Supplementary Data

Yeast strains, plasmids and media

S. cerevisiae strains used in this study are listed in Table 1.

Table 1: Yeast strains

yeast strain TOY	name	genotype	origin
	RRN3-HA_3	MATa ade2-101 ura3-52 lys2-801 trp1-Δ63 his1-Δ200 leu2-Δ1 RRN3-HA_3(HIS)	this work, derived from YPH499
685	RRN3-TAP	MATa ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 lys2-801 ade2-101 prc1-1 RRN3-TAP(URA3)	this work, derived from YWO365 (1)
657	YWO365-RPA43-TAP-RRN3-HA_3	MATa ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 lys2-801 ade2-101 prc1-1 RPA43-TAP(URA3) RRN3-HA_3(HIS)	this work, derived from YWO365 (1)
652	cim3-1-RRN3-TAP	MATa ura3-52 his3-Δ200 leu2-Δ1 prc1-1 cim3-1 RRN3-TAP(URA3)	this work, derived from cim3-1 (YWO366, (1))
660	cim3-1-A43-TAP-RRN3-HA_3	MATa ura3-52 his3-Δ200 leu2-Δ1 prc1-1 cim3-1 RPA43-TAP(URA3) RRN3-HA_3(HIS)	this work, derived from cim3-1 (YWO366,(1))
	RRN3-Prot.A	MATa ade2-1 can1-100 his3Δ200 leu2-3,112 trp1-1 ura3-1 RRN3-TEV-Prot.A-HIS_7(HIS)	(2) derived from BSY420
667	$pTet_7$-RRN3-Prot.A	MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn3::HIS3 pCM185-RRN3-Prot.A(TRP1)	this work, derived from NOY604 (3)
	pNOP1-RRN3-Prot.A	MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn3::HIS3 pNOP1-RRN3-Prot.A(LEU2)	this work, derived from NOY604 (3)
684	pNOP1-RRN3-Prot.A-RPA43-HA_3	MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn3::HIS3 RPA43-HIS_6-HA_3(kanMX6) pNOP1-RRN3-Prot.A(LEU2)	this work, derived from pNOP1-RRN3-Prot.A
	pNOP1-RRN3-ΔN-Prot.A	MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn3::HIS3 pNOP1-RRN3-Δ17-Prot.A(LEU2)	this work, derived from NOY604 (3)
685	pNOP1-RRN3-ΔN-Prot.A-RPA43-HA_3	MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn3::HIS3 RPA43-HIS_6-HA_3(kanMX6) pNOP1-RRN3-Δ17-Prot.A(LEU2)	this work, derived from pNOP1-RRN3-ΔN-Prot.A

Unless otherwise indicated, yeast cells were cultured in YPD medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose). *In vivo* pulse labelling was performed in YPAD medium (YPD medium containing 100 mg/l adenine). To induce growth arrest, cells were either transferred from YPD medium to a synthetic medium lacking essential amino acids or treated in YP(A)D medium with 200 ng/ml of rapamycin (Sigma-Aldrich). Amino acid drop out media consisted of SDC-Leu (0.67 % (w/v) YNB+nitrogen, 0.063 % (w/v) CSM-His-Leu-Trp w/20 mg/l Ade, 2 % (w/v) glucose,

20 mg/l histidine, 50 mg/l tryptophane), SDC-Trp (0.67 % (w/v) YNB+nitrogen, 0.063 % (w/v) CSM-His-Leu-Trp w/20 mg/l Ade, 2 % (w/v) glucose, 20 mg/l histidine, 100 mg/l leucine) and SDC-His-Leu-Trp (0.67 % (w/v) YNB+nitrogen, 0.063 % (w/v) CSM-His-Leu-Trp w/20 mg/l Ade, 2 % (w/v) glucose).

Construction of yeast strains and plasmids

General yeast methods were performed according to (4). Plasmids used in this study were derived by standard cloning techniques (5).

S. cerevisiae strains carrying chromosomally integrated TAP- and HA_3-tags were constructed as described in (6,7).

To generate the RRN3 shuffle strain NOY604-RRN3-URA (MATα ade2-1 ura3-1 his3-11 trp1-1 leu2-3,112 can1-100 rrn3::HIS3 YCplac33-wt-RRN3(URA3)), a RRN3 ORF and promoter containing DNA fragment was PCR-amplified from yeast genomic DNA and cloned into vector pRS315 (10). From the resulting construct pRS315-wt-RRN3 the RRN3 ORF-promoter sequence was subcloned into the centromeric vector YCplac33 (containing the URA3 marker, (11)) yielding plasmid YCplac33-wt-RRN3. Strain NOY604-RRN3-URA was obtained by transforming strain NOY604 (3) with YCplac33-wt-RRN3.

To construct yeast strain $pTet_7$-RRN3-Prot.A, the RRN3 ORF and a C-terminally fused TEV-Prot.A-HIS_7-tag coding sequence were amplified from strain BSY420-RRN3-Prot.A (2) by colony PCR and cloned into the centromeric vector pCM185, containing the TRP1 marker (8). After transforming the resulting construct pCM185-RRN3-Prot.A into yeast strain NOY604-RRN3-URA (see below), strain $pTet_7$-RRN3-Prot.A was obtained by shuffling out the URA3-containing plasmid using 5-FOA.

For construction of yeast strain pNOP1-RRN3-Prot.A, the RRN3 ORF and a C-terminally fused TEV-Prot.A-HIS_7 coding sequence were PCR-amplified from the genomic DNA of strain BSY420-

RRN3-Prot.A and cloned into the bacterial expression vector pQE60 (Qiagen). From the resulting construct pQE60-RRN3-Prot.A the RRN3-Prot.A sequence was subcloned into the centromeric yeast vector pNOP-GFP carrying the LEU2 marker (9), yielding plasmid pNOP1-RRN3-Prot.A.

To construct yeast strain pNOP1-RRN3-ΔN-Prot.A, the WT RRN3 sequence in vector pQE60-RRN3-Prot.A was replaced by a PCR-generated RRN3-Δ17 sequence. Details on the cloning strategy are available on request. The resulting construct pQE60-RRN3-Δ17-Prot.A encodes for a Rrn3p-Prot.A fusion protein lacking the first 17 amino acids.

From this plasmid, the RRN3-Δ17-Prot.A fragment was subcloned into vector pNOP-GFP yielding pNOP1-RRN3-Δ17-Prot.A. Note that the expression of the plasmid-encoded RRN3 alleles is driven by the Nop1 promoter. We also observe a slight overexpression of the ΔN-mutant when compared to Rrn3p-Prot.A (approximately 1.9-fold, see also suppl. Fig 4B).

To obtain yeast strains pNOP1-RRN3-Prot.A and pNOP1-RRN3-ΔN-Prot.A, plasmids pNOP1-RRN3-Prot.A and pNOP1-RRN3-Δ17-Prot.A were transformed in strain NOY604-RRN3-URA, respectively. Loss of plasmid YCplac33-wt-RRN3 was promoted by incubating the transformants in the presence of 5'-FOA..

Western blotting and antibodies

Protein samples mixed with SDS sample buffer were denaturated for 5 min at 95 °C, before they were separated on 8 or 10 % SDS polyacrylamide gels and blotted onto a PVDF membrane (Immobilon™-P, Millipore). Immunostaining was performed according to (5). Signals were developed with the BM Chemiluminescence Blotting Substrate (POD) from Roche and visualized in a Fujifilm LAS-3000 chemiluminescence imager (Raytest).

PAP (peroxidase anti peroxidase) immuno complexes (rabbit, polyclonal, DakoCytomation) were used to detect Prot.A- and TAP-tagged proteins. HA_3-tagged proteins were analysed either with monoclonal mouse (12CA5, kindly provided by Elisabeth Kremmer; 16B12, BabCO) or with monoclonal rat antibodies (3F10, 100 µg/ml, Roche Diagnostics). Pol I specific polyclonal

antibodies (α-A43 and α-A135) were kindly provided by Christophe Carles and Michel Riva (Gif-sur-Yvette). HRP-labeled secondary anti-rabbit, anti-mouse and anti-rat antibodies were purchased from Jackson IR/Dianova.

Co-immunoprecipitations

To analyse the Pol I-Rrn3p interaction in yeast strains YWO365-RPA43-TAP-RRN3-HA_3 and cim3-1-RPA43-TAP-RRN3-HA_3, whole cell extracts (WCEs) were first cleared by centrifugation (40 min, 100,000 g, 4 °C). 1.5 mg of the corresponding supernatants were adjusted to 750 mM potassium acetate, 0.5 % (v/v) Nonidet P40, 0.05 % (v/v) Triton X-100 and incubated for 2 h with 50 µl IgG sepharose (Amersham) to enrich the TAP-tagged Pol I subunit A43. After immunoprecipitation, the beads were washed once with buffer TST (50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.05 % (v/v) Triton X-100, 1 mM PMSF, 2 mM benzamidine), three times with 5 mM ammonium acetate (pH 5.0), three times with 1.5 M potassium acetate, 0.5 % (v/v) Nonidet P40 followed by a final washing step with buffer TST. Immunoprecipitated proteins were eluted from the beads with SDS sample buffer and analysed by Western blotting.

Quantification was performed as described in Materials and Methods.

Suppl. Fig. 1, related to Fig. 1 A fraction of cellular Rrn3p-TAP, but not the Pol I-specific subunit A135 is constitutively polyubiquitylated.

A) The same experiment as described in Fig. 1C, however using the yeast strain RRN3-Prot.A [pGal1-Myc_3-Ubi [G76A]] in which Rrn3p carries a Prot.A tag and YPG to induce ubiquitin-Myc3 expression. Retention of subunit A135 on the GST-Dsk2p-beads was analysed using A135-specific antibodies (gift from Drs. Christophe Carles and Michel Riva, Saclay). Ubiquitylation was analysed using anti-Myc-antibodies.

B) The proteasome ts-mutant strain (*cim3-1*) expressing a chromosomally TAP-tagged Rrn3p was grown to mid-log phase in YPD at 24 °C, before the cells were shifted to 37 °C for 2 hours. Half of

the culture was collected and lysed (t = 0 min), whereas incubation at 37 °C was continued for 1 hour with the remainder of the cells in the presence of 200 ng/ml rapamycin before harvest and lysis. Same amounts of WCEs (20 mg) were incubated with either recombinant GST-Dsk2p, or recombinant GST immobilized on 50 µl of glutathione sepharose. After washing, proteins bound to the beads were eluted with SDS sample buffer. 90% of the eluate (E) was analyzed by Western blotting using antibodies directed against the Prot.A-tag of Rrn3p. At longer exposure times Rrn3p species of higher molecular weight were detected (polyubiquitinylated Rrn3p). Note that in similar experiments other C-terminal Prot.A-tagged proteins were not found to be ubiquitylated which indicates that the Prot.A-tag is not necessarily a substrate of ubiquitylation (data not shown).

C) RRN3 cDNA levels drop drastically upon rapamycin treatment for 20 minutes. Yeast strain y622 (12)was cultured in YPD at 30°C to exponential phase. The culture was split in two and DMSO was added to one half of the culture, whereas rapamycin in DMSO was added to the other half to a final concentration of 200 ng/ml. Incubation was continued at 30°C for another 20 minutes, before cells were sedimented and total RNA was prepared as described (13). After cDNA synthesis according to the instructions of the iScript cDNA Synthesis kit (BIO-RAD), relative cDNA amounts of the RRN3 gene were measured by quantitative PCR using primer pair 1853/1854. The data was normalized to the relative cDNA amounts of the ACT1 gene (nucleotides 532-726) measured with primer pair 1524/1525. Primer pair 1526/1527, 1671/1672, and 1673/1674 amplifying another region of the ACT1 cDNA (nucleotides 437-559), or of the mitochondrial ATP6 and 21S rRNA cDNAs, respectively, were used as controls.

Oligonucleotides used for qPCR:

name	sequence	locus
1524	TGTCACCAACTGGGACGATA	ACT1
1525	AAACGGCTTGGATGGAAAC	ACT1
1526	ATGGTCGGTATGGGTCAAAA	ACT1
1527	TTTTCCATATCGTCCCAGTTG	ACT1
1671	GCTTAAAGGACAAATTGGAGGT	ATP6
1672	CCAGCAGGTACGAATAATGAGA	ATP6
1673	TGTGAACTCTGCTCCATGCT	21S
1674	TGCATAGGGTCTTTCCGTCT	21S
1853	CCGAGCAAGAATCCTGAAAG	RRN3

1854	GAAACGTCTTGCCACCATTT	RRN3

Suppl. Fig. 2, related to Fig. 3 Fractionation of whole cell extracts into nuclei and cytoplasm. Cells from logarithmically growing or amino acid depleted yeast strains pNOP1-RRN3-Prot.A (WT) and pNOP1-RRN3-ΔN-Prot.A (ΔN) were harvested, lysed and fractionated into nuclei and cytoplasm. 30 µg of whole cell extracts and 5% of the resulting nuclear and cytoplasmic fraction were analysed by Western blotting. Phosphoglycerate kinase (PGK) and Nop1p were used as marker proteins for cytoplasmic and nucleolar fractions, respectively. Proteins were detected by antibodies raised against ProteinA, PGK and Nop1p.

Suppl. Fig. 3, related to Fig. 4 Stabilizing cellular Rrn3p levels in a proteasome ts-mutant strain attenuates the reduction in initiation competent Pol I-Rrn3p complexes observed upon nutrient depletion.

A. Gelfiltration analysis. The proteasome ts-mutant strain (*cim3-1*) expressing a chromosomally TAP-tagged Rrn3p (TOY 652) or the isogenic *CIM3* WT strain (TOY 651) were grown to mid-log phase in YPD at 24 °C, before the cells were starved at 37 °C in SDC-Leu medium (- Leu). After lysis, same amounts of WCE (900 µg) were separated on a Superose-6® column in a buffer containing 1.5 M potassium acetate. 250 µl of the collected 500 µl fractions were TCA precipitated and analyzed by Western blotting together with the "Load" (30 µg). Antibodies used were directed against the Prot.A-tag of the Rrn3p versions and the Pol I subunit A135, respectively. The gel filtration fractions containing the transcriptionally competent Pol I-Rrn3p complexes are labelled in red.

B. Co-immunoprecipitations. Yeast strains TOY 657 (WT) and *cim3-1*- TOY 660 (*cim3-1*), both expressing chromosomally HA_3-tagged Rrn3p and a TAP-tagged Pol I subunit A43, were grown in YPD at 24 °C to mid-log phase and shifted to 37 °C. Half of the cells was harvested and lysed (t = 0

min). The remainder of the cells was starved in SDC-Leu (- Leu) for 2 h and treated as described above (t= 120 min). The TAP-tagged Pol I subunit A43 was immunoprecipitated from 250 µl of extracts (Inputs) with IgG sepharose. 50 % of the TAP-IPs as well as 1,6 % of the inputs were analyzed by Western blotting using antibodies directed against the HA-tag of the Rrn3p versions and the TAP-tag of the Pol I subunit, respectively. As a control an identical co-immunoprecipitation experiment was performed using extracts from yeast strain RRN3-HA_3 which does not express the TAP-tagged Pol I subunit A43 (ctr.). Western blot signal intensities were measured, and quantified using the LAS 3000 imaging system and the AIDA software. Rrn3p/A43 ratios were calculated, and the ratio of the 120 min samples was normalised to the ratio of the respective 0 min samples which was set to 100%. Numbers calculated are given below each lane. Note, that, in contrast to the experiment described in Figure 4B, cells have not been crosslinked with formaldehyde before extract preparation. This may account for the lower recovery of Rrn3p co-precipitating with the Pol I subunit A43.

Suppl. Fig. 4, related to Fig. 4 The number of actively transcribed rRNA genes is slightly increased in the ΔN- mutant

A. Comparison of nucleolar regions from logaritmic growing yeast cells of WT and ΔN strain by Millers spread analysis. Both an overview of nucleolar chromatin (upper panel) and a representative single transcribed rDNA gene (lower panel) are shown for each strain. Scale bars are indicated.

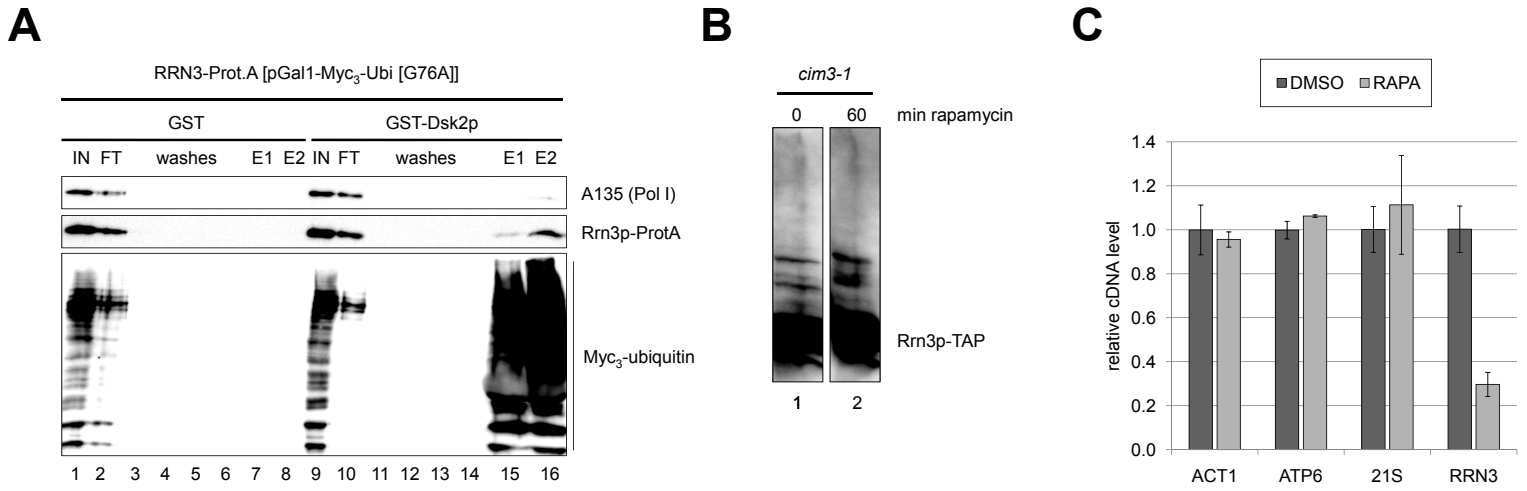
B. Psoralen cross-linking was performed using nuclei isolated from yeast strains pNOP1-RRN3-Prot.A (WT) and pNOP1-RRN3-ΔN-Prot.A (ΔN), grown in YPD at 30°C to midlog phase. DNA was isolated, digested with EcoRI, and analysed in a Southern blot using a probe detecting a 1.9kb 18S rDNA EcoRI restriction fragment. The graph on the right shows the intensity of the radioactive signal in the different lanes of the blot plotted against the migration distance in the gel. The signal intensity in each lane was normalized to the respective peak value. The positions of EcoRI

fragments derived from transcriptional inactive and actively transcribed rRNA genes are indicated. The additional open genes in the ΔN-mutant were approximated measuring the different peak areas. *Lower panel* Same amounts of WCE (15 µg) were analyzed by Western blotting, using antibodies directed against subunit A43 and the Prot.A-tag of the Rrn3p, respectively. The Rrn3p/A43 ratio is indicated at the bottom. The ratio in the WT was arbitrarily set to 1.

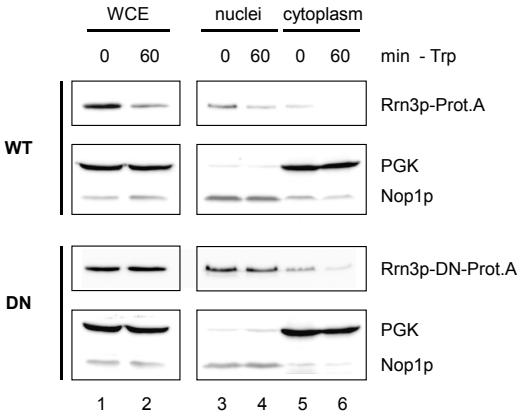
References

1. Hiller, M.M., Finger, A., Schweiger, M. and Wolf, D.H. (1996) ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science*, 273, 1725-1728.
2. Fath, S., Milkereit, P., Peyroche, G., Riva, M., Carles, C. and Tschochner, H. (2001) Differential roles of phosphorylation in the formation of transcriptional active RNA polymerase I. *Proc Natl Acad Sci U S A*, *98*, 14334-14339.
3. Yamamoto, R.T., Nogi, Y., Dodd, J.A. and Nomura, M. (1996) RRN3 gene of Saccharomyces cerevisiae encodes an essential RNA polymerase I transcription factor which interacts with the polymerase independently of DNA template. *EMBO-J.*, 15, 3964-3973.
4. Burke, D., Dawson, D. and Stearns, T. (2000) *Methods in Yeast Genetics*, Cold Spring Harbor.
5. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbour Laboratory Press, New York.
6. Knop, M., Siegers, K., Pereira, G., Zachariae, W., Winsor, B., Nasmyth, K. and Schiebel, E. (1999) Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast*, 15, 963-972.
7. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol*, 17, 1030-1032.
8. Gari, E., Piedrafita, L., Aldea, M. and Herrero, E. (1997) A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae. *Yeast*, 13, 837-848.
9. Milkereit, P., Gadal, O., Podtelejnikov, A., Trumtel, S., Gas, N., Petfalski, E., Tollervey, D., Mann, M., Hurt, E. and Tschochner, H. (2001) Maturation and intranuclear transport of pre-ribosomes requires Noc-proteins. *Cell*, 105, 499-509.
10. Sikorski, R.S. and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics*, 122, 19-27.
11. Gietz, R.D. and Sugino, A. (1988) New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene*, 74, 527-534.
12. Merz, K., Hondele, M., Goetze, H., Gmelch, K., Stoeckl, U. and Griesenbeck, J. (2008) Actively transcribed rRNA genes in S. cerevisiae are organized in a specialized chromatin

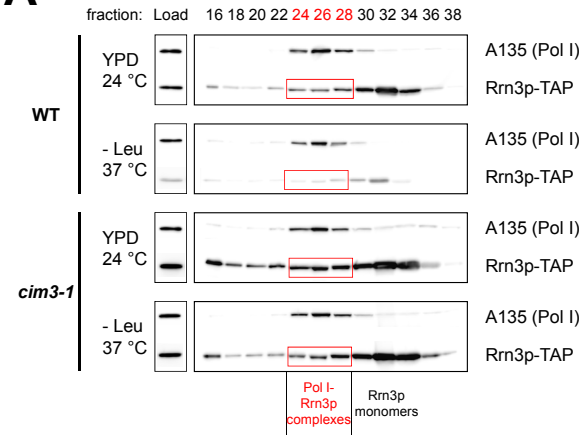
associated with the high-mobility group protein Hmo1 and are largely devoid of histone molecules. *Genes Dev*, 22, 1190-1204.
13. Ferreira-Cerca, S., Poll, G., Gleizes, P.E., Tschochner, H. and Milkereit, P. (2005) Roles of eukaryotic ribosomal proteins in maturation and transport of pre-18S rRNA and ribosome function. *Mol Cell*, 20, 263-275.

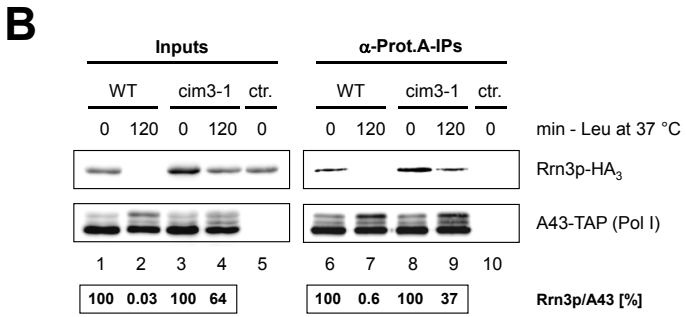

A
RRN3-Prot.A [pGal1-Myc3-Ubi [G76A]]
GST
GST-Dsk2p
IN FT washes E1 E2 IN FT washes E1 E2
A135 (Pol I)
Rrn3p-ProtA
Myc3-ubiquitin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
B
cim3-1
0 60 min rapamycin
Rrn3p-TAP
1 2
C
DMSO RAPA
relative cDNA level
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ACT1 ATP6 21S RRN3

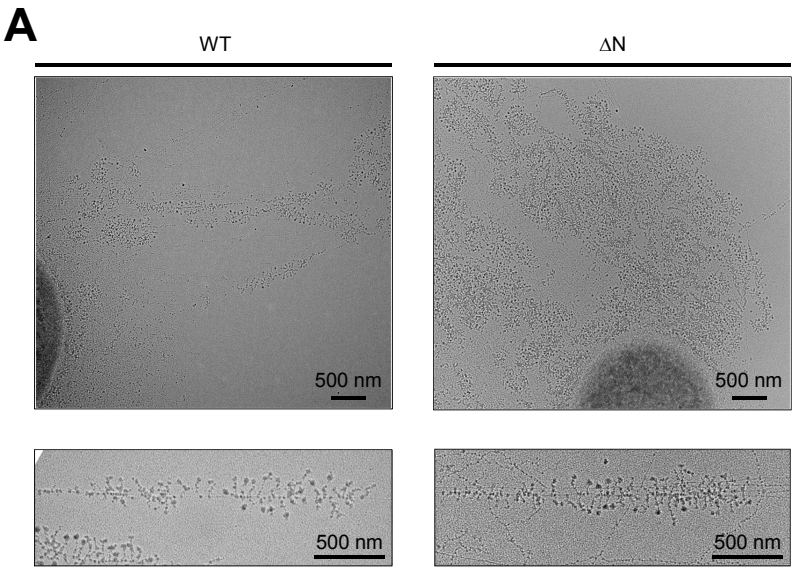
Suppl. Fig. 2

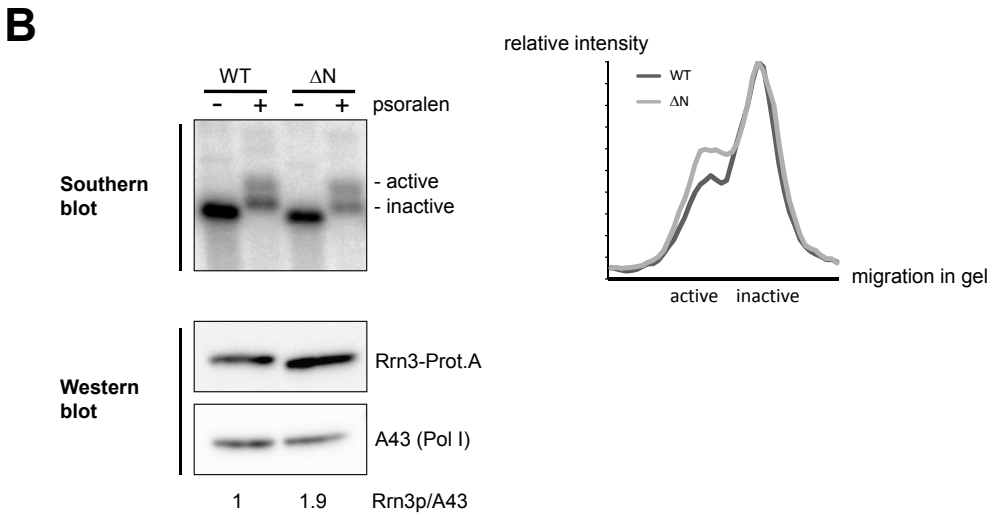

WCE
nuclei
cytoplasm
0 60 0 60 0 60 min - Trp
WT
Rrn3p-Prot.A
PGK
Nop1p
DN
Rrn3p-DN-Prot.A
PGK
Nop1p
1 2 3 4 5 6

Suppl. Fig. 3


A
fraction: Load 16 18 20 22 24 26 28 30 32 34 36 38
WT
YPD 24 °C
A135 (Pol I)
Rrn3p-TAP
- Leu 37 °C
A135 (Pol I)
Rrn3p-TAP
cim3-1
YPD 24 °C
A135 (Pol I)
Rrn3p-TAP
- Leu 37 °C
A135 (Pol I)
Rrn3p-TAP
Pol I-Rrn3p complexes
Rrn3p monomers


B
Inputs
α-Prot.A-IPs
WT cim3-1 ctr.
WT cim3-1 ctr.
0 120 0 120 0
0 120 0 120 0
min - Leu at 37 °C
Rrn3p-HA3
A43-TAP (Pol I)
1 2 3 4 5
6 7 8 9 10
100 0.03 100 64
100 0.6 100 37
Rrn3p/A43 [%]


A
WT
ΔN
500 nm
500 nm
500 nm
500 nm


B
WT
ΔN
- + - +
psoralen
Southern blot
- active
- inactive
relative intensity
WT
ΔN
migration in gel
active
inactive
Western blot
Rrn3-Prot.A
A43 (Pol I)
1
1.9
Rrn3p/A43