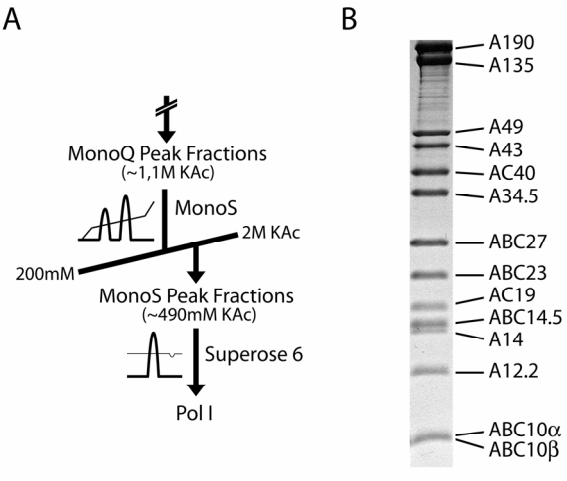
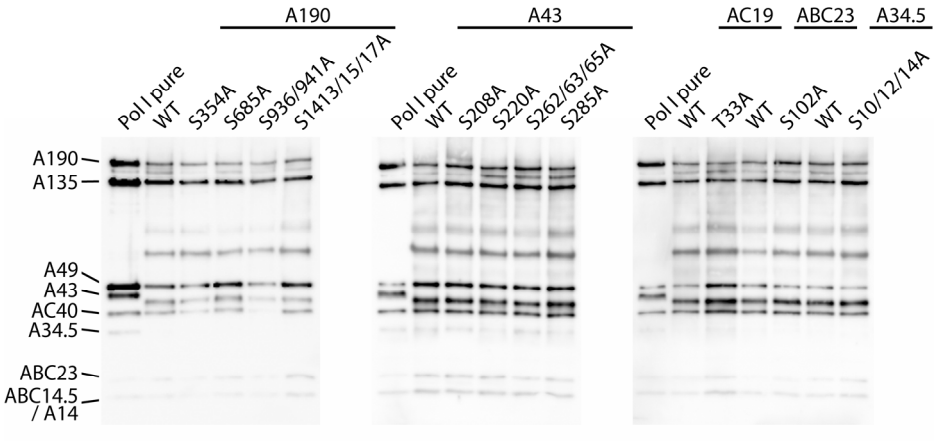
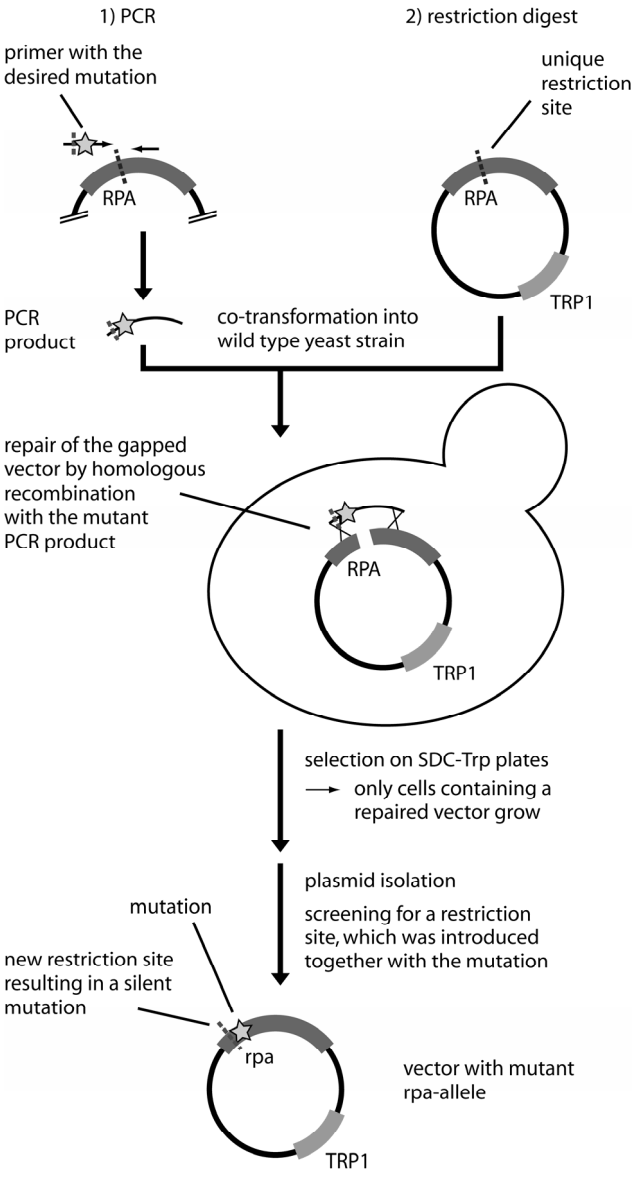
Supplementary Data



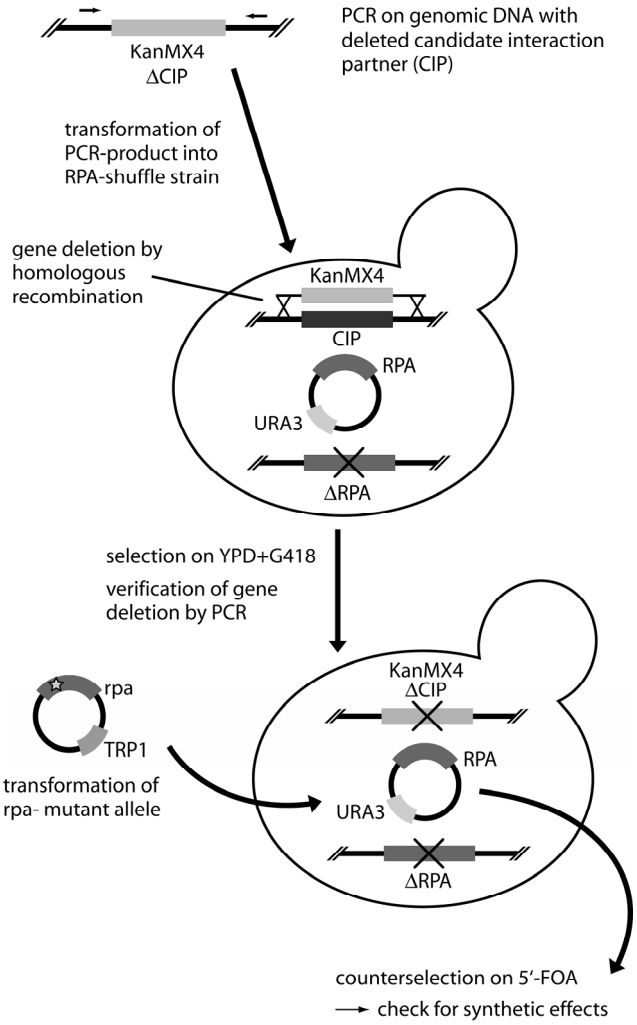
Supplementary Figure S1 RNA polymerase I purification (extended). (**A**) Extended Pol I purification scheme including an additional cation exchange chromatography step to obtain highest purity. This purification method was used for the structure determination by cryo electron microscopy (1) (**B**) Coomassie stained SDS-gel of the purified Pol I.



Supplementary Figure S2 Immunoprecipitation of mutant Pol I under high-salt conditions. Mutant Pol I and the corresponding wildtypes were immunoprecipitated from whole cell extracts via a Protein A-TEV-tag on subunit A135 under high salt conditions. The indicated subunits were detected on the western blots with an anti-Pol I antiserum. Purified Pol I (Pol I pure) was loaded on each western blot for direct comparison. Note that the A43 subunit of the purified Pol I is shifted due to the His_6-HA-tag, while the Protein A-tag on subunit A135 of immunoprecipitated Pol I was cleaved during elution.



Supplementary Figure S3 Strategy for site-directed mutagenesis.



Supplementary Figure S4 Scheme of the SL-screen. Counterselection on 5'-FOA, used for the derivates of strains D101-I2, JAY444 and DLY200 (RPA43-, RPO21- and RPC19 shuffle strains, respectively), is shown as an example. Derivates of NOY222 (RPA190 shuffle strain) were tested for synthetic effects by counterselection on L-canavanine and strains derived form T4-1c (RPA34 knockout strain) were checked after transformation of the mutant vectors on SDC-Trp plates.

Supplementary Methods

Strains and Templates

Supplementary Table S1 Yeast Strains.

Name	Genotype	Origin
GPY2	*leu2-Δ1 ade2-101 trp1-Δ63 ura3-52 his3-Δ200 lys2-801 RPA43Δ::LEU2 pAS22 (TRP1)*	(2)
NOY222	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pNOY20 (LEU2 CANs)*	(3)
ToY605	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-RPA190 (TRP1)*	this study
ToY778	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S354A (TRP1)*	this study
ToY781	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S354D (TRP1)*	this study
ToY779	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S685A (TRP1)*	this study
ToY780	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S685D (TRP1)*	this study
ToY677	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S936/941A (TRP1)*	this study
ToY1030	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S936D (TRP1)*	this study
ToY1031	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S941D (TRP1)*	this study
ToY1032	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S936E (TRP1)*	this study
ToY1033	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S941E (TRP1)*	this study
ToY245	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 pRS314-rpa190-S1413/1415/1417A (TRP1)*	this study
D101-I2	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 yCPA43 (URA3)*	(4)
GPY9	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pGP5 (TRP1)*	(5)
ToY523	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S208A (TRP1)*	this study
ToY612	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S208D (TRP1)*	this study

ToY678	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S220A (TRP1)*	this study
ToY1183	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S220D (TRP1)*	this study
ToY679	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S208/220A (TRP1)*	this study
ToY561	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S262A (TRP1)*	this study
ToY1178	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S262D (TRP1)*	this study
ToY1179	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S263D (TRP1)*	this study
ToY1180	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S262/263D (TRP1)*	this study
ToY611	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S262/263/265A (TRP1)*	this study
ToY562	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S285A (TRP1)*	this study
ToY1182	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S285D (TRP1)*	this study
ToY1181	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 pRS314-rpa43-S262/285D (TRP1)*	this study
T4-1C	*ura3-52 ade2-1 lys2-801 his3-Δ200 trp1-Δ1 rpa34-Δ::HIS3*	(6)
ToY1034	*ura3-52 ade2-1 lys2-801 his3-Δ200 trp1-Δ1 rpa34-Δ::HIS3 pRS314-RPA34*	this study
ToY1035	*ura3-52 ade2-1 lys2-801 his3-Δ200 trp1-Δ1 rpa34-Δ::HIS3 pRS314-rpa34-S10/12/14A (TRP1)*	this study
JAY444	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 pRPO26 (URA3)*	(7)
ToY607	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 pRS314-RPO26 (TRP1)*	this study
ToY568	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 pRS314-rpo26-S102A (TRP1)*	this study
ToY1036	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 pRS314-rpo26-S102D (TRP1)*	this study
DLY200	*ura3-52 his3-Δ200 trp1Δ lys2-801 ade2-101 rpc19::HIS3 pLS135 (URA3)*	(8)
ToY608	*ura3-52 his3-Δ200 trp1Δ lys2-801 ade2-101 rpc19::HIS3 pRS314-RPC19 (TRP1)*	this study
ToY609	*ura3-52 his3-Δ200 trp1Δ lys2-801 ade2-101 rpc19::HIS3 pRS314-rpc19-T33A (TRP1)*	this study

ToY531	*trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 can r rpa190Δ::URA3 RPA135-ProtA::kanMX6 pNOY20 (LEU2 CANs)*	this study
ToY532	*rpa43::LEU2 ade2-101ura3-52 lys2-801 trp1-Δ63 his3-Δ200 leu2-Δ1 RPA135-ProtA::kanMX6 yCPA43 (URA3)*	this study
ToY534	*ura3-52 ade2-1 lys2-801 his3-Δ200 trp1-Δ1 rpa34-Δ::HIS3 RPA135-ProtA::kanMX6*	this study
ToY535	*CAN1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1 rpo26::LEU2 RPA135-ProtA::kanMX6 pRPO26 (URA3)*	this study
ToY783	*ura3-52 his3-Δ200 trp1Δ lys2-801 ade2-101 rpc19::HIS3 RPA135-ProtA::kanMX6 pLS135 (URA3)*	this study
BSY420 (1n) [1]	*ade2-1 can1-100 his3Δ200 leu2-3,112 trp1-1 ura3-1*	(9)

[1] Wildtype yeast strain BSY420 was used as a cloning tool in site-directed mutagenesis

Yeast strains used in this study are listed in Supplementary Table S1. Genes for mutational analysis were cloned into vector pRS314 (TRP1) (10) including their endogenous promoter and terminator regions. RPA190 was subcloned from plasmid pNOY16 (3). RPA34, RPO26 and RPC19 were cloned via PCR from yeast genomic DNA (strain S288C, Invitrogen). Plasmid pGP5 (RPA43 cloned into pRS314) (5) was used for analysis of the A43 subunit. Chromosomal tagging of RPA135 with a C-terminal TEV-Protein A-tag was performed using plasmid pYM8 as previously described (11). Standard yeast genetic techniques and media were used for plasmid transformation and shuffling (12).

Protein identification

Samples of the purified Pol I preparations were precipitated by Methanol/Chloroform and loaded on a 4-12 % NuPAGE™ Bis-Tris gel (Invitrogen). After electrophoresis using NuPAGE™ MOPS SDS running buffer (Invitrogen), the gel was stained with SimplyBlue™ SafeStain (Invitrogen) and the bands of interest were excised. The proteins were digested in-gel with modified sequencing grade Trypsin (Roche). Prior to analysis, the resulting peptides were desalted using ZipTip™ C_{18} pipette tips (Millipore). Peptide mass fingerprints and MS/MS analyses were performed on an Applied Biosystems 4700 Proteomics Analyzer™ MALDI-TOF/TOF mass spectrometer using CHCA matrix and evaluated by

searching the NCBInr protein sequence database with the Mascot™ search engine implemented in the GPS Explorer™ software (Applied Biosystems).

Site-directed mutagenesis

A scheme of the mutagenesis strategy is given in Supplementary Figure S3. PCR reactions were performed with primers carrying the desired mutations on plasmids containing the gene for the respective Pol I subunit. The length of the PCR product was chosen to span a unique restriction site, which in turn was used to cut another aliquot of the same plasmid. 45-75 ng of the PCR product were transformed together with 15 ng of the linearized plasmid into a BSY420 (1n) wildtype yeast strain. Transformants were selected for the TRP1 marker contained on the vector. As a consequence only cells carrying a plasmid repaired by homologous recombination could form colonies. After plasmid isolation, vectors containing the mutation were selected by restriction analysis screening for a new restriction site, which was also introduced in the PCR primer, but results in a silent mutation. All constructs were verified by sequencing. The finished vectors, carrying the mutant alleles of the RPA-genes, were shuffled into the corresponding yeast shuffle-strains to obtain the strains listed in Supplementary Table S1.

SL-screen

The general strategy used in the SL-screen is depicted in Supplementary Figure S4. PCR products from genomic DNA, prepared from yeast strains carrying the desired deletion (EUROSCARF collection of *S. cerevisiae* gene deletion strains), were used to knock-out the corresponding genes in the strains NOY222, D101-I2, T4-1c, JAY444 and DLY200 by homologous recombination. Transformants were screened on YPD+G418 plates for the KanMX4 marker originally used to create the knock-outs. Gene deletions were verified via PCR. The resulting strains were transformed with the various phosphorylation site mutants of the respective Pol I subunit and screened for synthetic lethality or growth defects on SDC-Arg+Can for variants of NOY222 or SDC-Trp+5'FOA for the other shuffle-strains, respectively. T4-1c transformants were screened for genetic interactions on SDC-Trp plates.

6AU phenotyping

Equally sized colonies of yeast strains carrying the phosphorylation site mutations or the corresponding wild type genes were resuspended in H_2O and serial dilutions were spotted on SDC-Ura plates with increasing amounts of 6-Azauracil (6AU) and on YPD plates as control. Phenotypes were monitored after incubation at either 30 °C or 37 °C for 2 days (plates without 6AU) or 4-6 days (plates with 6AU).

Alkaline phosphatase treatment

Samples of the purified Pol I were incubated with alkaline phosphatase (Roche) or heat-inactivated alkaline phosphatase (95 °C, 30 min) for 20 min at 37 °C in buffer AP (250 mM KAc, 20 mM HEPES pH 7.8, 1 mM $MgCl_2$, 10 % glycerol). Afterwards Pol I was bound to Ni-NTA agarose (Qiagen) via the His_6/HA-tag on A43 for 2 h at 4 °C and the supernatant carefully removed for analysis. After washing with buffer AP1 (1.5 M KAc, 20 mM HEPES pH 7.8, 1 mM $MgCl_2$, 10 % glycerol, 0.5 % NP40, 10 mM imidazole, 1 mM PMSF, 2 mM benzamidine) and 20 mM HEPES pH 7.8, Pol I was eluted by incubation with SDS-sample buffer for 5 min at 95 °C. Supernatant and eluate were analyzed by western blotting after electrophoresis on 4-12 % NuPAGE™ Bis-Tris Gels (Invitrogen). Immunodetection of the Pol I subunits was performed using an anti-Pol I antiserum and visualized on a LAS-3000 chemiluminescence imager (Fujifilm).

Immunoprecipitation of Protein A-tagged Pol I

Yeast strains containing the various Pol I phosphorylation site mutations and a C-terminal Protein A-TEV-tag on A135 were cultured in 500 ml YPD to OD_{600} = 1. Cells were lysed with glass beads in buffer IP1 (400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 150 mM HEPES pH 7.6, 20 % glycerol, 5 mM β-mercaptoethanol, 1 mM PMSF, 2 mM benzamidine). The resulting extracts were cleared by centrifugation at 100,000 g for 35 min (Kontron TFT 55.38, with custom made adapters for use with Beckman 1.5 ml Microfuge™ tubes). 5 – 10 mg total protein was incubated with 40 µl IgG Sepharose™ 6 FF (GE Healthcare) for 2 h at 4 °C on a turning wheel. Washing steps were performed in 1 ml Mobicol columns (MoBiTec) using

buffers IP2 (1 M NaCl, 20 mM HEPES pH 7.6, 0.5 % NP40, 0.05 % Triton X-100) and IP3 (100 mM NaCl, 50 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 0.15 % NP40, 0.5 mM DTT). Pol I was eluted from the IgG-beads by cleavage of the tag with TEV protease for 2 h at 16 °C in buffer IP3 and analyzed by western blotting as described for the alkaline phosphatase treated Pol I samples.

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