, protease inhibitor cocktail) by bead beating. Chromatin was fragmented using a bath sonicator (Diagenode). Cell lysates were centrifuged at high speed and the supernatant was pre-cleared with normal rabbit IgG followed by Protein A beads (GE Life Sciences). Supernatant was collected and 10 μL of this lysate was taken as input. Immunoprecipitation of chromatin was performed by incubating the lysate with anti-GST antibody overnight at 4°C followed by Protein A beads for 4 h. Beads were washed twice each in wash buffer I (50 mM HEPES pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, protease inhibitor cocktail), wash buffer II (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.75% NP-40, 0.75% sodium deoxycholate), and TE buffer. Chromatin was eluted in 100 μL of elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) and incubated at 65°C overnight to reverse the cross linking. DNA was extracted using a PCR purification kit (Qiagen). PCR reactions were set up with primers 5’GCTAAGATTAAAACTGGAATAGC3’ and 5’GCCTACTGAATTCGTTTCC3’ to amplify the rDNA promoter, and primers 5’TCAAACGTTGGAGAGAAGTGC3’ and 5’ACCAATGGAATCGCAAGATGC3’ to amplify the 5’ETS. Real-time PCR was performed using Mesa Green 2X PCR MasterMix (Eurogentec) in a 20 μL reaction volume using 1 μL from the input sample and 3 μL from the immunoprecipitated sample (Applied Biosystems). Ct values of the immunoprecipitated samples were normalised to the adjusted Ct values of input, and data were plotted using GraphPad Prism.
### Table S1. *S. cerevisiae* strains used in this study and their corresponding genotypes.

| Strain         | Genotype                                                                 | Reference          |
|----------------|---------------------------------------------------------------------------|--------------------|
| BY4741 WT      | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0                                         | [12]               |
| kcs1Δ          | BY4741 kcs1Δ::kanMX4                                                      | [3]                |
| vip1Δ          | BY4741 vip1Δ::kanMX4                                                      | [13]               |
| kcs1Δ / pKCS1  | BY4741 kcs1Δ::kanMX4, pKCS1                                              | [14], this study   |
| kcs1Δ / pkcs1  | BY4741 kcs1Δ::kanMX4, pkcs1-S887A/L888A/L889A (URA3)                     | [14], this study   |
| rpa34Δ         | BY4741 rpa34Δ::kanMX4                                                    | [13]               |
| rpa43Δ         | BY4741 rpa43-S322A/S323A/S325A::nat1                                     | This study [3]     |
| DDY1810 WT     | MATa leu2-3, 112 trp1-Δ901 ura3-52 prb1-1122 pep4-3 prc1-407             | [1]                |
| kcs1Δ          | DDY1810 kcs1Δ::LEU2                                                      | [1]                |
| vip1Δ          | DDY1810 vip1Δ::loxP                                                      | [1], this study    |
| kcs1Δ/ddp1Δ    | DDY1810 kcs1Δ::loxP, ddp1Δ::LEU2                                         | This study         |
| kcs1Δ / pKCS1  | DDY1810 kcs1Δ:: LEU2, pKCS1                                              | [14], this study   |
| kcs1Δ / pkcs1  | DDY1810 kcs1Δ:: LEU2, pkcs1-S887A/L888A/L889A (URA3)                     | [14], this study   |
| NOY222         | MATα trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pNOY20 (LEU2 CANs, RPA190) | [15]               |
| RPA190 WT      | MATα trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-RPA190 (TRP1) | [4]                |
| rpa190Δ        | MATα trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S1413/1415/1417A (TRP1) | [4]                |
| NOY222 rpa34Δ  | MATα leu2Δ ura3Δ can r rpa190Δ::URA3 rpa34Δ::kanMX4 pNOY20 (LEU2 CANs, RPA190) | This study         |
| RPA190 WT      | MATα leu2Δ ura3Δ can r rpa190Δ::URA3 rpa34Δ::kanMX4 pRS314-RPA190 (TRP1) | This study         |
| rpa190Δ        | MATα leu2Δ ura3Δ can r rpa190Δ::URA3 rpa34Δ::kanMX4 pRS314-rpa190-S1413/1415/1417A (TRP1) | This study         |
| NOY222 rpa34Δ  | MATα leu2Δ ura3Δ can r rpa190Δ::URA3 rpa34Δ::kanMX4 pRS314-rpa190-S1413/1415/1417A (TRP1) | This study         |
| RPA190 WT      | MATα leu2Δ ura3Δ can r rpa190Δ::URA3 rpa34Δ::kanMX4 pRS314-rpa190-S1413/1415/1417A (TRP1) | This study         |
**Table S2.** Primers used in this study to clone RNA Pol I subunits in pYesGex6p2 yeast expression vector [16]. *Inserts generated by overlap-extension PCR.

| Plasmid | Primers used to amplify insert |
|---------|--------------------------------|
| pYesGex6p2 | Yeast expression vector [16] |
| Uaf30FL | Uaf30-F: CTGATGGATCCATGGCTGAATTAACGATTATAGTAC  
Uaf30-R: CATGAGCCGCCTCAAATGCGCCTTTCGATCGATGTTG |
| A135 (1-112) | A135-F: TGATCGGATCCATGCGAAGCATAACCAGTATGAC  
A135-R: CATGAGCCGCCTCAAATGCGCCTTTCGATCGATGTTG |
| A190 (1-556) | A190-F: CTGATGGATCCATGGATATTTCTTAACCC  
A190-R: CATGACTCGAGCTATGCTAGCTTTACATTTAGT |
| A190 (557-1100) | A190-557-F: CTGATGGATCTAGCTTTTTAGATTACACTTGACAC  
A190-1100-R: CATGACTCGAGCTATGCTAGCTTTACATTTAGT |
| A190 (1101-1664) | A190-1101-F: CTGATGGATCCACTTGAATACAGAAGGAAGAA  
A190-R: CATGAGCCGCCTCAAATGCGCCTTTCGATCGATGTTG |
| A190 (1338-1448) | A190-1338-F: ATCTGGGATCCAGAAGCATAACCAGTATGAC  
A190-1448-R: CATGACTCGAGCTATGCTAGCTTTACATTTAGT |
| A43FL (1-326) | A43-F: TGATCGGATCCATGCAAGAATCAGCTGACGACCTGAC  
A43-R: CATGAGCCGCCTCAAATGCGCCTTTCGATCGATGTTG |
| A34FL (1-233) | A34-F: TGATCGGATCCATGCAAGAATCAGCTGACGACCTGAC  
A34-R: CATGAGCCGCCTCAAATGCGCCTTTCGATCGATGTTG |
| A34 (Δ10-17a.a) | A34-del-F: TGATCGGATCCATGCAAGAATCAGCTGACGACCTGAC  
A34-R: CATGAGCCGCCTCAAATGCGCCTTTCGATCGATGTTG |
| A34 (1-204) | A34-F: TGATCGGATCCATGCAAGAATCAGCTGACGACCTGAC  
A34-R: CATGAGCCGCCTCAAATGCGCCTTTCGATCGATGTTG |
| *A34FL (1-233)S205,206/A | A34-F: TGATCGGATCCATGCAAGAATCAGCTGACGACCTGAC  
A34-Sa-R: CATGAGCCGCCTCAAATGCGCCTTTCGATCGATGTTG |

*A Inserts generated by overlap-extension PCR.*
**Table S3. Doubling time of yeast strains.** Growth of the indicated yeast strains at 30°C in rich medium (YPD), synthetic complete (SC) medium or SC medium without uracil (SC-Ura) was monitored up to 48 h by measuring OD$_{600}$ of the culture at regular intervals. The doubling time was calculated from the logarithmic phase of growth. Data are mean ± SEM (n=4).

| S. cerevisiae (BY4741) | Growth condition | Doubling time (h) |
|------------------------|------------------|-------------------|
| WT                     | YPD              | 1.72 ± 0.08       |
| kcs1Δ                  | YPD              | 2.15 ± 0.01       |
| WT                     | SC               | 1.83 ± 0.03       |
| kcs1Δ                  | SC               | 2.96 ± 0.16       |
| vip1Δ                  | SC               | 2.0 ± 0.01        |
| kcs1Δ / pKCS1          | SC-Ura           | 1.9 ± 0.02        |
| kcs1Δ / pkcs1$^{SLL\rightarrow AAA}$ | SC-Ura         | 2.97 ± 0.20       |

**Table S4.** Primers used in this study to introduce site-specific genomic mutations in *rpa43* to replace codons encoding Ser 322, 323 and 325 with codons encoding Ala.

| Fragment name | Primers used to amplify the fragment |
|---------------|-------------------------------------|
| A43 3’        | A43-gen-F: AGATCGTATACGAGGAAACACCAAGTGGAAGCAATGATGGTGAAGCAGCTGATGCTGATTAGGACGGGAAAATGAG |
|               | A43-gen-R: CACGGCAGCTAGCAGGGAAGATTTGTCGAATTGTCGATATGGCCACGAGCTTTAGC |
| NAT 5’        | NAT-5-F: GCTAAACCGTGGCAAATTCGACAATCTTCCGCTGCTAGCCGCAGCCCCTG |
|               | NAT-5-R: TCTGTTCCAACCAGAAATAG |
| NAT 3’        | NAT-3-F: GTCTACTACTTTGGATGATAC |
|               | NAT-3-R: GTCACAAGCAGATGTTTCCGCGCAAGCAAGTTCCTTATTC |
| Ubc11 5’      | UBC11-F: GATATCGGAACCTTCGTCAGGCGGCCGACACAAAAACATGCTTCTGAC |
|               | UBC11-R: ATTATCGTCTACGGAAACGCAC |
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Figure S1. Viability and cell mass are unaltered in \textit{kcs1\textDelta} yeast. \textit{(A)} Yeast cells equivalent to 5 OD\textsubscript{600} were harvested from log phase and stationary phase cultures and dry weight was measured. Data are mean ± range (n=2). \textit{(B)} Cells from log phase and stationary phase cultures were counted using a Neubauer chamber and represented as total number of cells present in 1 OD\textsubscript{600} of yeast culture. Data are mean ± SEM (n=3). \textit{(C)} Cells from log phase and stationary phase cultures were stained with trypan blue. Dead cells were counted and represented as a percentage of total cell count. Data are mean ± SEM (n=3). \textit{(D)} Cells from log phase and stationary phase cultures were plated on YPD-agar plates and colonies were counted to determine the number of viable cells per 1 OD\textsubscript{600} unit (CFU colony forming units). Data are mean ± SEM (n=4). P values are from a 2-tailed paired \textit{t} test (n.s. not significant)
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Figure S2. Amino acid sequences of RNA Pol I components tested for pyrophosphorylation by IP7. (A) Sequence of full length Uaf30, a subunit of upstream activating factor (UAF). (B) Sequence of a fragment of A135 (amino acids 1-112), the second largest subunit of RNA Pol I. (C) Sequence of a fragment of A190 (amino acids 1338-1448), corresponding to the extended loop of RNA Pol I. (D) Sequence of full length A43. (E) Sequence of full length A34.5. All sequences are annotated as follows: previously determined phosphorylated serines (curated in PhoshoGRID) are underlined, serine residues within an acidic serine motif are coloured red, and pyrophosphorylated serine residues identified in this study are highlighted in yellow.
Figure S3. Phenotypic analysis of RNA Pol I mutants: (A) 5-fold serial dilutions of the indicated S. cerevisiae strains were plated on SC-Ura medium containing 6-azauracil (6AU), and incubated for 2-4 days at 30°C. (B) Protein synthesis was measured in the indicated S. cerevisiae strains by pulse-labeling cells for 5 min with [35S]Met/Cys. Radioactivity incorporated into total protein expressed as counts per min (cpm) was normalised to the optical density (OD$_{600}$) of the labeled culture. Data are mean ± SEM (n=4). P values are from a 2-tailed paired t test (n.s. not significant, P > 0.05). See Supplementary Table S1 for a detailed description of the S. cerevisiae strains used in these analyses.