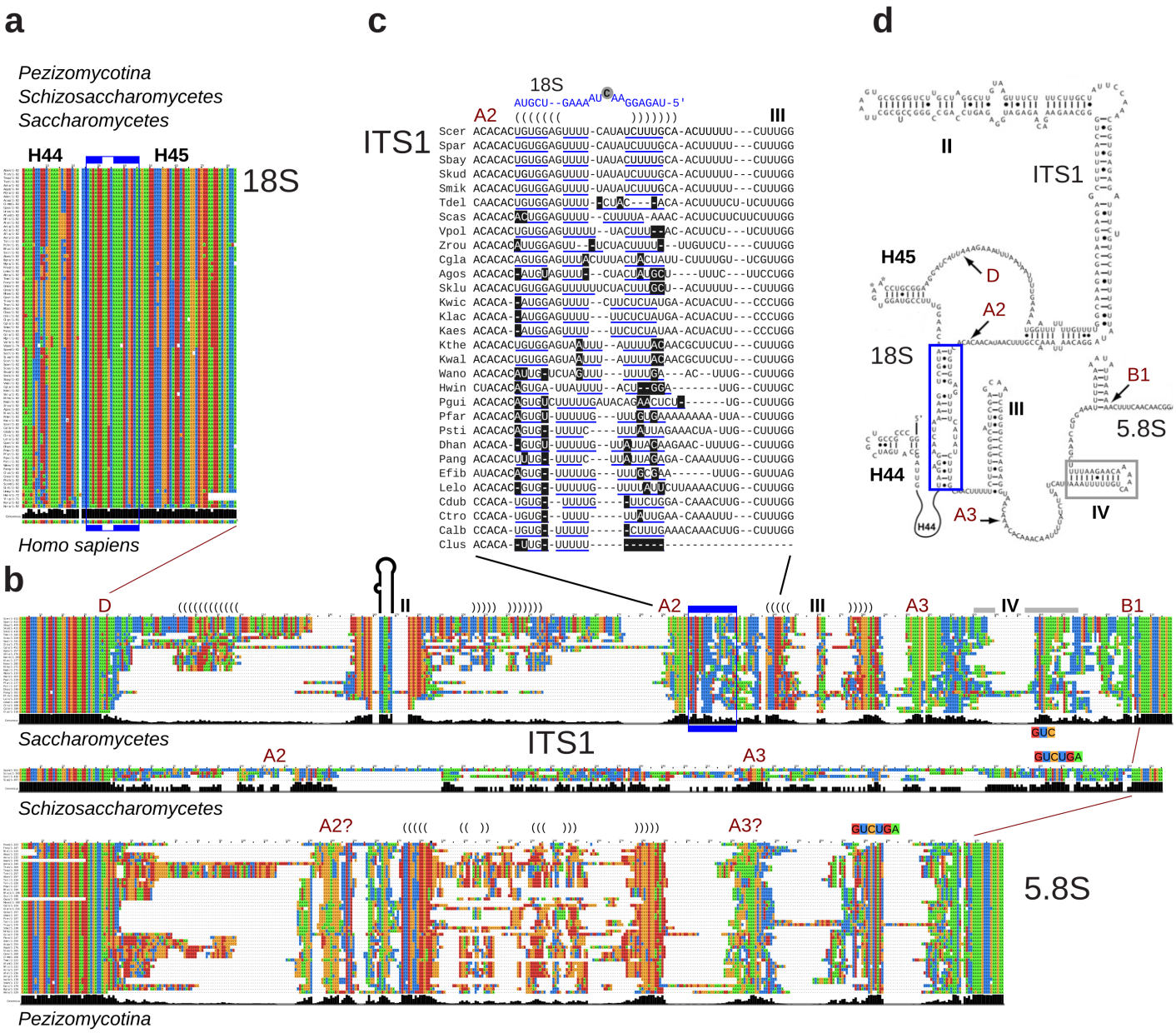
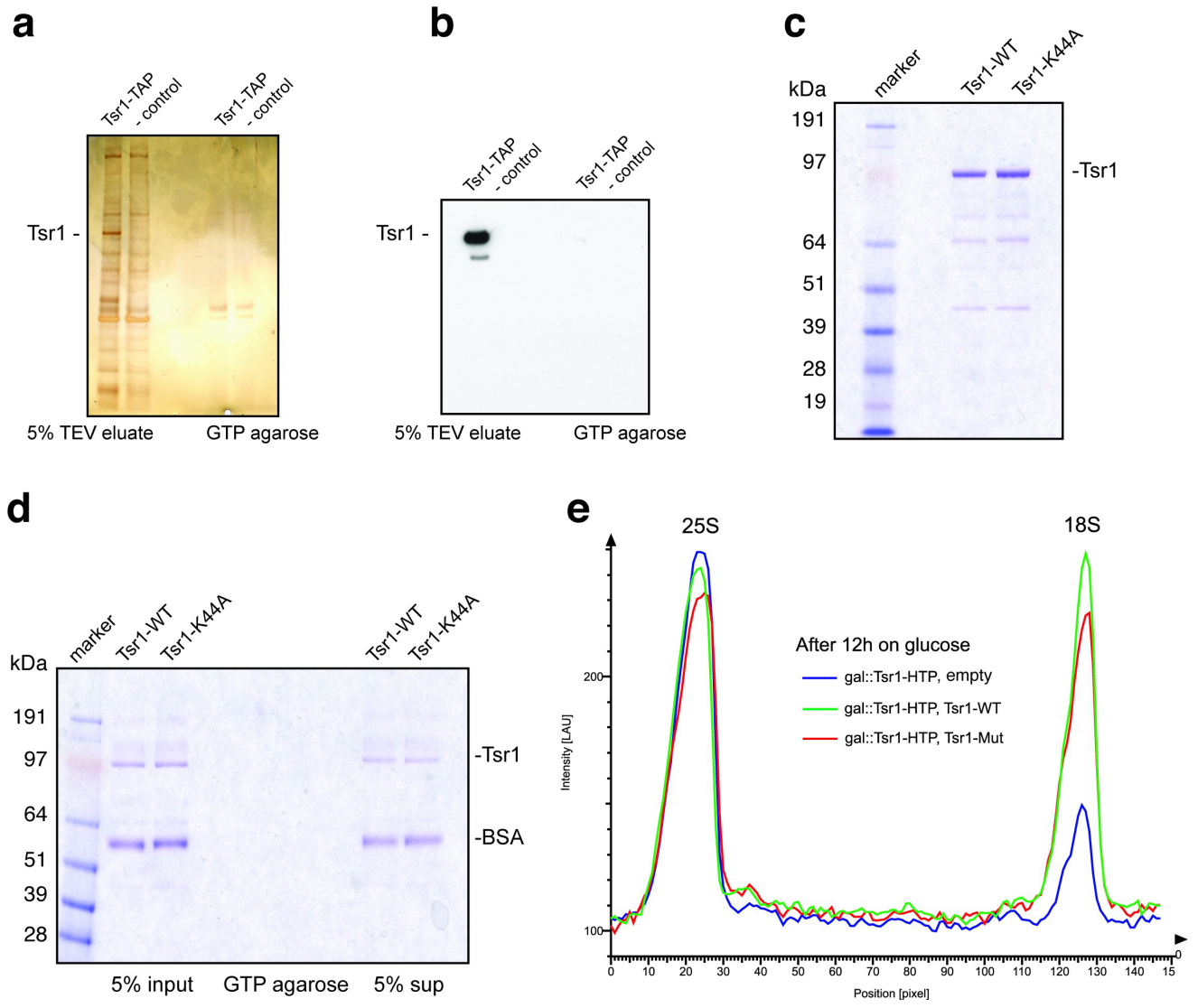
Supplementary information

Proof reading of pre-40S ribosome maturation by a translation initiation factor and 60S subunits

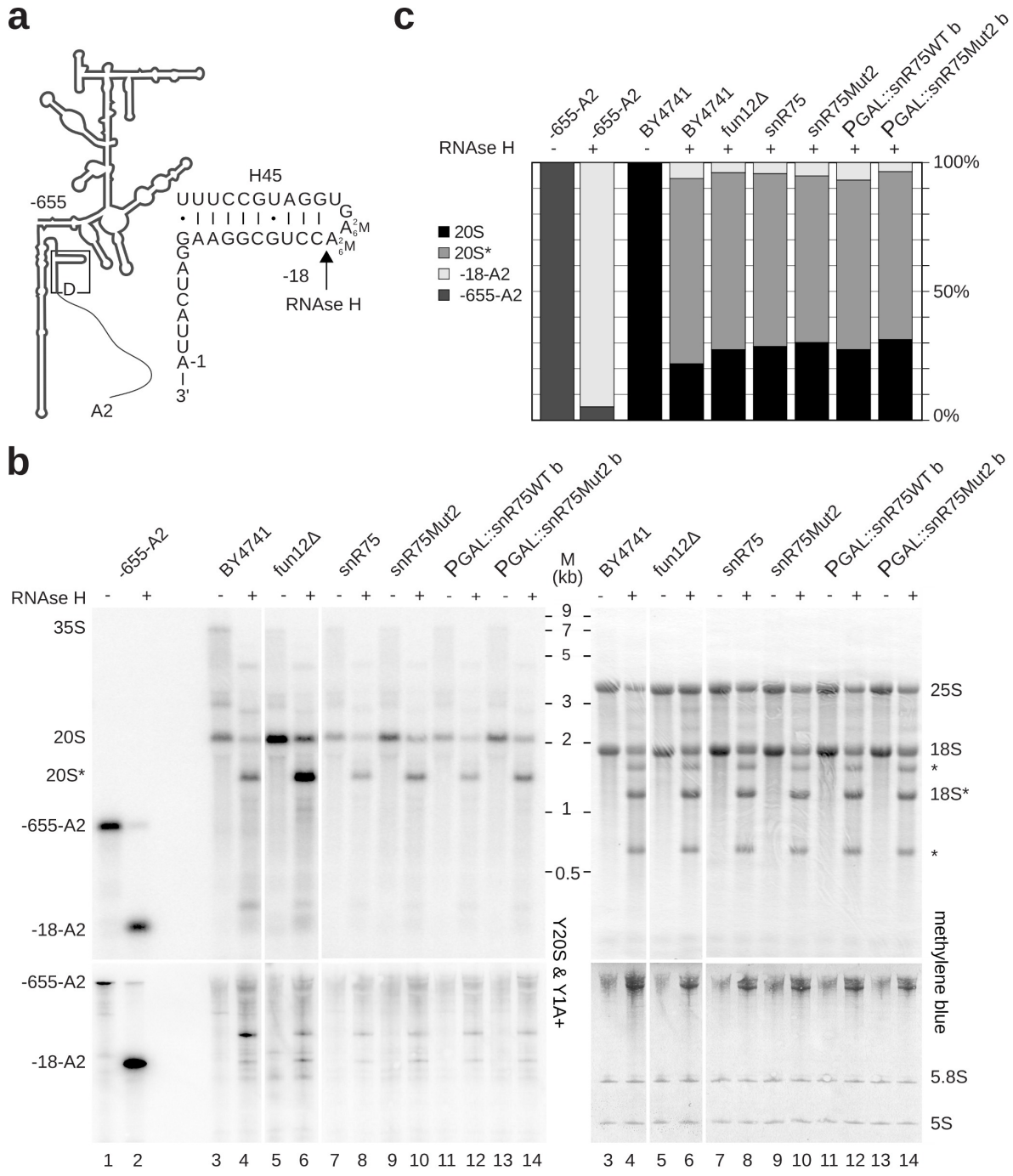
Simon Lebaron, Claudia Schneider, Robert W. van Nues, Agata Swiatkowska, Dietrich Walsh, Bettina Böttcher, Sander Granneman, Nicholas J. Watkins, David Tollervey



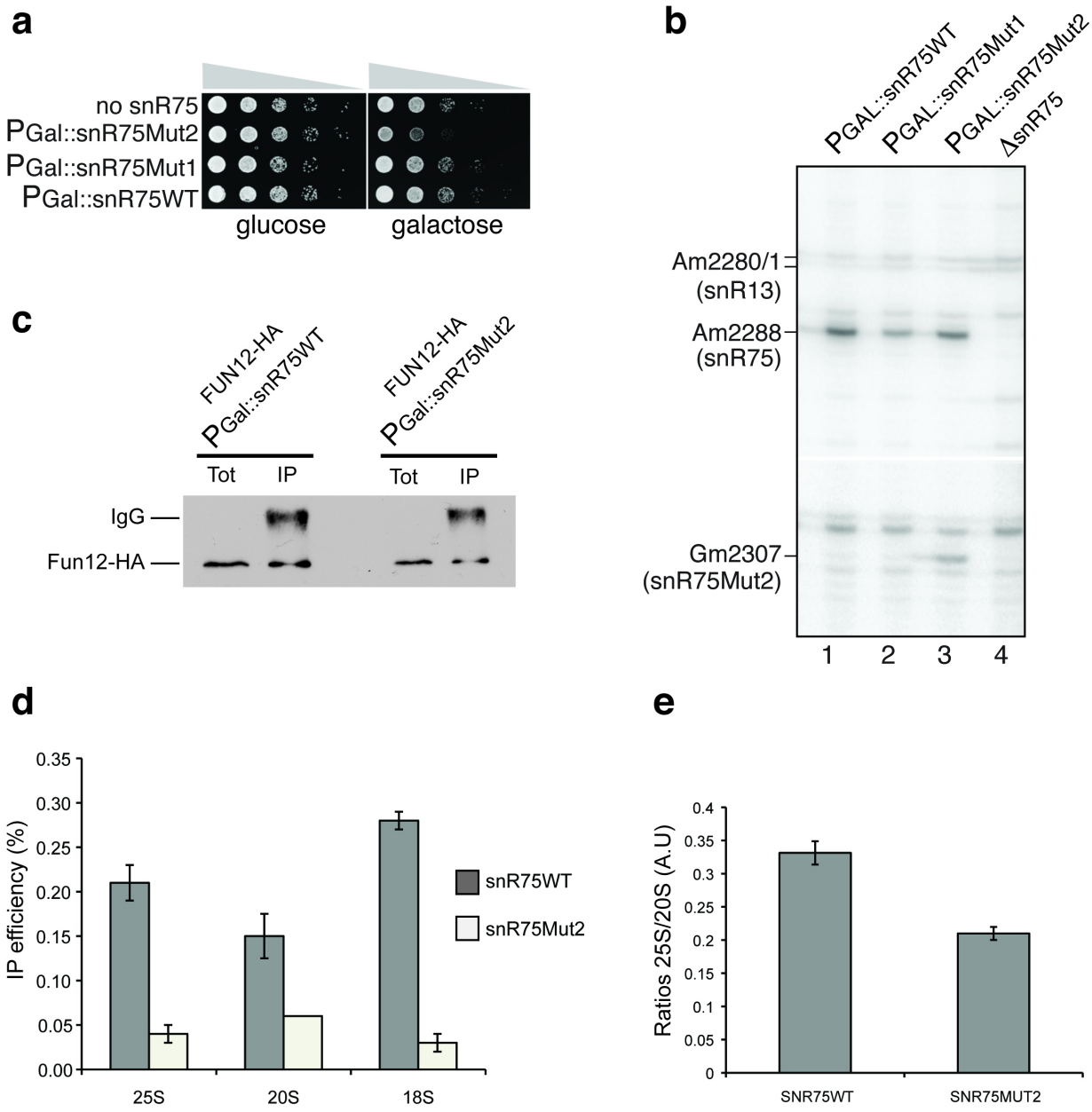
Supplementary Figure 1 Conservation of ITS1 sequence and predicted folding. Two related models have previously been proposed for interactions between a highly conserved 18S rRNA region within helix 44 and the ITS1 region downstream of cleavage site A2 [1,2]. This putative 18S-ITS1 base-pairing is, however, poorly conserved in *Saccharomycetes* and absent in more distant fungi or higher eukaryotes including humans. (**a**) The proposed 18S interaction regions (indicated by blue bars) are almost 100% identical among all fungi examined. (**b**) In contrast, the corresponding ITS1 sequences (indicated by blue bars) are poorly conserved. Nucleotides A, U/T, G, and C are colored green, blue, red, and orange, respectively. Brackets indicate secondary structures; structural elements and processing sites are indicated. (**c**) The putative 18S-ITS1 base-pairing potential is weakly conserved in *Saccharomycotina* and was not found in more distant fungi. **(d)** For reference, the recently described pre-rRNA refolding model [2,3] is shown with proposed 18S-ITS1 interactions indicated by the blue box. Species names are explained in on-line Materials and Methods.



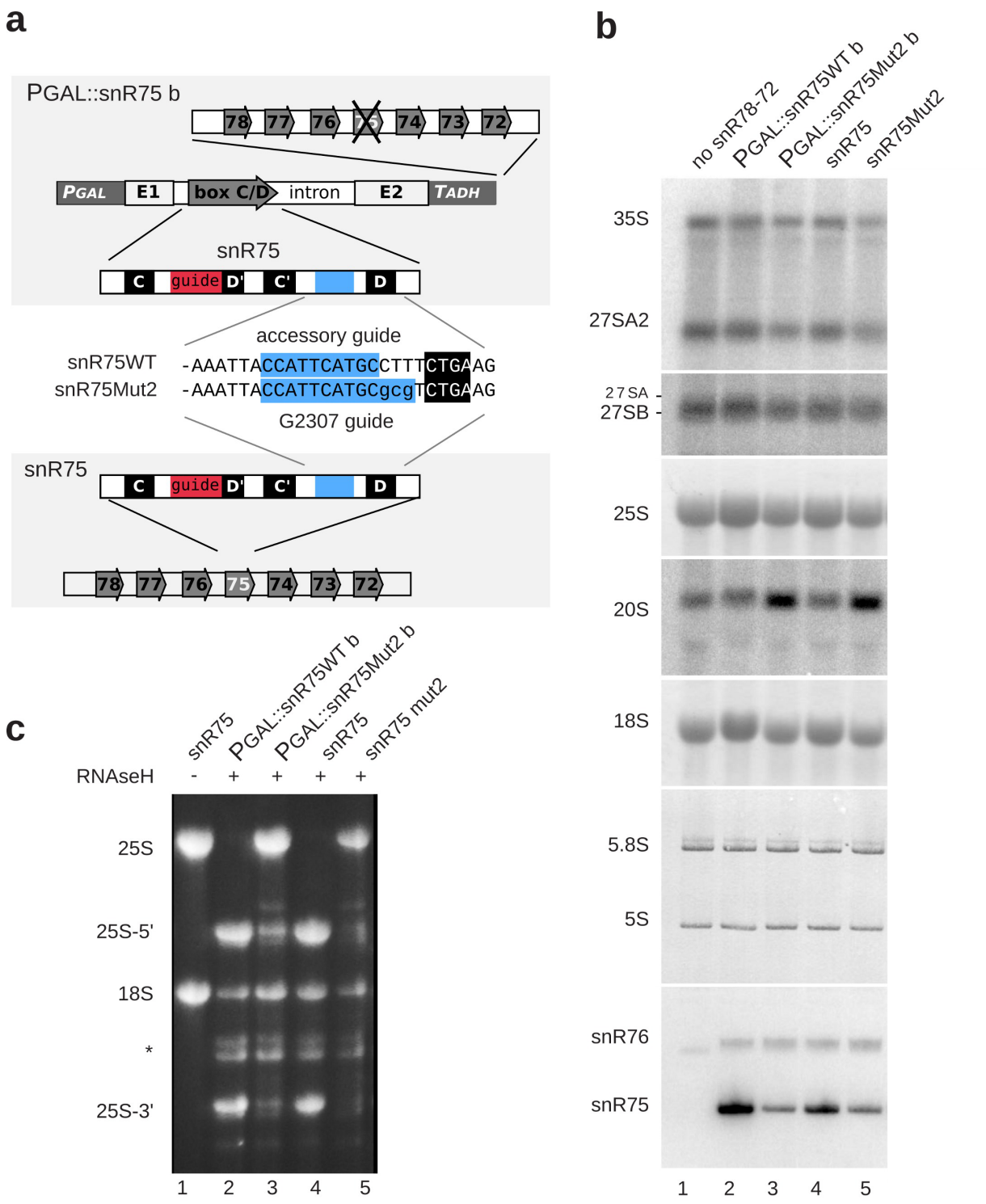
Supplementary Figure 2 Tsr1 lacks apparent GTP binding and GTPase activity. (**a**,**b**) HTP-tagged Tsr1 can be purified using IgG as shown by silver staining (**a**) and western blot with anti-TAP antibody (**b**), but not by GTP-agarose affinity purification. (**c**) Recombinant GST-fusion proteins, purified from *E.coli*, of either Tsr1-WT or Tsr1-K44A. The mutation is in the Tsr1 GTGK domain, which is similar to the GSGK p-loop involved in nucleotide binding by active GTPases. (**d**) The GST-fusion proteins did not purify on GTP-agarose beads which indicated a lack of GTP binding for both Tsr1 WT and the Tsr1 mutant. (**e**) Analysis of 25S and 18S rRNA, isolated after 12 h depletion of 3HA-TSR1-HTP, from strains transformed with empty plasmid (blue), or plasmids expressing Tsr1-WT (green) or Tsr1-Mut(K44A) (red). Depletion of endogenous Tsr1 results in a decrease of 18S rRNA (blue) whereas expression of only Tsr1 carrying the putative active-site point mutant supports normal pre-rRNA processing (red).



Supplementary Figure 3 20S pre-rRNA accumulating when Fun12 is absent or when its 25S rRNA binding is impaired has undergone cytoplasmic methylation. (**a**) Overview of the 3' domain of 18S rRNA with helix 45 (boxed) enlarged showing A1781 and A1782 that are dimethylated in the cytoplasm. (**b**) Dimethylation of A1781 and A1782 was probed by site-directed RNase H cleavage with oligo A2M for BY4741 (lanes 3, 4), fun12Δ (lanes 5, 6) or BY4741ΔsnR72-78 expressing the indicated snoRNA genes (lanes 7-14; see Supplementary Fig. 5a). Unmodified 20S accumulating in the nucleus would be cleaved producing an -18-A2 fragment (numbering is relative to site D) as observed with an in vitro synthesized -655-A2 fragment (mixed with *E. coli* RNA; lanes 1, 2). RNAs, separated on 1.2 % agarose (top) or 8% PAA/Urea (bottom), were visualized with probes Y1A+ and Y20S (left panels) or methylene blue (right panels). Nonspecific cleavage within 18S at ~600 nt upstream of site D yielded 18S* and 20S*. An RNA ladder was run alongside (M). (**c**) 20S derived species were quantified in comparison to -655-A2. In contrast to over 90% cleavage of in vitro synthesized RNA, less than 10% -18-A2 fragment was released from 20S pre-rRNAs indicating almost complete modification.



Supplementary Figure 3 25S methylation at G2307 affects growth and Fun12 ribosome association. (**a**) Analysis of the effects of expression of wild-type or mutant snR75 on cell growth. YPHΔsnR78-72 cells transformed with plasmids lacking snR75 or expressing the galactose inducible expression cassettes for snR75, snR75Mut1 or snR75Mut2 were spotted in ten-fold serial dilutions and incubated for two days at 30°C. (**b**) Analysis of RNA extracted from strain expressing different snR75 mutants. Methylation was analyzed by primer extension with reduced dNTP concentrations, which induces arrest at sites of 2'-O-methylation. (**c**) Mutations in snR75 do not affect immunoprecipitation of Fun12-HA with anti-HA from a strain in which deletion of the endogenous copy of SNR75 was complemented by snR75 or snR75Mut2 (as in panel (**a**)). (**d**) Coprecipitation of pre-rRNA and mature rRNAs with Fun12 from cells expressing snR75 or snR75Mut2 were quantified using northern hybridization data. (**e**) Ratios between 25S and 20S rRNAs co-precipitated with PTH-NOB1 from cells expressing snR75WT or snR75Mut2. Amounts of 25S and 20S co-purified were quantified by EtBr staining and normalized according to their relative lengths.



Supplementary Figure 4 Accumulation of 20S pre-rRNA by 25S methylation at G2307 does not depend on the context of expression of snR75Mut2. (**a**) The snR78-72 cluster was reinstated into the strains in which it had been disrupted. For this, the cluster lacking the gene for snR75 was inserted into the plasmids expressing the galactose-inducible expression cassettes for snR75 or snR75Mut2 (top) or the complete cluster was cloned into pRS416 (bottom) with the Mut2 mutation introduced after sitedirected mutagenesis. (**b**) Expression of snrR75Mut2 leads to a block of 20S pre-rRNA processing as shown by northern blot analysis with probe Y1a (detecting 20S, 35S pre-RNAs), when compared to 27SA2 or 27SB pre-rRNAs (with Y27SA2, or Y2a, respectively). The levels of snoRNAs were determined with probes snR75-5' and snR76. (**c**) Methylation of G2307 by snR75Mut2 fully protects 25S rRNA against site-specific RNase H cleavage with oligo aG2307rnh as 25S rRNA remains intact and no significant amounts of specific cleavage products (25S-5' and 25S-3') were formed. Mature rRNAs were visualized by EtBr (**b**) or methylene blue staining (**c**).

Supplementary Table 1 Strains used in this study.

Name	Description	Genotype	Source
ySLD34	GAL::NOB1	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; GAL1::3HA::NOB1; Kan^R*	This Study
YSLD11	GAL::NOB1, PTH-NOB1	*MATa; his3Δ1; met15Δ0; ura3Δ0; GAL1::3HA::NOB1; pRS415-PTH-NOB1; Kan^R*	This Study
YSLD57	GAL::FUN12	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; GAL1::3HA::FUN12; Kan^R*	This Study
YSLD67	GAL::FUN12, FUN12-HTP	*MATa; his3Δ1; met15Δ0; ura3Δ0; GAL1::3HA::FUN12; pRS415-FUN12-HTP; Kan^R*	This Study
YSLD68	GAL::FUN12, FUN12$_{T439A}$-HTP	*MATa; his3Δ1; met15Δ0; ura3Δ0; GAL1::3HA::FUN12; pRS415-FUN12$_{T439A}$-HTP; Kan^R*	This Study
YSLD69	GAL::Fun12, PTH-NOB1	*MATa; met15Δ0; ura3Δ0; GAL1::3HA::NOB1; GAL1::3HA::FUN12; pRS415-PTH-NOB1; Kan^R*	This Study
YSLD73	GAL::Fun12, PTH-NOB1, empty	*MATa; met15Δ0; GAL1::3HA::NOB1; GAL1::3HA::FUN12; pRS415-PTH-NOB1; pRS426; Kan^R*	This Study
YSLD72	GAL::Fun12, PTH-NOB1, fun12-high	*MATa; met15Δ0; GAL1::3HA::NOB1; GAL1::3HA::FUN12; pRS415-PTH-NOB1; pRS426-ADH1::FUN12; Kan^R*	This Study
YSLD74	GAL::Fun12, PTH-NOB1, fun12$_{D533N}$-high	*MATa; met15Δ0; GAL1::3HA::NOB1; GAL1::3HA::FUN12; pRS415-PTH-NOB1; pRS426-ADH1::FUN12$_{D533N}$; Kan^R*	This Study
YSLD70	GAL::Fun12, PTH-NOB1, fun12-low	*MATa; met15Δ0; GAL1::3HA::NOB1; GAL1::3HA::FUN12; pRS415-PTH-NOB1; pRS416-Met25::FUN12; Kan^R*	This Study
YSLD71	GAL::Fun12, PTH-NOB1, fun12$_{D533N}$-low	*MATa; met15Δ0; GAL1::3HA::NOB1; GAL1::3HA::FUN12; pRS415-PTH-NOB1; pRS416-Met25::FUN12$_{D533N}$; Kan^R*	This Study
YSLD75	YPHΔsnR78-72	*MATa ura3-52 lys2-801_amber ade2-101_ochre leu2Δ1 his3-Δ200 trp1-Δ63; snr78-72::NAT; NatR*	van Nues et al. 2011
YSLD80	ΔsnR78-72	*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; snr78-72::NAT; NatR*	This Study
YSLD81	ΔsnR78-72, snr75, FUN12-HA	*MATa; leu2Δ0; met15Δ0; snr78-72::NAT; pRS416 pGAL1-snR75; FUN12-HA::HIS3 ; Nat^R*	This Study
YSLD82	ΔsnR78-72, snr75Mut2, FUN12-HA	*MATa; leu2Δ0; met15Δ0; snr78-72::NAT; pRS416 pGAL1-snR75Mut2; FUN12-HA::HIS3; Nat^R*	This Study
D1089	TSR1-HTP	*MATa; his3Δ1; leu2Δ0; met15Δ0; TSR1-HTP*	Granneman et al. 2010
D1100	GAL::TSR1-HTP	*MATa; his3Δ1; leu2Δ0; met15Δ0; GAL1::Tsr1-HTP, Kan^R*	This Study
D1101	GAL::TSR1-HTP 3HA-TSR1$_{WT}$	*MATa; his3Δ1; leu2Δ0; met15Δ0; GAL1::Tsr1-HTP, p3HA-TSR1, Kan^R*	This Study
D1102	GAL::TSR1-HTP 3HA-TSR1$_{K44A}$	*MATa; his3Δ1; leu2Δ0; met15Δ0; GAL1::Tsr1-HTP, p3HA-TSR1$_{K44A}$, Kan^R*	This Study

All strains generated in this study were derived from BY4741 (*MATa; his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) except YSDL75, which is derived from YPH499.

Supplementary Table 2 Oligonucleotides used in this study.

Name	Sequence (5'-3')
Tsr1 S1	CAGAAATTTATTTGTTAGTTGAAGAGCGGTAGTTTTACGCAGGCATCAGAATGCGTACGCTGCAGGTCGAC
Tsr1 S2	TAAGATTTGTGTCCGTTTTTTAATGATGACCTGTGTGAATGACCTGCCATCGATGAATTCTCTGTCG
Fun12 F2	GGCTGCTATTGAAGAAGCTGAAGGTCGTTTTCGGCATCGAACGGATCCCCGGGTTAATTAA
Fun12 R1	GTAGAATGTGATTGGGTTGACAAGTCAGCGTATGCCATGCGCATAGGCCACTAGTGGATC
Fun12 F5	CACACCGTAATATCCCATCTTAAAAGTGGAAAACTCTTATGAATTCGAGCTCGTTTAAA
Fun12 R5	CATCCCAGTAGTTCTGTTGGTTCTTTTTACTCTTTTTCGCGCACTGAGCAGCGTAATCTG
FUN12 Xma1 a	GGGGGCCCGGGCTGCGAAAAAGAGTAAAAAGAACCAACAGAACTACTGGG
Fun12 Xma1 b	GGGGGCCCGGGTCATTCGATGCCGAAAACGACCTTCAGCTTCTTCAATAG
Fun12T439A1	GGTGGTGAAGCTGGTGGCATCGCCCAACAGATTGGTGCCAC
Fun12T439A2	GTGGCACCAATCTGTTGGGCGATGCCACCAGCTTCACCACC
Fun12D533N1	CATTTGTCGTTGCCCTAAACAAAATTAATAGATTATATGACTGGAAAGCCATTC
FUN12D533N2	GAATGGCTTTCCAGTCATATAATCTATTAATTTTGTTTAGGGCAACGACAAATG
Probe 004	CGGTTTTAATTGTCCTA
Probe 005	ATGAAAACTCCACAGTG
Probe 006	AGATTAGCCGCAGTTGG
Probe 011	TCTCTTCCAAAGGGTCG
Probe 012	GCACCGAAGGTACCAG
ITS1RT	CCATCTCTTGTCTTCTTGCCCAG
Y1a	TTAAGCGCAGGCCCGGCTGG
Y27SA2	CCGATTGCTCGAATGCCCAAA
Y58	CAAACAGGCATGCCCCCT
Y2a	GTATCACTCACTACCAAACAGAATG
snR76	TTTCTAGGCCCGCTAAAGCATTGTCA
snR75-5'	GTCATCTATAAATATCTCATCATA
map75	CTAGATAGTAGATAGGGACAGTGG
aG2307rnh	mAmUmGCGCGmUmCmAmCmUmAmAmUmUmAmGmA
Y20S	GAAATCTCTCACCGTTTGGAATAGCA
Y1A+	ACTTAAGCGCAGGCCCGGCTGGACT
A2M	mAmGmGTTCAmCmCmUmAmCmGmGmAmAmAmC
ITS1FISH	TTGCACAGAAATCTCTcy3CACCGTTTGGAATcy3AGCAAGAAAGAAACTcy3TACA

Supplementary Note The potential for a conformational switch involving helix 44 in 18S rRNA and ITS1 is not conserved.

As shown in Supplementary Fig. 1 no significant conservation of the complementarity to the implicated 18S regions can be discerned in the ITS1 from *Pezizomycotina* or *Schizo-saccharomycetes* (**b**) or in an alignment of 11 mammalian, ~1 kb long, very GC-rich spacer sequences (data not shown) in which the proposed 18S-interacting regions or A2 cleavage sites in rat or human ITS1 [2] do not line up. The ITS1 regions in the three groups of fungi do not appear to share a common secondary structure [1,4–6], while the ITS1 from *Verticillium albo-atrum*, a plant-pathogen belonging to the *Pezizomycotina,* blocks 35S pre-rRNA processing when substituted for the normal ITS1 in a *S. pombe* pre-rRNA transcript [7] suggesting incompatible processing machineries between these groups of fungi. Sequences nearby A2 or containing A3 in *S. pombe* ITS1 [6] appear conserved among *Schizo-saccharomycetes* whereas in the *Pezizomycotina* ITS1, AC-rich sequences flank a branched stem-loop, reminiscent of the *S. cerevisiae* A2-A3 organization. For all *Saccharomycetes* apart from *Clavispora lusitaniae* (Clus) three structural elements can be modeled: a helical domain II with a conserved tip, a stem-loop downstream of A2, and helix III with a fairly well conserved base (indicated by brackets in panels (**b**) or (**c**)). The whole of domain II, rather than its conserved tip (which is protected against DMS modification by the presence of Nob1 or Rrp5 in vitro [3]) is required for normal growth but not pre-rRNA processing [4]. The proposed stem-loop 3' to site A2 is phylogenetically and experimentally supported [1,4,5] (data not shown). The helix IV (gray box in panel (**d**) and gray lines above ITS1 alignment in panel (**b**)) is neither common nor essential [4]. However, as revealed by this pile-up, this region harbors the remnant of an element (GUCUGA) conserved in the other two groups of fungi. As shown in panel (**c**), in *S. cerevisiae* the previously proposed ITS1-18S base-pairing required either multiple G-U interactions [2,3] or non-conserved base-pairing [1,4] (data not shown). The high conservation of the 18S sequence among *Saccharomycetes* limits the potential for compensatory base changes. The only nucleotide altered, a C (against gray background) to a G, is not expected to interact with ITS1. This provides, however, a compensatory change for maintaining perfect base-pairing within helix 44 itself (data not shown). Several ITS1 nucleotides that could potentially form interactions (blue underlined) are not aligned with the residues in *S. cerevisiae* and the internal bulges in the resulting ITS1/18S helices are not predicted to be favorable for helix formation. In most cases, mismatches (in reverse contrast) interrupt the putative base-pairing. Only among the *Sensu Strictu Saccharomyces* species, *S. mikatae* (Smik), *S. bayanus* (Sbay), *S. kudravezii* (Skud), and *S. paradoxus* (Spar), is the proposed 18S-ITS1 interaction fully conserved. Overall, the phylogenetic comparisons indicate that a conformational switch involving ITS1 and 18S rRNA [2] is unlikely in organisms that are not closely related to *S. cerevisiae*.

Supplementary References

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