2.1 Materials

2.1.1 Strains and plasmids
All *S. cerevisae* and bacterial strains and plasmids used in this study are listed in Table 2.1 and 2.2.

2.1.2 Antibodies
Antibodies used in this study are listed in Table 2.3.

2.1.3 Oligonucleotides
Oligonucleotides used in this study were designed manually by examining the relevant DNA sequences. Oligonucleotides were commercially synthesised at MWG Biotech Pvt. Ltd., Bangalore, India or Ocimum biosolutions, Hyderabad, India. Oligonucleotides used in this study are listed in Table 2.4 and 2.5.

2.1.4 Chemicals, kits and culture medium components
Agarose, phenol, dimethyl sulphoxide (DMSO), sodium acetate, sodium chloride, sodium carbonate, sodium bicarbonate, sodium dodecyl sulphate (SDS), formamide, calcium chloride, ethylenediaminetetraacetic acid (EDTA), glycerol, polyethylene glycol, ammonium persulphate, N, N, N’, N’-Tetramethylethylenediamine (TEMED), acrylamide, dithiothreitol (DTT), bis-acrylamide, chloroform, formaldehyde, lithium chloride, lithium acetate, isopropanol, nuclease free water, diethylpyrocarbonate (DEPC), Tween-20, acid washed glass beads, trisodium citrate dehydrate, β-mercaptopetoethanol, 0.4% trypan blue solution, yeast protease inhibitor cocktail, magnesium chloride, manganese chloride and phosphatase inhibitors were purchased from Sigma-Aldrich Chemicals. Agar, uracil, leucine, lysine, histidine, tryptophan, methionine, yeast extract, peptone, tryptone, and sorbitol were obtained from HiMedia. Dextrose, sucrose, potassium chloride, sodium hydroxide, hydrochloric acid, Tris and glycine were from Fisher Scientific. [14C]-labelled uracil was from Oogene Systems. γ[32P]ATP, [35S]Met/Cys in vivo protein twin label mix, α[32P]UTP and Taq DNA polymease were from JONAKI/BRIT, Ultima flow liquid scintillation fluid was obtained from Perkin-Elmer. Hybond-N+ and Hybond-P membranes for nucleic acid and protein transfer respectively, and protein A beads were purchased from GE Life Science. NuPAGE gradient gels, MES running buffer and 4X LDS sample buffer were purchased from Invitrogen. Super Signal West pico chemiluminiscent substrate was from Thermo Scientific. Different restriction enzymes used for cloning and knock-out generation were purchased from New England Biolabs (NEB). High-fidelity Phusion
Taq polymerase was from Thermo Scientific. Plasmid DNA purification, PCR purification, gel extraction and reaction clean up kits were procured from Qiagen. Medium components for growth of *S. cerevisiae*, namely, YPD, yeast nitrogen base, and yeast nitrogen base without ammonium sulphate were purchased from BD (Becton, Dickinson and Company, USA). Yeast synthetic dropout media supplement without uracil / histidine were obtained from Sigma-Aldrich.
Table 2.1. *S. cerevisiae* strains used in this study and their corresponding genotypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BY4741 WT</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>(Brachmann et al., 1998)</td>
</tr>
<tr>
<td><em>kcs1Δ</em></td>
<td>BY4741 kcs1Δ::kanMX4</td>
<td>(Saiardi et al., 2002)</td>
</tr>
<tr>
<td><em>vip1Δ</em></td>
<td>BY4741 vip1Δ::kanMX4</td>
<td>(Giaever et al., 2002)</td>
</tr>
<tr>
<td><em>kcs1Δ/pKCS1</em></td>
<td>BY4741 kcs1Δ::kanMX4, pKCS1(URA3)</td>
<td>(Dubois et al., 2002), this study</td>
</tr>
<tr>
<td><em>kcs1Δ/pkcs1</em></td>
<td>BY4741 kcs1Δ::kanMX4, pkcs1-S887A/L888A/L889A (URA3)</td>
<td></td>
</tr>
<tr>
<td><em>rpa34Δ</em></td>
<td>BY4741 rpa34Δ::kanMX4</td>
<td>(Giaever et al., 2002)</td>
</tr>
<tr>
<td><em>rpa43</em></td>
<td>BY4741 rpa43-S322A/S323A/S325A::nat1</td>
<td>This study, (Saiardi et al., 2002)</td>
</tr>
<tr>
<td>DDY1810 WT</td>
<td>MATa leu2-3, 112 trp1-Δ901 ura3-52 prb1-1122 pep4-3 prc1-407</td>
<td>(Onnebo and Saiardi, 2009)</td>
</tr>
<tr>
<td><em>kcs1Δ</em></td>
<td>DDY1810 kcs1Δ::LEU2</td>
<td>(Onnebo and Saiardi, 2009), this study</td>
</tr>
<tr>
<td><em>vip1Δ</em></td>
<td>DDY1810 vip1Δ::loxP</td>
<td></td>
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<tr>
<td><em>kcs1Δ/ddp1Δ</em></td>
<td>DDY1810 kcs1Δ::loxP, ddp1Δ::LEU2</td>
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</tr>
<tr>
<td><em>kcs1Δ/pKCS1</em></td>
<td>DDY1810 kcs1Δ::LEU2, pKCS1(URA3)</td>
<td>Werner, 2010 #510), this study</td>
</tr>
<tr>
<td><em>kcs1Δ/pkcs1</em></td>
<td>DDY1810 kcs1Δ::LEU2, pkcs1-S887A/L888A/L889A (URA3)</td>
<td></td>
</tr>
<tr>
<td>NOY222</td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pNOY20</td>
<td>(Wittekind et al., 1988)</td>
</tr>
<tr>
<td><em>RPA190 WT</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190</td>
<td>(Gerber et al., 2008)</td>
</tr>
<tr>
<td><em>rpa190</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-S1413/1415/1417A (TRP1)</td>
<td></td>
</tr>
<tr>
<td>NOY222 <em>rpa34Δ</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-S1413/1415/1417A (TRP1)</td>
<td>This study</td>
</tr>
<tr>
<td><em>RPA190 WT</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-TRP1</td>
<td></td>
</tr>
<tr>
<td><em>rpa34Δ</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-TRP1</td>
<td></td>
</tr>
<tr>
<td>NOY222 <em>rpa34Δ</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-TRP1</td>
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</tr>
<tr>
<td><em>RPA190 WT</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-TRP1</td>
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</tr>
<tr>
<td><em>rpa34Δ</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-TRP1</td>
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</tr>
<tr>
<td>NOY222 <em>rpa34Δ</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-TRP1</td>
<td></td>
</tr>
<tr>
<td><em>rpa43</em></td>
<td>MATa trp1-Δ1 his4-Δ401 leu2-3,112 ura3-52 canr rpa190Δ::URA3 pRS314-RPA190-TRP1</td>
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Materials and methods
### Table 2.2 Plasmids used in this study

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<th>Plasmid name</th>
<th>Representation in the text</th>
<th>Reference</th>
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<tr>
<td>pTOPO</td>
<td>Topo</td>
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</tr>
<tr>
<td>pTOPO NTS2</td>
<td>NTS</td>
<td></td>
</tr>
<tr>
<td>pTOPO-rDNA start</td>
<td>rDNA start</td>
<td></td>
</tr>
<tr>
<td>pTOPO-5'ETS</td>
<td>5'ETS</td>
<td></td>
</tr>
<tr>
<td>pTOPO-25S</td>
<td>25S</td>
<td></td>
</tr>
<tr>
<td>pRS314-RPA190 WT</td>
<td>RPA190 WT</td>
<td>(Gallagher et al., 2004)</td>
</tr>
<tr>
<td>pRS314-RPA190 S1413/1415/1417A</td>
<td>RPA190 WT S1413/1415/1417A</td>
<td>(Gerber et al., 2008)</td>
</tr>
<tr>
<td>pRS314-RPA43</td>
<td>pGP5</td>
<td></td>
</tr>
<tr>
<td>pFL38-KCS1</td>
<td>Kcs1 wild type</td>
<td>(Dubois et al., 2002)</td>
</tr>
<tr>
<td>pFL38-kcs1mut</td>
<td>Kcs1 mutant</td>
<td></td>
</tr>
<tr>
<td>pTOPO-ACT1</td>
<td>Not used in this study</td>
<td>This study</td>
</tr>
<tr>
<td>pUC-5S</td>
<td>Not used in this study</td>
<td>Dr. Purnima Bhargava</td>
</tr>
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### Table 2.3 Antibody used in this study with appropriate dilutions used.

<table>
<thead>
<tr>
<th>Name</th>
<th>Dilution used</th>
<th>Clonality</th>
<th>Company</th>
<th>Catalogue number</th>
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<tr>
<td><strong>Primary</strong></td>
<td></td>
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</tr>
<tr>
<td>Rabbit Anti GST Antibody</td>
<td>1:5000</td>
<td>Polyclonal</td>
<td>AbCam</td>
<td>19256</td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td></td>
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<tr>
<td>Goat Anti rabbit IgG</td>
<td>1:20,000</td>
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<td>Southern Biotech</td>
<td>4030-05</td>
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<tr>
<td><strong>Antibody used in Chip</strong></td>
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</tr>
<tr>
<td>Rabbit Anti GST Antibody</td>
<td>5 µg</td>
<td>Polyclonal</td>
<td>AbCam</td>
<td>19256</td>
</tr>
<tr>
<td>Normal rabbit IgG</td>
<td>3 µg</td>
<td></td>
<td>Invitrogen</td>
<td>10500C</td>
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</table>
Table 2.4. Primers used in this study to clone RNA Pol I subunits in pYesGex6p2-GST yeast expression vector.

<table>
<thead>
<tr>
<th>Name of the overexpression plasmid</th>
<th>Primers used to clone</th>
<th>Template and method used to generate.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pYesGex6p2-GST</strong></td>
<td></td>
<td>Bhandri, 2007</td>
</tr>
<tr>
<td><strong>pYesGex6p2-GST-RPA190 (1-556)</strong></td>
<td>Rpa190Nter1FP:</td>
<td>Template-pRS314 RPA190WT</td>
</tr>
<tr>
<td></td>
<td>CTGATGGATCCATGGATATTTTCAACC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rpa190Nter1RP:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CATGACTCGGCTATGCTTTACGTTGCTTAC</td>
<td></td>
</tr>
<tr>
<td><strong>pYesGex6p2-GST-RPA190 FRAG (557-1100)</strong></td>
<td>Rpa190Nter2FP:</td>
<td>Template-pRS314 RPA190WT</td>
</tr>
<tr>
<td></td>
<td>CTGATGGATCCCTTATGCTAACAACTATGGACAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rpa190Nter2RP:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CATGACTCGGCTACTTTTTAGAGTAGTACTTTCAAG</td>
<td></td>
</tr>
<tr>
<td><strong>pYesGex6p2-GST-RPA190 (1101-1664)</strong></td>
<td>Rpa190FP:</td>
<td>BY4741 WT gDNA</td>
</tr>
<tr>
<td>(pYesGexRPA190 1.7kb)</td>
<td>CTGATGGATCCACTTTGAATACAGAAGAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rpa190RP:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CATGACGGGCGCTTAAGCAGCGATTTGGAACC</td>
<td></td>
</tr>
<tr>
<td><strong>pYesGex6p2-GST-RPA190 (1338-1448)</strong></td>
<td>A190jawFP:</td>
<td>Template-pYesGex6p2-GST-RPA190 (1101-1664)</td>
</tr>
<tr>
<td>(jaw)</td>
<td>ATCTGGGATCCAGACTACAGACGACATTTG</td>
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<tr>
<td></td>
<td>A190jawRP:</td>
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<tr>
<td></td>
<td>CTATGCTCGGAGATTATTGTAGTTAGTCCACAATATCTC</td>
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<tr>
<td><strong>pYesGex6p2-GST-RPA190 (1338-1448)</strong></td>
<td>A190jawFP:</td>
<td>Template-pYesGex6p2-GST-RPA190 (1101-1664)</td>
</tr>
<tr>
<td>S1413, 1415, 1417/A (jaw S/A)</td>
<td>ATCTGGGATCCAGACTACAGACGACATTTG</td>
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<tr>
<td></td>
<td>MUT190UPRP:</td>
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<tr>
<td></td>
<td>CGACGTAGCATCAGCTTCTTTATCGGAATCGATACTCTTTCGTCAGAAG</td>
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<td>MUT190DNFP:</td>
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<tr>
<td></td>
<td>GATTCGGATAAAGAAGCTGATGCTGAGGCACAGCGATGTTGACATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A190jawRP:</td>
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</tr>
<tr>
<td></td>
<td>CTATGCTCGGAGATTATTGTAGTTAGTCCACAATATCTC</td>
<td></td>
</tr>
<tr>
<td><strong>pYesGex6p2-GST-RPA190 (1338-1448)</strong></td>
<td>A190jawFP:</td>
<td>By overlap extension PCR method</td>
</tr>
<tr>
<td>S1413, 1415, 1417/A (jaw S/A)</td>
<td>ATCTGGGATCCAGACTACAGACGACATTTG</td>
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<tr>
<td></td>
<td>MUT190UPRP:</td>
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<tr>
<td></td>
<td>CGACGTAGCATCAGCTTCTTTATCGGAATCGATACTCTTTCGTCAGAAG</td>
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<td></td>
<td>MUT190DNFP:</td>
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<tr>
<td></td>
<td>GATTCGGATAAAGAAGCTGATGCTGAGGCACAGCGATGTTGACATG</td>
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<tr>
<td></td>
<td>A190jawRP:</td>
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<tr>
<td></td>
<td>CTATGCTCGGAGATTATTGTAGTTAGTCCACAATATCTC</td>
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</tr>
<tr>
<td>Sequence Name</td>
<td>Primer Sequences</td>
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</tr>
<tr>
<td>--------------</td>
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| pYesGex6p2-GST-RPA34 FL (1-233) | RPA34FP: TAGATCGGATCCATGTCAAAGCTTTTCGAAAG  
RPA34RP: CATGAGC GCCCGCTCAATCTCTATGTTTCCTTTTC |
| pYesGex6p2-GST-RPA34 del (Δ 10-17a.a) | RPA34delFP: TAGATCGGATCCATGTCAAACCTTTCTAAAGGACTACGTGACGATGAAGTGATATCAAACGAGTTACG  
RPA34delRP: CATGAGCGGCCGCCTCAATCTCTATGTTTCCTTTTC |
| pYesGex6p2-GST-RPA34 1-144 (Δ 10-17a.a) (RPA34 N-frag) | RPA34delFP: TAGATCGGATCCATGTCAAACCTTTCTAAAGGACTACGTGACGATGAAGTGATATCAAACGAGTTACG  
A34FRAG_RP: CATGAGCGGCCGCCTATATTAGCGGTTCGCTGACGAAAAATAC |
| pYesGex6p2-GST-RPA34 1-144 (Δ 10-17a.a) S113,115,118/A | RPA34delFP: TAGATCGGATCCATGTCAAACCTTTCTAAAGGACTACGTGACGATGAAGTGATATCAAACGAGTTACG  
A34UP SDM_RP: TTTAAGGCTTCTCTAGTTCCGGCAGGAACAAATAGCTATATTAC  
A34DN SDM_RP: TTAATGGTTCTCTGCGGAAAGCTAGGACTCTTTAAATTGCTAGCTGGCAAG  
A34FRAG_RP: CATGAGCGGCCGCCTATATTAGCGGTTCGCTGACGAAAAATAC |
| pYesGex6p2-GST-RPA34 (1-204) (RPA34 C-trunc) | RPA34FP: TAGATCGGATCCATGTCAAAGCTTTTCGAAAG  
A34trunC-RP: CATGACTCGAGTCACTCTCCTTTCATCGTCGTGATGT |
| pYesGex6p2-GST-RPA34 FL (1-233) S205,206/A (RPA34 S-A) | RPA34FP: TAGATCGGATCCATGTCAAAGCTTTTCGAAAG  
A34FL S-A UPRP: CATTCTCTTTTTCTTTCTTTTCAGCAGCCCTCTCTTCATCGTCGTGATGT  
A34FL S-A DNF: CAAAGAAGCGGTTCATACGACGATGAAAGAGGAGGCTGCTGAAAAGAAGAAAAAGAAGAAG  
RPA34RP: CATGAGCGGCCGCCTCAATCTCTATGTTTCCTTTTC |

Materials and methods
<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYesGex6p2-GST-RPA34 FL (1-233) S205.206/D (RPA34 S-D)</td>
<td>RPA34FP: TGATCGGATCCATGTCCAGCTTCGAAAG A34FL_SDMS_DNFP: CCAAGAAAGCGTTCAATCATCAGCAGATGAAGGAGGATGATGAAAGAAGAAAGAAGAAGAAGG</td>
</tr>
<tr>
<td>pYesGex6p2-GST RPA43 FL (1-326)</td>
<td>RPA43FP: TGATCGGATCCATGTCAACAGTAAAAGAGCC RPA43RP: CATGACGCGCCGCTTAATCAGATC</td>
</tr>
<tr>
<td>pYesGex6p2-GST-RPA43 del (1-314)</td>
<td>RPA43FP: TGATCGGATCCATGTCAACAGTAAAAGAGCC MUT43RP: TACTGCCGTCGAGCTGTTTCCTCGATAGTACCATC</td>
</tr>
<tr>
<td>pYesGex6p2-GST-RPA43 FL S-A (S322,323,325/A)</td>
<td>RPA43FP: TGATCGGATCCATGTCAACAGTAAAAGAGCC RPA43_S-A_RP: CCATGACGCGAGCTTAATCAGATCAGAGCGCTTCACACCACATGGTTTCAGCTG</td>
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<tr>
<td>pYesGex6p2-GST-RPA43 frag (248-326)</td>
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<td>Description</td>
<td>Primer Sequence</td>
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| **pYesGex6p2-GST-RPA43 FL S-D (S322,323,325/D)** | RPA43FP: TGATCGGATCCATGTCACAAGGAAAGAGCC  
RPA43 S-D_RP: CATGACTCGAGCTAAATCATCAGATCGTCCTACCACATATTGGCTTTTCCTG | **Template pYesGex6p2-GST-RPA43 FL (1-326)** |
| **pYesGex6p2-GST-RPA135 (1-112)** | RPA135FP: TGATCGGATCCATGTCACAAGGAAAGAGCC  
RPA135RP: CATGACTCGAGCTAAATCATCAGATCGTCCTACCACATATTGGCTTTTCCTG | **BY4741WT gDNA** |
| **pYesGex6p2-GST-UAF30** | Uaf30FP: CTGATGGATCCATGTCACAAGGAAAGAGCC  
Uaf30RP: CATGACTCGAGCTAAATCATCAGATCGTCCTACCACATATTGGCTTTTCCTG | **BY4741WT gDNA** |
| **p416GPD-GST-RPA43** | By cut paste method from **pYesGex6p2-GST-RPA43 FL (1-326)** | **Template pYesGex6p2-GST-RPA43 FL (1-326)** |
| **p416GPD-V5-RPA34 FL (1-233)** | V5A34FP: TGATCGGATCCATGTCACAAGGAAAGAGCC  
V5A34RP: CATGACTCGAGCTAAATCATCAGATCGTCCTACCACATATTGGCTTTTCCTG | **Template pYesGex6p2-GST-RPA34 FL (1-233)** |
| **p416GPD-V5-RPA34 (1-233) S205,206/A (RPA34 S-A)** | V5A34FP: TGATCGGATCCATGTCACAAGGAAAGAGCC  
V5A34RP: CATGACTCGAGCTAAATCATCAGATCGTCCTACCACATATTGGCTTTTCCTG | **Template pYesGex6p2-GST-RPA34 FL (1-233)** |
| **pYesGex6p2-GST-RPA190 (86aa)** | A19086aa-FP: CTGATGGATCCATGTCACAAGGAAAGAGCC  
A19086aa-RP: CATGACTCGAGCTAAATCATCAGATCGTCCTACCACATATTGGCTTTTCCTG | **Template pYesGex6p2-GST-RPA190 (1-556)** |
| **Sequencing primers** | Rpa190FPseq: GAAATGCAGCTCGTTCGTATG  
Rpa190RPsseq: GATGACTGATTTATGATAT | **Template pYesGex6p2-GST-RPA190 (1-556)** |
<p>| | pYesGex6p2RP (name pGex6p2RP is printed on the side but the original name yesGex6p2RP is written on the white label on the top) | <strong>Template pYesGex6p2-GST-RPA190 (1-556)</strong> |
| | p426GPDRP: GCGTGAATGTAAGCGTGAC | <strong>Materials and methods</strong> |</p>
<table>
<thead>
<tr>
<th>Primer not used</th>
<th>A43_251_RP: CATGACTCGAGCTAACCGTCCACGAAACAACCCTTCC</th>
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<tr>
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<td>RPA49RP:</td>
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|           | A43gen-NAT-3R (primer6)  
|           | GTCAGAAGCATTTTTTGCTCGCGCCGCTGACGAAAGTTTCTATTC | Template - NAT plasmid |
| Product 4 | A43gen-4F (primer7)  
|           | GAATAGGAACTCTCGTACGCGCCGACGACAAACATGTCTTCTGAC  
|           | A43-ubc-4R (primer8)  
|           | ATATCGTCTACGGGAAACGCAC | Template - pGP5 pRS314 RPA43 plasmid obtained from Dr. Herbert Tschochner |
Table 2.5 Primers and probes used in rRNA analysis

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2.1.5 Media

2.1.5.1 Yeast media (Media composition was followed as described by Sigma product data sheet)

**Yeast extract Peptone Dextrose (YPD)**
- 1% Yeast extract
- 2% Peptone
- 2% Dextrose

**Yeast synthetic complete medium (SC)**
- 0.67% Yeast Nitrogen Base with amino acids
- 2% Dextrose
- 1.92 g/L Yeast Synthetic Drop-Out media supplement without Uracil
- 76 mg/L uracil

**Yeast synthetic complete medium without histidine (SC-His)**
- 0.67% Yeast Nitrogen Base without amino acids
- 1.92 g/L Yeast Synthetic Drop-Out media supplement without histidine
- 2% Dextrose

**Yeast synthetic complete medium without uracil (SC-Ura)**
- 0.67% Yeast Nitrogen Base without amino acids
- 1.92 g/L Yeast Synthetic Drop-Out media supplement without Uracil
- 2% Dextrose

**Yeast synthetic complete medium without methionine (SC-Met)**
- 0.67% Yeast Nitrogen Base without amino acids
- 380 mg/L Leu
- 76 mg/L His
- 76 mg/L Ura
- 2% Dextrose

**Yeast synthetic complete medium without tryptophan (SC-Trp)**
- 0.67% Yeast Nitrogen Base without amino acids
- 380 mg/L Leu
- 76 mg/L His
- 76 mg/L Ura
- 76 mg/L Met
- 2% Dextrose
Materials and methods

Yeast synthetic complete medium without leucine (SC-Leu)

- 0.67% Yeast Nitrogen Base without amino acids
- 76 mg/L His
- 76 mg/L Ura
- 76 mg/mL Trp
- 76 mg/mL Met
- 2% Dextrose

Yeast sporulating medium

- 1% Potassium acetate
- 0.05% Dextrose

2.1.5.2 Luria-Bertani (LB) medium for bacterial growth

- 0.5% Yeast Extract
- 1% Tryptone
- 1% NaCl

LB-ampicillin plates

- LB medium
- 100 μg/mL ampicillin

Media and solutions were sterilized either by routine autoclaving at 121°C and 15 psi for 20 min or by filtration through membrane of 0.22 μm porosity.

For yeast and bacterial growth, plates were prepared by adding 2% to the medium before autoclaving.

2.1.6 Buffers and solutions

2.1.6.1 Common buffers

Phosphate Buffered Saline (PBS)

- 137 mM NaCl
- 2.7 mM KCl
- 10 mM Na$_2$HPO$_4$
- 2 mM KH$_2$PO$_4$

pH was adjusted to 7.3 using HCl and NaOH before autoclaving. PBS was prepared as a 10X stock solution and diluted to 1X concentration before autoclaving.
EDTA (pH 8.0)
186.1 g of EDTA.2H2O was dissolved into 800 mL of water stirred vigorously and the pH was adjusted with NaOH pellets. When the pH of the solution reached 8.0 EDTA dissolved completely and was made upto 1000 mL with water.

Tris-HCl buffer (1M)
121.1 g of Tris base was dissolved in 800 mL of water and pH was adjusted to 7.2 using concentrated HCl.

Tris-EDTA (TE) buffer
10 mM Tris-HCl, pH 8.0
1 mM EDTA

Tris-Acetic acid EDTA (TAE) buffer
40 mM Tris base
1mM EDTA
pH was adjusted to 8.4 with glacial acetic acid. TAE buffer was prepared as a 50X stock solution and used at 1X concentration.

Tris-Saline
20 mM Tris-HCl, pH 7.2
0.9% NaCl

2.1.6.2 Buffers for extraction and analysis of genomic DNA and RNA

Yeast lysis buffer for genomic DNA extraction
50 mM Tris-HCl, pH 8.0
10 mM EDTA
150 mM NaCl
1% Triton-X
1% SDS

AE buffer for RNA extraction
50 mM Sodium acetate, pH 5.3
1 mM EDTA, pH 8.0
Solution was made in DEPC treated water. 0.2% diethyl pyrocarbonate (DEPC) was added to the water and stirred for 12 h. To remove DEPC, water was autoclaved twice.

DNA sample loading buffer (6X)
15.25 mg Bromophenol blue
15.25 mg Xylene cyanol
Materials and methods

30% Glycerol
Made in 100 mL.

RNA sample loading buffer (10X)

- 50% glycerol
- 10 mM EDTA
- 0.025% Bromophenol blue
- 0.025% Xylene cyanol

Inoue transformation buffer, pH 6.7 (125 mL, prepared just before use)

- 10 mM PIPES
- 15 mM CaCl$_2$.2H$_2$O
- 250 mM KCl
- 55 mM MnCl$_2$.4H$_2$O (1.361 g is dissolved in 10 mL of water separately)

PIPES (0.307 g), CaCl$_2$.2H$_2$O (0.275 g) and KCl (2.325 g) were added to 80 mL of sterile water while mixing with a magnetic stirrer and the pH was adjusted to 6.8 with 1 N KOH. After attaining the appropriate pH, MnCl$_2$ solution was added slowly in aliquotes of 300 µL over 10 min, while stirring to avoid a brown precipitate.

MOPS buffer (10X)

- 0.2 M MOPS, pH 7.2
- 20 mM CH$_3$COONa
- 10 mM EDTA

Buffer was made in DEPC treated water

Yeast transformation reagents

- 1 M Lithium acetate
- 50% Polyethylene glycol
- 2 mg/mL Salmon sperm carrier DNA
- Dimethyl sulfoxide (DMSO)

Zymolyase cocktail buffer for yeast colony PCR

- 2.5 mg/mL Zymolyase (Zymo Research)
- 1.2 M Sorbitol

Zymolyase buffer was prepared in 1X PBS.

2.1.6.3 Buffers for ribosome and polysome analysis

Lysis buffer

- 10 mM Tris, pH 7.4
Materials and methods

100 mM NaCl
30 mM MgCl₂
50 μg/mL cycloheximide
200 μg/mL heparin

All the components were made in DEPC treated water.

**Gradient buffer**

**10% sucrose gradient buffer**

50 mM Tris-HCl, pH 7.4
50 mM NH₄Cl
12 mM MgCl₂
1 mM DTT
0.1% DEPC
10% sucrose solution

To analyse individual ribosome subunits, MgCl₂ was eliminated from the gradient buffer.

**30% sucrose gradient buffer**

50 mM Tris-HCl, pH 7.4
50 mM NH₄Cl
12 mM MgCl₂
1 mM DTT
0.1% DEPC
30% sucrose

To analyse individual ribosome subunits, MgCl₂ was eliminated from the gradient buffer.

**50% sucrose gradient buffer**

50 mM Tris-HCl, pH 7.4
50 mM NH₄Cl
12 mM MgCl₂
1 mM DTT
0.1% DEPC
50% sucrose

To analyse individual ribosome subunits, MgCl₂ was eliminated from the gradient buffer.

**37% sucrose gradient buffer**
Materials and methods

50 mM Tris-HCl, pH 7.4
50 mM NH₄Cl
12 mM MgCl₂
1 mM DTT
0.1% DEPC
37% sucrose solution

2.1.6.4 Buffers for transcription run on analysis

TMN buffer
10 mM Tris-HCl, pH 7.4
5 mM MgCl₂
100 mM NaCl

Permeabilization buffer
950 µL of cold water
50 µL of 10% (wt/vol) sodium N-lauroyl sarcosine

Transcription assay buffer (100 µL)
50 mM Tris-HCl, pH 7.4
100 mM KCl
5 mM MgCl₂
1 mM MnCl₂
2 mM dithiothreitol
0.5 mM ATP
0.25 mM GTP
0.25 mM CTP
10 mM phosphocreatine
2.4 units creatine phosphokinase
100 µCi [α-³²P] UTP (3,000 Ci/mmol)

Alkaline denaturing solution for DNA for membrane preparation
0.5 M NaCl
0.25 M NaOH
Volume was adjusted to 20 mL with sterile water.

Saline Sodium Citrate (SSC) buffer (20X)
3.0 M Sodium chloride
0.3 M Sodium citrate
Volume was adjusted with water to 1 L and solution was sterilized by autoclaving.

**Pre-hybridization / hybridization buffer (Modified Church and Gilbert buffer)**

- 0.5 M phosphate buffer (134 g of Na$_2$HPO$_4$.7H$_2$O, 4 mL of 85%H$_3$PO$_4$), pH7.2
- 7% (w/v) SDS
- 10 mM EDTA

Volume was adjusted to 1 L with DEPC treated sterile water. Buffer was aliquoted into 50 mL RNase free conical tubes (Corning) and stored in -20°C.

**Post hybridization wash buffers**

- **Wash buffer 1**
  - 2X SSC
  - 0.1% SDS

- **Wash buffer 2**
  - 1X SSC
  - 0.1% SDS

- **Wash buffer 3**
  - 0.5X SSC
  - 0.1% SDS

Buffers were prepared with sterile DEPC treated water.

**2.1.6.5 Buffers for chromatin immunoprecipitation**

**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 (added fresh)

**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
Materials and methods

Wash buffer II

10 mM Tris-HCl, pH 8.0
1 mM EDTA
250 mM LiCl
0.75% NP-40
0.75% sodium deoxycholate
Protease inhibitor cocktail

Elution buffer II

50 mM Tris-HCl, pH 8.0
10 mM EDTA
1% SDS

2.1.6.6 Buffers for IP_7 purification

Buffer A

1 mM EDTA in HPLC grade water (Fisher Scientific)

Buffer B

1 mM EDTA
(NH₄)₂HPO₄ 1.3 M, pH 3.8
171.6 g of (NH₄)₂HPO₄ was dissolved in 750 mL of HPLC grade water. pH was adjusted to 3.8 with 75 mL of H₃PO₄ by continuous stirring and the volume was made upto 1000 mL.

Both buffers were filtered through a 0.22 µm filter (Millipore) using vacuum filter apparatus (Tarsons) and degassing was performed at least for 20 min using a vacuum pump.

2.1.6.7 Buffers for protein purification and IP_7 reaction

Buffer A

20 mM HEPES pH 6.8
100 mM NaCl
2 mM EDTA
5 mM DTT
Yeast protease inhibitor cocktail and phosphatase inhibitor cocktail (added fresh to the buffer A)

Buffer B

20 mM HEPES pH 6.8
100 mM NaCl
Materials and methods

2 mM EDTA
5 mM DTT
1% Triton-X
Yeast protease inhibitor cocktail and phosphatase inhibitor cocktail (added fresh to the buffer B)

Buffer C
20 mM HEPES
500 mM NaCl
2 mM EDTA
1% Triton-X
Yeast protease inhibitor cocktail and phosphatase inhibitor cocktail (added fresh to the buffer C)

IP\textsuperscript{7} reaction buffer (10X)
250 mM HEPES, pH 7.4
500 mM NaCl
60 mM MgCl\textsubscript{2}
10 mM DTT (1 M stock was made separately, aliquoted into 100 µL and stored at -20°C).
10X buffer was made and stored at 4°C. An appropriate amount was added to the reaction mix to get a final concentration of 1X.
DTT was added fresh to the reaction buffer just before use.

2.1.6.8 Buffers for SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

Whole cell lysis buffer for yeast (Homogenizing buffer)
50 mM Tris-HCl, pH 7.5
2 mM EDTA
yeast protease inhibitor cocktail

SDS-PAGE 30% Acrylamide solution
29 g acrylamide 1 g bis-acrylamide dissolved in 100 mL H\textsubscript{2}O.

10% sodium dodecyl sulfate (SDS)
10 g SDS in 100 mL H\textsubscript{2}O

Stacking gel mix (6%) (5 mL)
3.4 mL H\textsubscript{2}O
0.63 mL 30% acrylamide:bisacrylamide (29:1) mix
0.83 mL 1.5 M Tris-HCl, pH 6.8
50 μL 10% SDS
50 μL 10% Ammonium persulfate (APS)
8 μL N, N', N'-Tetramethylethylenediamine (TEMED)

**Resolving gel mix (12%) (20 ml)**
6.6 mL H₂O
8 mL 30% acrylamide: bisacrylamide (29:1) mix
5 mL 1.5 M Tris-HCl, pH 8.8
200 μL 10% SDS
200 μL 10% Ammonium persulfate (APS)
8 μL N, N', N'-Tetramethylethylenediamine (TEMED)
2.2 Methods

2.2.1 Strains and culture conditions

*S. cerevisiae* strains were routinely grown either in rich YPD medium or synthetic complete medium (SC) (Section 2.1.5.1) at 30°C with continuous shaking at 200 rpm unless otherwise stated. In general, *S. cerevisiae* frozen glycerol stocks were revived on 2% YPD medium by streaking and allowed to grow for 1-2 days. *S. cerevisiae* strains harbouring a plasmid containing the *URA3* gene as the auxotrophy selection marker were revived on synthetic complete medium lacking uracil (SC-Ura). To prepare liquid cell culture, a single colony of each *S. cerevisiae* strain was inoculated either in YPD or SC-Ura medium and grown for 14-16 h. *S. cerevisiae* strains streaked on plates were sealed with paraffin film (parafilm M) and stored at 4°C for a maximum period of 2 weeks. Protein overexpression in yeast was carried in presence of galactose instead of glucose as the carbon source, as the plasmid pYesGex 6p2 carries the GAL promoter under which yeast proteins were expressed.

2.2.2 Growth analysis and determination of generation time

For growth analysis of *S. cerevisiae* strain, single colony was inoculated in appropriate broth medium and grown over night. This culture was used to inoculate the test medium to an initial OD600 of 0.1. Cultures were transferred to a shaker incubator set at 30°C and 200 rpm. Absorbance of cultures was measured using Ultraspec 2100 pro UV/visible spectrophotometer (Amersham Biosciences) at 600 nm at regular time-intervals till 72 h. Absorbance values were plotted with respect to time and the generation time was calculated from the logarithmic phase of the growth curve, by plotting $A_{600}$ vs. time on a semi-logarithmic scale, using GraphPad Prism5 for curve fitting analysis.

2.2.3 Cell mass, cell number and viability assessment

To determine yeast cell mass, cells equivalent to 5 OD$_{600}$ were harvested from mid-log and overnight grown cultures, and washed twice 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 OD$_{600}$ was calculated.
To monitor yeast cell death 1 OD$_{600}$ equivalent cells from mid-log and overnight grown cultures were pelleted, washed in PBS and the cell pellet was suspended in 100 µL of PBS. 20 µL of 0.4% trypan blue solution was added to 20 µL of cell suspension and incubated for 10 min. 20 µL of this suspension was placed on a slide, covered with a cover slip, and cell death was measured by scoring dead cells that take up the dye.

To monitor cell viability, cells equivalent to 10$^{-5}$ OD$_{600}$ 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 OD$_{600}$.

### 2.2.4 Drug sensitivity analysis

Analysis of sensitivity to translation inhibitors was conducted in the DDY1810 S. cerevisiae strain background, which does not contain the kan$^r$ selection marker. Sensitivity to 6-azauracil (6AU) was monitored in the DDY1810, BY4741 or NOY222 strain backgrounds (Table 2.1). As uracil is a competitive inhibitor of 6AU, the plasmid p416GPD, carrying the URA3 gene (Mumberg et al., 1995) was introduced into BY4741-derived strains whereas DDY1810 derived yeast strains were supported by the pYesGex plasmid carrying the URA3 gene.

Yeast strains were grown in YPD or SC-Ura medium, for 14-16 h at 30°C under continuous shaking at 200 rpm. Cultures were diluted to 0.25 OD$_{600}$, followed by 5 fold serial dilutions, and 3µL of each dilution was spotted on a YPD-agar plate containing the translation inhibitors G418 (8 µg/mL), paromomycin (100 µg/mL or 200 µg/mL), or hygromycin B (8 µg/mL), or an SC-Ura agar plate, containing 6AU 50 µg/mL or 100 µg/mL and growth was monitored at 30°C or 37°C for 2-3 days.

To perform an analysis of 6AU sensitivity with yeast carrying pYesGex6p2 plasmid, cells were grown overnight in SC-Ura medium and the serial dilutions were plated on SC-Ura medium containing 6AU, with galactose instead of glucose to express proteins under the GAL4 promoter.

### 2.2.5 Protein synthesis analysis

Wild type and knock out yeast strains were grown in YPD (Difco) whereas synthetic complete medium without uracil was used for the KCS1 complemented strains. Overnight grown yeast were subcultured in appropriate medium at 0.2 OD$_{600}$ and
Materials and methods

grown till the OD$_{600}$ reached 0.8-1.0. Cells equivalent to 1 OD$_{600}$ of each culture was taken for the labelling of total protein. Cells were washed in methionine-free synthetic complete medium (SC-Met) twice, suspended in SC-Met medium containing 25 μCi/mL of $^{35}$S Met-Cys twin label mix and incubated for 1 min, 5 min and 15 min. Cells were washed twice in ice-cold SC-Met medium twice and suspended in 500 μL of Tris-saline containing protease inhibitor cocktail. To this, 300 μL of glass beads (0.45-0.6 mm diameter) were added and cells were lysed for 10 min by bead beating (with intervals of 1 min on time and 30 sec off time). The lysate was centrifuged at high speed for 15 min at 4°C. To the supernatant, sodium deoxycholate was added to a final concentration of 0.1 mg/mL and incubated on ice for 30 min. To this solution, 20% trichloroacetic acid was added to a final concentration of 6%, incubated for 1 h on ice, and centrifuged at high speed for 20 min at 4°C. The pellet was suspended in 300 μL of Tris-saline and counted in a liquid scintillation counter (Perkin Elmer-Tricarb 2900). The cpm values obtained were plotted using GraphPad Prism5.

2.2.6 $^{35}$S-Met uptake assay

Yeast were grown in YPD (Difco) overnight, and sub-cultured at 0.2 OD$_{600}$. Cells were harvested at 0.6-0.8 OD$_{600}$. 1 OD$_{600}$ of each culture was used for the labelling. Cells were washed in SC-Met medium twice, suspended in SC-Met medium containing 25 μCi/mL of $^{35}$S Met-Cys and pulsed for 15 min. Cells were washed twice in methionine-free medium and suspended in 300 μL of Tris-saline. Cell suspension was counted in a liquid scintillation counter (Perkin Elmer-Tricarb 2900). The cpm values obtained were plotted using GraphPad Prism5.

2.2.7 Ribosome profiles

The method to analyse ribosome profiles was adapted from (Lee et al., 1992). Yeast cells were grown to 0.2 to 0.8 OD$_{600}$ at 30°C in 200 mL of YPD, and cycloheximide was added to this media at 50 μg/mL final concentration. The culture was placed and mixed continuously on an ice and salt mixture for 2-5 min and centrifuged immediately at 4,000 x g. The culture was not allowed to stay for a longer time on the ice and salt mixture to avoid freezing. The cell pellet was suspended in 1 mL of lysis buffer (Section 2.1.6.3), and transferred to a 2 mL microfuge tube, to which 1 mL glass beads (0.45-0.6 mm diameter) were added, and lysed by bead beating for 10 min with intervals (30 sec on time and 1min off time). During the 1 min off time, tubes were
Materials and methods

placed on ice. The samples were centrifuged at 10,000 x g for 10 min at 4°C and the supernatant was extracted by avoiding the glass beads. Absorbance of the lysate was measured at 260 nm, and it was divided into 200 µL aliquots and frozen at -80°C. No difference in the ribosome profiles were detected between these samples and fresh samples analysed immediately.

Polysomes were analysed by centrifugation through a 10-50% sucrose continuous gradient. The gradient was prepared by pipetting, gradient buffers (Section 2.1.6.3), 5 mL of each layer onto the bottom of an 11 mL open top centrifuge tube, covering the tube with paraffin film and by placing it horizontally on a flat surface at 4°C for 2 h. The ribosome sample (cell lysate) was loaded on top, and gradient was centrifuged at 100,000 x g at 4°C for 6 h in an SW41 rotor (Beckman). An amount of lysate equivalent to 10 A_{260} units was loaded on the gradient. Ribosome levels were measured by the gradient analysis with an ISCO UV-6 gradient collector with continuous monitoring at A_{254}.

Polysome purification was performed by layering the cell lysate on 37% sucrose solution in an 11 mL open top centrifuge tube, and centrifuged at 100,000 x g for 14 h in an SW41 rotor (Beckman). The pellet was suspended in 50 to 100 µL of lysis buffer. An amount of lysate equivalent to 0.7 A_{260} units was loaded on the gradient. Ribosome levels were measured by gradient analysis with an ISCO UV-6 gradient collector with continuous monitoring at A_{254}.

Ribosome subunits were analysed by centrifugation through a 10-30% sucrose continuous gradient. The gradient was prepared by pipetting 2.2 mL of each layer onto the bottom of a 5 mL open top centrifuge tube, sealing it with paraffin film and placing it horizontally on a flat surface at 4°C for 2 h. The ribosome sample was loaded on top, and gradient was centrifuged at 100,000 x g and 4°C for 4.5 h in an SW55 rotor (Beckman). Ribosome subunit levels were measured by gradient analysis with an ISCO UV-6 gradient collector with continuous monitoring at A_{254}. Ribosome profiles with yeast cell lysates and purification of ribosomes from yeast were performed with the help of Mr. Aluri Srinivas and Dr. Umesh Varshney.
2.2.8 RNA extraction by hot-phenol method

Yeast were grown till mid-log phase 0.6-0.8 OD<sub>600</sub> in an appropriate medium and 10 mL of culture was pelleted at 2500 x g. Cells were suspended in 350 μL of AE buffer, mixed with 50 μL of 10% sodium dodecyl sulphate and 400 μL acid phenol (pH 4.3) and immediately shaken vigorously on a dry bath (Eppendorf) at 65°C for 15 min. The tube was then quickly chilled on ice and centrifuged at 12000 x g for 15 min to separate the aqueous phase from the phenol. After centrifugation, the aqueous phase was transferred to a new tube and extracted with an equal volume of chloroform. RNA was precipitated by adding 50 μL of 3 M sodium acetate (pH 5.3) and equal volume of 100% ethanol followed by incubation at -20°C for 2 h, and centrifugation at maximum speed for 30 min at 4°C. The pellet obtained was washed in 70% ethanol, dried at room temperature and dissolved in an appropriate volume of DEPC-treated water. The concentration of RNA was estimated by measuring A<sub>260</sub> using a Nano Drop Spectrophotometer (ND1000). To monitor different classes of rRNA levels, 10 μg of total RNA from each strain was resolved on a 1.2% formaldehyde-agarose gel.

2.2.9 Preparation of formaldehyde agarose gel (50 mL) and RNA sample

A mixture of 40 mL of DEPC treated water and 0.6 g of agarose was melted by boiling. After cooling down the temperature to 55°C, 8.4 mL of formaldehyde (final concentration 2.2 M) and 5 mL of 10X MOPS were added, mixed well and poured into a boat to cast a gel.

<table>
<thead>
<tr>
<th>RNA sample preparation for loading:</th>
<th>Volume (for 30 μL)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>5.5</td>
<td>2.2 M</td>
</tr>
<tr>
<td>Formamide</td>
<td>15</td>
<td>50%</td>
</tr>
<tr>
<td>10X MOPS buffer</td>
<td>1.5</td>
<td>0.5X</td>
</tr>
<tr>
<td>RNA</td>
<td>Volume of water and RNA are interchangeably, and up to 8 μL.</td>
<td></td>
</tr>
<tr>
<td>water</td>
<td>200 ng final concentration</td>
<td></td>
</tr>
</tbody>
</table>

RNA samples were denatured by heating at 55°C for 15 min, 3 μL 10X RNA loading dye was added and loaded into the gel.
2.2.10 Capillary transfer of RNA on to the membrane

After the RNA samples were resolved, the gel was placed in a tray and incubated for 5-10 min in DEPC treated water followed by 10X SSC buffer. A nylon membrane (N+ Hybond, GE Life Sciences) was cut to the size of the gel and it was rinsed in 10X SSC buffer for 5 min. In the same tray, 10X SSC buffer was filled and a gel running boat (15 cm length) was placed in an inverted position. Whatman number 3 filter paper (wick) was cut to an appropriate size and placed on the inverted boat in such a way that the longer edges of the paper should touch the buffer. The gel was placed upside down on the wick and care was taken not to allow any air bubbles between the gel and wick. On top of the gel, three Whatman number 3 papers that were cut to the size of gel were placed by avoiding the air bubbles. A bundle of blotting sheets was placed on top of this, on which appropriate weight was placed and was allowed the transfer to take place for 15 – 16 h.

2.2.11 [14C]uracil labelling of total RNA

Overnight grown yeast were sub-cultured at 0.2 OD$_{600}$ and grown till 0.8 OD$_{600}$. Cells equivalent to 1 OD$_{600}$ were harvested and washed twice with SC-Ura medium to remove any residual uracil from the cells. Cells were incubated in SC-Ura medium containing 3 μCi/mL [14C]uracil for 1, 5, 10 and 20 min, and RNA was extracted as described in Section 2.2.8. Equal total RNA was resolved on a formaldehyde agarose gel and transferred to an N+ Hybond membrane (GE Life Sciences). Radiolabeled rRNA was detected using a phosphorimager scanner (Fujifilm FLA-9000).

2.2.12 Pulse-chase analysis of rRNA synthesis

Overnight grown yeast were sub-cultured at 0.2 OD$_{600}$ and grown till 0.8 OD$_{600}$. Cells equivalent to 1 OD$_{600}$ were harvested and were washed in SC-Ura medium, suspended in 1 mL of SC-Ura medium containing 3 μCi/mL of [14C]uracil and pulse labelled for 15 min at 30°C. After incubation the cells were pelleted and a chase was performed by adding SC medium containing 240 mg/L unlabelled uracil. Samples were taken at 0, 1, 5, 10 and 20 min after the chase, centrifuged at 12000 x g for 1 min at 4°C, and total RNA was isolated from cells by the hot-phenol technique described in Section 2.2.8. Equal total RNA was loaded on a 1.2% formaldehyde-agarose gel. After transfer of
labelled RNAs to N⁺ Hybond membrane (GE Life Science), the radiolabelled rRNA was detected using a phosphorimager (Fuji Film FLA-9000).

2.2.13 Uracil uptake assay

Cells were grown in YPD to an OD$_{600}$ of 0.5-0.7. Cells equivalent to 1 OD$_{600}$ were washed with synthetic complete medium without uracil twice and suspended in SC-Ura containing 3 μCi/mL of $[^{14}\text{C}]$ uracil for 5 min. Cells were pelleted and washed with SC-Ura medium twice and suspended in 0.5 mL of AE solution (Section 2.1.6.2). 1 OD$_{600}$ of this cell suspension was counted in a liquid scintillation counter (Perkin Elmer- Tricarb 2900). The cpm values obtained were and converted into moles based on the specific activity of $[^{14}\text{C}]$ uracil and plotted using GraphPad Prism5.

2.2.14 Dot blot membrane preparation

The following plasmids (Table 2.2) were used as probes to detect the transcribed RNA (Fig. 2.1). The TOPO plasmids containing rDNA start (+1 to +177), 5’ETS (+351 to +610), end 5’ETS (+611 to +952), 25S (+5270 to +5630) and NTS2 (gifted by Dr. Susan J Baserga) (Gallagher et al., 2004); ACT1 cDNA (+175 to +701) cloned into TOPO vector; pUC12 plasmid containing 5S rDNA construct (gifted by Dr. Purnima Bhargava). Empty TOPO plasmid and genomic DNA extracted from wild type yeast were used as controls. Plasmids and gDNA were extracted, quantitated and denatured in alkaline denaturing solution. 10 μg of each plasmid and gDNA in replicates were denatured in alkali, blotted on a Hybond N⁺ membrane using a 96-well Dot Blot

Figure 2.1 Diagramatic representation of a single rDNA unit. The *S. cerevisiae* rDNA locus consists of approximately 150 copies of rDNA units, where each unit is 9.1 kb. Each unit consists of NTS (non-transcribed sequences), ETS (external transcribed sequences) ITS (internal transcribed sequences), 18S, 5.8S and 25S sequences, whereas 5S is outside the rDNA unit and it separates two rDNA units. Angled arrows represent direction of transcription and straight arrows indicate the probes used for hybridization.
apparatus. Membranes were neutralised in 2X SSC, and denatured plasmids were cross-linked to Hybond-N+ membranes using a UV cross linker at 2000 J/inch square energy for 2 min.

2.2.15 Transcription run on analysis

Yeast were grown in appropriate medium while the logarithmic phase and 1 OD₆₀₀ cells were harvested and chilled on an ice (Elion and Warner, 1986). All subsequent steps, unless specified, were carried out at 4°C or on ice. Cells were collected by centrifugation at 2500 g for 6 min and washed with 2 mL of TMN (Section 2.1.6.4). Cells were suspended in 1 mL of ice cold permeabilization buffer (Section 2.1.6.4) and incubated for 15 min. Cells were pelleted and incubated with 100 μL of transcription assay buffer (Section 2.1.6.4) containing [α-³²P]UTP. After incubation for 10 min at 30°C and 300 rpm in a shaking dry bath (Eppendorf), 1 mL of cold TMN containing 1 mM nonradioactive UTP was added, the cells were collected by centrifugation, and RNA was prepared by the hot phenol method as described in Section 2.2.8. Equal counts of labelled RNA were used for hybridization. The membrane was pre incubated with 50 mL of hybridization buffer for 20-30 min, and hybridization was performed with labelled RNA at 65°C for 15 h in 20 mL of hybridization buffer.

2.2.16 Post-hybridization washes

After 14-16 h incubation, hybridization buffer was decanted to a radioactive liquid waste container. Membranes were washed twice with 2X SSC (saline-sodium citrate) containing 0.1% SDS for 15 min at 55°C followed by two washes with 1X SSC containing 0.1% SDS for 15 min at room temperature. Post washes, membranes were rinsed with 1X SSC buffer at room temperature exposed to a phosphorimager screen for 1 h and scanned using a phosphorimager (Fuji Film FLA-9000). The data were analysed by densitometry using Fuji Film Multigauge software V3.11 and graphs were plotted using GraphPad Prism5 software.

Note: Depending on signal saturation or non-specificity, high stringency washes were performed starting from 0.5X SSC followed by 0.2X SSC or 0.1X SSC wash buffers containing 0.1% SDS at room temperature.
2.2.17 Genomic DNA isolation by glass bead lysis method

The desired *S. cerevisiae* strain was grown overnight in YPD liquid medium and yeast cells were harvested by centrifugation at 2,500 x g in 15 mL polypropylene tubes. Yeast cells were washed with PBS, suspended in 500 μL lysis buffer (Section 2.1.6.2) and were transferred to a 1.5 mL microcentrifuge tube. Yeast cells were incubated for 15 min on a thermomixer set at 65°C and 750 rpm. After incubation, a volume equivalent to 500 µL of glass beads (0.5 mm) were added and cells were lysed on a beating apparatus by mixing three times for 45 seconds each with intermittent cooling on ice to prevent overheating. Cell lysates were centrifuged at 12,000 x g for 5 min and upper aqueous phase was transferred carefully to a new 1.5 mL microcentrifuge tube, to which 275 µL of 3M sodium acetate was added. To this solution, 500 µL of chloroform was added, mixed well, and centrifuged at 12,000 x g for 5 min at 4°C (this step was repeated twice). Supernatant was transferred to a new 1.5 mL microcentrifuge tube and 500 µL of isopropanol was added and mixed well by inverting the tube 3-4 times. To precipitate genomic DNA, the suspension was centrifuged at 12000 x g for 15 min at 4°C. Precipitated genomic DNA was washed with 70% ethanol and dried at room temperature. The genomic DNA pellet was dissolved either in 100 μL of Sigma molecular biology grade water or TE buffer supplied with Qiagen plasmid mini prep kit, and add 1 μL of RNase solution (30 mg/mL) was added to this and incubated at 37°C for 1 h. The extracted genomic DNA was checked for integrity on a 0.8% agarose gel by electrophoresis and stored at -20°C.

2.2.18 Chromatin immuno precipitation (ChIP)

Yeast strains carrying p416GPD GST-*RPA43* were grown in SC-Ura medium overnight and sub cultured at 0.2 OD<sub>600</sub>. 45 mL of mid-log phase yeast cultures were subjected to cross linking with 1% formaldehyde for 15 min at room temperature (Szijgyarto *et al*., 2011). 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 (Section 2.1.6.5) by bead beating. Chromatin was fragmented using a Diagenode bath sonicator, 15 sec on time and 30 sec off time, for 15 min. Cell lysates were centrifuged at high speed and the supernatant was pre-cleared with 3 µg of normal rabbit IgG followed by 30 µL of 1:1 suspension of Protein A beads. Supernatant was collected and 10 μL of this lysate was taken as input.
Immunoprecipitation of chromatin was performed by incubating the lysate with 3 µg of anti-GST antibody overnight at 4°C followed by 50 µL of 1:1 suspension of Protein A beads for 4 h. Beads were washed twice each in wash buffer I, wash buffer II, and TE buffer (Section 2.1.6.5). Each wash was performed for 15 min at 4°C by rotating on a Lab-net end over mixer. Chromatin was eluted in 100 µL of elution buffer (Section 2.1.6.5) by rigorous mixing on a Thermo mixer (Eppendorf) for 30 min at 65°C. 90 µL of this eluted sample was incubated overnight at 65°C to reverse the cross linking. 10 µL lysate taken as input was diluted to 90 µL with elution buffer and incubated overnight at 65°C to reverse the crosslinking. DNA was extracted from the input and immunoprecipitated samples into a final volume of 40 µL using a PCR purification kit (Qiagen).

2.2.19 Quantitative PCR (q-PCR) reaction

PCR reactions were set up with the immunoprecipitated DNA by using the primers that amplify the 5’ETS and promoter regions. 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 in an RT-PCR machine (Applied Biosystems). Ct values of the immunoprecipitated samples were normalised to the adjusted Ct values of the input sample, and the data were plotted as ‘immunoprecipitated DNA as a percentage of input DNA (% Input)’, as described in (Haring et al., 2007; Livak and Schmittgen, 2001).

Calculation of % Input

The amount of DNA taken for q-PCR analysis is inversely proportional to the Ct value

% Input = 2^{-Ct(IP)} / 2^{-Ct(Input)} \times 100, (\% of gDNA immunoprecipitated from the total amount of gDNA taken)

Ct(IP) = C_t value of immunoprecipitated DNA

Ct(Input) = C_t value of Input DNA

In this experiment, 10 µL of this lysate was taken as input and this has to be adjusted to the total volume of the lysate. Therefore, the formula would be,

% Input = 2^{-Ct(IP)} / 2^{-Ct(adjusted Input)} \times 100
Materials and methods

2.2. Cloning of *S. cerevisiae* ORFs

The nomenclature system for RNA Pol I subunits, gene and protein name is given in Table 2.4 and The *S. cerevisiae* RNA Pol I subunits Uaf30, A34.5, A43 full length and fragments of A135 (1-112 a.a) and A190 (1101-1664 a.a) were PCR amplified from genomic DNA of the wild-type strain using high fidelity DNA Taq polymerase, with primers carrying restriction sites for BamHI and NotI or BamHI and XhoI (Table 2.4).

Amplified fragments were cloned downstream of the GAL4 promoter in the pYesGex6p2 plasmid (Werner *et al.*, 2010). These plasmids were generated by a colleague, Mr. Unnikannan CP, in the laboratory.

**Table. 2.6 The gene name and corresponding protein name for RNA Pol I subunits.**

RPA represents RNA polymerase I and protein name starts with A which represents that the subunit is present in RNA pol I.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Protein name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>RPA190</em></td>
<td>A190</td>
</tr>
<tr>
<td><em>RPA43</em></td>
<td>A43</td>
</tr>
<tr>
<td><em>RPA34.5</em></td>
<td>A34.5</td>
</tr>
<tr>
<td><em>RPA135</em></td>
<td>A135</td>
</tr>
</tbody>
</table>

Fragments and mutant versions of full length RNA Pol I subunits A34.5 and A43 were generated using above plasmids as templates (Table 2.3). A190 N-terminal (1-556 a.a)

Calculation of adjusted input

- Volume of cell lysate: 500 μL
- Volume set aside as input: 10 μL, eluted into a final volume of 40 μL
- Volume of lysate taken for IP: 490 μL; eluted into 100 μL after IP, of which 90 μL was eluted into a final volume of 40 μL.

Therefore, volume of lysate for IP corresponds to (90/100)*490 = 440 μL (approx. 500 μL)

1 μL of input sample, and 3 μL of immunoprecipitated sample was taken from 40 μL of eluted volumes, for q-PCR reaction.

The input sample taken for q-PCR = 1/40 x10 μL= 0.25 μL

The immunoprecipitated DNA taken for q-PCR = 3/40 x 500 = 37.5 μL

Therefore, the Ct values obtained for input samples (Ct input) were from 0.25 μL out of 37.5 μL of total lysate

Adjusted Ct = [Ct(Input)-log₂(input dilution factor)]

The dilution factor for input sample was 37.5/0.25 = 150

Adjusted Ct = [Ct(Input)-log₂(150)] = Ct(Input)-7.23

2.2.20 Cloning of *S. cerevisiae* ORFs
and A190 middle (557-1100 a.a) fragments were amplified from the plasmid pRS314RPA190 gifted by Dr. Herbert Tschochner with primers containing BamHI and XhoI (Table 2.4). The mutant versions of A34 and A190 fragments were generated by overlap extension PCR based method.

p416GPD GST-RPA43 used in ChIP was generated by extracting the GST-RPA43 fragments from pYesGex6p2 GST-RPA43 by restriction digestion with HindIII and XhoI followed by ligation into p416GPD. Clones were verified by bacterial colony PCR, sequencing and western blot.

For colony PCR a small amount of bacterial colony was taken with the help of toothpick and touch the bottom of the 0.2 mL PCR tube to which PCR reaction mix was added and performed PCR.

2.2.21 Preparation of E. coli DH5α ultracompetent cells

A single colony of E. coli DH5α strain was inoculated in 10 mL LB medium and incubated at 37°C overnight. Overnight culture was subcultured in 250 mL of LB medium of about 0.1 OD and incubated at 18°C for 36 h till the OD600 reached approximately 0.5. Cultures were centrifuged at 2,500 x g for 10 min at 4°C and harvested cells were washed gently with 80 ml ice-cold Inoue transformation buffer (Section 2.1.6.2). Cells were collected by centrifugation at 2,500 x g for 10 min at 4°C and gently resuspended in 20 mL ice-cold Inoue transformation buffer. To this cell suspension, 1.5 ml sterile DMSO was added and swirled gently. The cell suspension was kept on ice for 30 min, and 100 μL volume was aliquoted into pre-chilled sterile microcentrifuge tubes. Cells were immediately snap-frozen in liquid nitrogen and stored at -80°C.

2.2.22 Bacterial transformation

E. coli DH5α strain was transformed with yeast plasmids carrying appropriate inserts that express S. cerevisiae proteins (Sambrook and Russell, 2001). Ultracompetent cells stored at -80°C were thawed on ice for 5-10 min. 20 μL ligated plasmid was added to 100 μL ultracompetent cells, and the cells were incubated on ice. After 30 min, competent cells were subjected to heat shock at 42°C for 90 seconds. Cells were immediately transferred to ice for 2-3 min. Next, 900 μL LB medium was added and cells were allowed to recover for 45 min on a shaker incubator set at 37°C. After the
recovery, cells were centrifuged at 2,500 x g for 4 min. The medium supernatant was discarded and cells were resuspended in 200 μL fresh sterile LB medium. Cells were plated on LB agar medium containing appropriate antibiotics. Plates were incubated at 37°C for 12-16 h to allow growth of individual colonies.

2.2.23 Protein expression and purification from yeast

GST fusions of yeast RNA Pol I subunits were purified as described in (Werner et al., 2010). Yeast strains over expressing GST tagged RNA Pol I proteins were grown overnight at 30°C in 10 mL of SC-Ura medium with 2% glucose medium. Cells were pelleted, washed in SC-Ura with galactose. Protein expression was induced by transferring the entire pellet into 200 mL of SC-Ura with 2% galactose to give a final OD$_{600}$ of 0.8-1.0. For proteins A190 and A43 that express at very low levels, the overnight culture volume and induction volume were doubled. Cells were cultured at 30°C harvested at 3.0-5.0 OD and washed with ice cold water. The cell pellet was suspended in 5 mL of ice cold Buffer A (Section 2.1.6.7), 750 μL of cell suspensions were aliquoted into 1.5 mL microfuge tubes and to this 500 μL glass beads were added. Cells were lysed by bead beating using a vortex mixer (VortexGenei -2 with mix-mate attachment), and the lysate was centrifuged at high speed for 15 min at 4°C.

Supernatants were dispensed into a 15 mL conical tube and Triton X-100 was added to a final concentration of 1%. Pre-swollen glutathione beads were washed in Buffer B (Section 2.1.6.7) from which 200 μL of 1:1 bead suspension was added to approximately 5 mL of A34 and A43 expressing cell lysate and 100 μL of 1:1 bead suspension was added to 5 mL of A190 cell lysate and incubated for 2 h at 4°C on a rotary mixer. Lysates were centrifuged at 5000 x g for 2 min and the beads were washed with ice cold Buffer C (Section 2.2.6.6) twice. Beads were further washed with ice cold Buffer B followed by ice cold 1X phosphate buffered saline (PBS) twice. Beads were suspended in an equal volume of 1X PBS with protease inhibitor cocktail (Sigma).

2.2.24 Synthesis of radiolabelled 5-IP$_7$

Synthesis of 5[β-32P]IP$_7$ was conducted as described earlier (Azevedo et al., 2010). An acrylic box was placed at a 37°C in a hybridization oven before setting up the reaction.
Materials and methods

to hold 1.5 mL microfuge tubes. Acrylic shield was used throughout to block the β radiation.

Preparation of IP₇ reaction buffer, pH 7.4 (5X)

<table>
<thead>
<tr>
<th>Stock concentration</th>
<th>Volume for 100 µL</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Tris pH 7.4</td>
<td>10 µL</td>
<td>100 mM</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>2 µL</td>
<td>100 mM</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>3 µL</td>
<td>30 mM</td>
</tr>
<tr>
<td>1 M DTT</td>
<td>0.5 µL</td>
<td>5 mM</td>
</tr>
<tr>
<td>Sigma water</td>
<td>84.5 µL</td>
<td></td>
</tr>
</tbody>
</table>

The Reaction Buffer was made fresh just before setting up the reaction.

IP₇ synthesis reaction was set up in 8 microfuge tubes, with 25 µL volume per reaction.

<table>
<thead>
<tr>
<th>Components</th>
<th>25 µL</th>
<th>Master mix (200 µL for 8 reactions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sigma water</td>
<td>2 µL</td>
<td>16 µL</td>
</tr>
<tr>
<td>5X buffer</td>
<td>5 µL</td>
<td>40 µL</td>
</tr>
<tr>
<td>BSA (1 mg/mL)</td>
<td>1 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>IP₆ (1 mM)</td>
<td>5 µL</td>
<td>40 µL</td>
</tr>
<tr>
<td>Mg-ATP (1 mM)</td>
<td>1 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>[γ-³²P]UTP (20 µCi/µL)</td>
<td>6 µL</td>
<td>48</td>
</tr>
</tbody>
</table>

The reaction mix was mixed well using a 200 µL pipette and 20 µL each was dispensed into eight 1.5 mL microfuge tubes. To this reaction mix 5 µL of purified hexa histidine tagged IP6K1 enzyme (2-3 µg) was added. Tubes were placed in an acrylic box and incubated at 37°C overnight in a hybridization oven. The next morning, reactions were pooled into two 1.5 mL microfuge tubes containing 100 µL each. 100 µL of 0.6 M perchloric acid was added to neutralise the reaction, the tubes were kept on ice for 1 min, and 33.5 µL of 1 M potassium carbonate with 5 mM EDTA was added and mixed by gentle tapping. CO₂ was liberated leaving a white precipitate, while tubes were kept open on ice for 1 h with gentle tapping at 15 min intervals. Tubes were centrifuged at 12000 x g for 2-5 min and the supernatant from each tube was pooled into a new 1.5 mL microfuge tube.
2.2.25 Purification of radiolabelled IP$_7$ by HPLC method

**HPLC set up**

A strong anion exchange partisphere SAX 4.6 mm diameter and 125 mm length column (Whatman) was fixed to the HPLC system (Waters 515 pumps). The column was equilibrated with buffer A (1 mM EDTA) (Section 2.1.6.6) overnight at 100 µL/min flow rate. Before starting the HPLC run, the fraction collector (BioRad 2110) was prepared by placing the outlet tube to vial 1 on the fraction collector. Fresh FACS tubes were placed at fraction numbers 40-65 on the fraction collector. The flow rate on the fraction collector was set to 1 mL/min and it was kept ready. The SAX column was allowed to equilibrate (warmup programme) with Buffer A by slowly increasing the flow rate from 0.1 to 1 mL/min over a period of 70 min.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL)</th>
<th>% Buffer A (1 mM EDTA)</th>
<th>% Buffer B, [1.3 M (NH$_4$)$_2$HPO$_4$, pH 3.8]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>70</td>
<td>1</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>80</td>
<td>1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>81</td>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>96.5</td>
<td>0.10</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>180</td>
<td>0.01</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

On the HPLC system (Waters Empower Software), the instrument method was set to the programme ‘tritium small coloumn’ and the method set was set to ‘tritium small column’. The ‘set up’ icon was selected and once the flow rate was 1 mL/min, the ‘prepare’ icon was selected. A gradient was generated by mixing buffer A and buffer B as described below (Section 2.1.6.6). The injector was moved to the load position and sample was injected using a 1 mL syringe. The injector was moved to the inject position and the ‘inject’ icon was pressed immediately. On the fraction collector, the ‘run’ button was pressed immediately. The syringe was rinsed 5 times with buffer A.

2.2.26 Preparation triethylammonium bicarbonate solution

During the HPLC run, a triethylammonium bicarbonate solution (1.5 M) was prepared. Triethylamine and water mixture generates heat, and therefore, approximately 100 mL
of ice cold deionized water (water was kept in the cold room overnight) was added to a 250 mL volumetric flask which was placed on ice. To the water, 52.1 mL of triethylamine solution (~7.2 M, Cat. No-T088, Sigma-Aldrich) was added with the help of a glass measuring cylinder, and mixed well. The volume of the solution was made up with ice cold water till the mark on the volumetric flask, and the flask was mixed well.

A pH meter (Eutech instruments 510) was calibrated using pH 7.0 and pH 10.0 solutions (Eutech instruments 510). Approximately 100 mL of 1.5 M triethylamine solution from the volumetric flask was added to a 250 mL conical flask that was placed on ice. A magnetic bead was placed in the flask and the ice bucket was placed on the magnetic stirrer. The pH measuring probe was immersed into the solution, and CO₂ was bubbled through the triethylamine solution and stirred until the pH reached 8.5. The conical flask was covered with paraffin film and kept on ice until the solution was used (within 1-4 h).

2.2.27 Purification of IP₇ by anion exchange cartridge

After the HPLC run, vials numbered from 50 to 65 were surveyed using a Geiger counter, 4 vials with high counts were pooled into one vial and 5 µL of this 4 mL solution was measured in a liquid scintillation counter (Perkin Elmer). To the remaining solution, 800 µL of 50% ammonia solution was added to neutralise the pH and the tube was kept on ice. In a 50 mL conical tube, 45 mL of chilled water was taken, to which the neutralised IP₇ solution was added and kept on ice.

A Sep-Pak cartridge [Waters, WAT020545] was equilibrated with 10 mL of ice cold deionized water using a 10 mL syringe. The Sep-Pak cartridge was attached to a 60 mL syringe and 50 mL of diluted IP₇ solution was passed slowly through it so that IP₇ would bind to the Sep-Pak column. The cartridge was washed with 8 mL of chilled water, followed by chilled 8 mL 0.2 M triethylammonium bicarbonate solution pH 8.5 (4 mL of 1.5 M triethylammonium bicarbonate, pH 8.5 + 26 mL chilled water). The bound IP₇ was eluted in 4 mL of 1.5 M triethylammonium bicarbonate solution, pH 8.5, into three 1.5 mL microfuge tubes. The eluted IP₇ was concentrated in a vacuum concentrator (Scanvac) at 2000 rpm, 25°C, to obtain 30-50 µL of a 1-2 µCi/µL solution of radiolabelled IP₇.
2.2.28 IP$_7$-mediated phosphorylation reaction

The pyrophosphorylation reaction was performed with proteins on beads in presence of IP$_7$ reaction buffer (Werner et al., 2010). 10X IP$_7$ reaction buffer (Section 2.1.6.7) was prepared, aliquoted and stored at 4°C. For the reaction, 30 μL of purified protein on GSH beads (1:1 beads in PBS suspension), 3.5 μL of 10X buffer and 1 μCi of 5[β-32P]IP$_7$ were added, and made up to a final volume of 35 μL, and incubated at 37°C for 15 min. A 50 μL reaction was performed for proteins with low expression levels such as A190. To the reaction mix, 4X LDS sample buffer (Invitrogen) was added to a final concentration of 1X and incubated at 95°C for 5 min. The reaction mix was centrifuged at high speed and resolved on a 4%-12% gradient gel by Nu-PAGE (Invitrogen) using 1X MES buffer (Invitrogen). Proteins were transferred to a Hybond-P membrane (GE Lifescience) and the radiolabelled proteins were detected using a phosphorimager (Fuji Film FLA-9000). The membrane was blocked with 5% non-fat dry milk (Rockland) in 1X PBST (pH 7.4) for 2 h at room temperature followed by washes with 1X PBST at room temperature for 10 min three times.

Proteins were detected by western blot using a rabbit anti-GST antibody. 1:5000 dilution of anti-GST antibody in 1X PBST containing 0.2% BSA, was added and incubated overnight at 4°C on a rotating platform. The membrane was washed in 1X PBST for 10 min three times, followed by incubation with HRP conjugated goat anti-rabbit IgG at 1:20,000 dilution in 5% non-fat dry milk (Rockland) in 1X PBST, for 1 h at room temperature. Membrane was washed with 1X PBST at room for 10 min three times. Protein bands were detected by using Super Signal West pico chemiluminescence substrate (Perkin Elmer).

2.2.29 Generation of yeast mutants

The *S. cerevisiae* strains used were BY4741 rpa34Δ and NOY222 (Table 2.1) (Gerber et al., 2008). NOY222 harbours a genomic deletion of RPA190 with the RPA190 encoded on pNOY20, a shuffle plasmid, as the genomic deletion is lethal. BY4741 rpa34Δ (MATa), and NOY222 (MATa) were mated by mixing these two strains and patched on the YPD agar. The resulting diploids were sporulated on yeast sporulating medium at 25°C for 2-3 weeks. The spores were treated with 5 U zymolyase and sonicated briefly in a bath sonicator to release the spores. The free spores were washed...
with 0.01% NP 40 in water, and were diluted serially and plated on rich medium (YPD). \textit{RPA34} in the genome was replaced with \textit{kanMx4} that provides resistance to G418 and \textit{RPA190} gene deletion was done by replacing it with \textit{URA3} gene that supports growth of the strain in the absence of uracil in the medium. \textit{pNOY20}, a shuffle plasmid harbours a \textit{LEU} gene which supports yeast growth in the absence of uracil. Therefore germinated spores were streaked on a selection medium lacking leucine and uracil and containing 200 μg/mL G418 to select the strain containing double mutation. The genotype of the strain was confirmed by growing the strain different combinations of media such as SC-Ura, SC-Leu, SC-Trp, SC-Met and SC-His. The procedure to generate \textit{RPA190} genome deletion in the background of \textit{rpa34Δ} (NOY222 \textit{rpa34Δ}) was conducted by a colleague, Ms. Sitalakshmi Thampatty, in the laboratory.

![Diagram](image)

**Figure 2.2** \textit{PCR based site directed mutagenesis of endogenous RPA43.} (A) Schematic representation of \textit{RPA43} and \textit{UBC11} gene arrangement. The arrows 1, 2, 7 and 8 represent the primers used to amplify the \textit{RPA43} 3’ region and \textit{UBC11} 5’ region. (B) Plasmid carrying \textit{nat} gene. The arrows 3, 4, 5 and 6 indicate the primers used to amplify overlapping region of the \textit{nat} gene. Green colour indicates the \textit{nat} gene and black colour indicates \textit{RPA43} and \textit{UBC11} genes. The 5’ regions primers 2 and 3 and primers 6 and 7 are complementary to each other, and were used to generate fusion products by overlap extension PCR. Vertical lines on primer 1 indicate the positions of the three Ser codons replaced with Ala.
A genomic mutation on the A43 protein coding gene RPA43 was inserted in the wild type BY4741 and NOY222 rpa34Δ strains. Plasmid pGP5 RPA43, gifted by Dr. Herbert Tschochner, and a plasmid harbouring the nourseothricin N-acetyltransferase (nat1) gene (Goldstein and McCusker, 1999) were used for a PCR based site directed mutagenesis to create a DNA fragment to generate a genomic mutant of RPA43 (Fig. 2.2) (Table 2.4). Using overlap extension PCR, the fragment encoding the mutated 3’ end of RPA43 was fused to the 5’ region of the nat1 gene. The 3’ region of the nat1 gene was in turn fused to the 5’ region of UBC11, the gene that is downstream of RPA43 in the genome. These two fragments were transformed into the NOY222 rpa34Δ strain using yeast transformation kit (Clontech) and selected on nourseothricin (NAT) 200 µg/mL to identify strains carrying mutant RPA43 generated by homologous recombination (Fig. 2.4) containing YPD. Colony PCR was performed using primers that amplify the merged region of RPA43 fragment and nat gene to verify the site of insertion.

Figure 2.3 Homologous recombination on RPA43 to generate genomic mutations. (A) Schematic representation of homologous recombination between transformed PCR amplicons and RPA43 and UBC11 gene. The crosses represent the site of recombination. (B) Schematic representation of the fusion product after homologous recombination. The three vertical line represent the three Ser to Ala. The black arrows indicate the primers used in PCR to confirm the site of insertion. This primer lies 5’ to the site of recombination on RPA43 and 3’ to the site of recombination on nat gene.
To conduct yeast transformation competent cells were made from relevant haploid yeast strain using Frozen EZ Yeast Transformation II Kit from Zymo Research Corporation, according to the manufacturer’s instructions.

To conduct plasmid shuffling in the NOY222, NOY222\textit{rpa34}\Delta and NOY222\textit{rpa34}\Delta \textit{RPA43}^{S/322/323/325/A} strains to generate native and S/A mutant versions of the \textit{RPA190} gene, the competent cells were transformed with either pRS314 \textit{RPA190} or pRS314 \textit{RPA190}^{S1413/1415/1417/A} harbouring wild type a mutant versions of \textit{RPA190} gene respectively, and transformants were selected by growing them on yeast nitrogen base containing leucine, G418 (200 \text{µg/mL}) and canavanine (6 \text{µg/mL}) but lacking uracil. The resulting strains have chromosomal deletions of \textit{RPA34} and \textit{RPA190} and harbour either wild type or mutant versions of \textit{RPA190} on pRS314 plasmids.

\textbf{2.2.30 Yeast colony PCR}

For each strain calls from a single colony, picked with a microtip from the appropriate medium were suspended in 10 \text{µL} zymolyase cocktail and incubated at 37\textdegree C for 90 min. 2 \text{µL} of zymolyase-treated cell suspension was used as the template in a 25 \text{µL} PCR reaction.