Eukaryotic ribosomes are assembled by a complex pathway that extends from the nucleolus to the cytoplasm and is powered by many energy-consuming enzymes\(^1-9\). Nuclear export is a key, irreversible step in pre-ribosome maturation\(^8,9\), but mechanisms underlying the timely acquisition of export competence remain poorly understood. Here we show that a conserved *Saccharomyces cerevisiae* GTPase Nug2 (also known as Nog2, and as NGP-1, GNL2 or nucleostemin 2 in human\(^9\)) has a key role in the timing of export competence. Nug2 binds the inter-subunit face of maturing, nucleo-plasmic pre-60S particles, and the location clashes with the position of Nmd3, a key pre-60S export adaptor\(^10\). Nug2 and Nmd3 are not present on the same pre-60S particles, with Nug2 binding before Nmd3. Depletion of Nug2 causes premature Nmd3 binding to the pre-60S particles, whereas mutations in the G-domain of Nug2 block Nmd3 recruitment, resulting in severe 60S export defects. Two pre-60S remodelling factors, the Rea1 ATPase and its co-substrate Rsa4, are present on Nug2-associated particles, and both show synthetic lethal interactions with *nug2* mutants. Release of Nug2 from pre-60S particles requires both its K\(^+\)-dependent GTPase activity and the remodelling ATPase activity of Rea1. We conclude that Nug2 is a regulatory GTPase that monitors pre-60S maturation, with release from its placeholder site linked to recruitment of the nuclear export machinery.

The conserved GTPase Nug2 (Extended Data Fig. 1) is associated with several pre-60S particles located in the nucleoplasm, but was not detected on particles with a known cytoplasmic location (see also Extended Data Fig. 2). The bacterial homologue of Nug2, YIqF, binds directly to ribosomal RNA\(^1,3\), and we therefore used the ultraviolet cross-linking and analysis of complementary DNA (CRAC) method to localize the binding site for yeast Nug2 within the pre-60S particle\(^12\). Direct contacts for Nug2 were identified only with the 25S rRNA, at sites in helices H38, H69, H81–83, H84–86, H89, H91–92 and H93 (Fig. 1a, c). Yeast three-hybrid analyses confirmed interactions between Nug2 and these rRNA helices (Fig. 1b). Mapping the major RNA crosslink sites of Nug2 onto the 60S subunit structure (Fig. 1d) showed a distinct cluster of the inter-subunit joining surface\(^13\). Nug2-binding sites overlap with regions occupied by the export factor Nmd3 in cryo-electron microscopy\(^14\). CRAC was therefore applied to Nmd3 to identify its binding sites more precisely, which were found to lie in H38, H69 and H89 of 25S rRNA (Fig. 1a, c, e). Notably, the Nug2- and Nmd3-binding sites overlapped in H38, H69 and H89 (Fig. 1c, f), suggesting that the binding of these two proteins is mutually exclusive. To test this, pre-60S particles were purified with tagged Nug2 and shown to lack detectable Nmd3, and vice versa (Extended Data Fig. 2). Nmd3 is an essential nuclear export factor that recruits the export receptor Crm1 to the nascent 60S subunits\(^15,16\). These observations suggested that Nug2 acts as a 'placeholder' to prevent premature recruitment of Nmd3 to early, export-incompetent pre-60S particles.

Like other GTP-binding proteins, Nug2 has characteristic G1, G3 and G4 motifs in its G-domain (Fig. 2a and Extended Data Fig. 1), suggesting that GTP-binding or hydrolysis\(^16\) might regulate dynamic interactions between Nug2 and the pre-ribosome. Dominant-negative mutations were previously described in two GTPases involved in ribosome biogenesis, the G1 motif of Lsg1 (Lys349Asn/Arg/Thr)\(^17\) and the G3 motif of Nog1 (Gly224Ala)\(^18\). Orthologous G1- and G3-motif mutants, *nug2(K328R)* and *nug2(G369A)*, respectively (Fig. 2a and Extended Data Fig. 1), each showed severe growth defect phenotypes (Fig. 2b), and were also dominant-negative when overexpressed in the presence of chromosomal *NUG2* (Fig. 2c). Pre-ribosome analysis by sucrose gradient centrifugation showed that the *Nug2(Lys328Arg)* and *Nug2(Gly369Ala)* proteins were efficiently assembled into pre-60S subunits, but induced a ‘half-mer’ polysome phenotype (in particular for *Nug2(Lys328Arg)*), characteristic of reduced 60S subunit synthesis (Fig. 2d). The reduced 60S levels were more apparent under low Mg\(^2+\) conditions that cause 80S ribosomes to dissociate into 60S and 40S subunits (Fig. 2d). The *nug2(K328R)* and *nug2(G369A)* strains showed nuclear accumulation of an enhanced green fluorescent protein (eGFP)-containing RpL25–eGFP reporter, but not RpS3–eGFP, revealing a specific block in pre-60S nuclear export (Fig. 2e). We conclude that mutations in the GTPase domain of Nug2 allow recruitment to the pre-ribosomes, but block nuclear export.

To determine the basis of the defects associated with *Nug2(Lys328Arg)* and *Nug2(Gly369Ala)*, we assayed *in vitro* guanine-nucleotide-binding activity and GTP hydrolysis. Nug2 from *S. cerevisiae* was unstable when expressed in *Escherichia coli* (data not shown). By contrast, good yields were obtained for wild-type and mutant Nug2 from the eukaryotic thermophile *Chaetomium thermophilum* (ctNug2, ctNug2(Lys339Arg) and ctNug2(Gly380Ala), respectively; Fig. 2f), the thermostable proteins of which have superior biochemical properties\(^19\). ctNug2 is highly homologous to yeast Nug2 (74% identity; Extended Data Fig. 1), and can complement, albeit not perfectly, a yeast *nug2* mutant (Extended Data Fig. 3). As Nug2 may act as a potassium-dependent GTPase\(^20\), we tested the cation requirement for GTP hydrolysis. The GTPase activity of ctNug2 was low in NaCl-containing buffer, but was substantially stimulated by KCl (Fig. 2f). By contrast, ctNug2(Lys339Arg) and ctNug2(Gly380Ala) exhibited only background GTPase activity (Fig. 2f). In binding assays, wild-type ctNug2 and ctNug2(Gly380Ala) readily bound the fluorescent nucleotides MANT-GTP or MANT-GDP, whereas ctNug2(Lys339Arg) did not (Fig. 2g). We conclude that ctNug2(Lys339Arg) is defective in GTP binding, whereas ctNug2(Gly380Ala) binds but cannot hydrolyse GTP. This *K*\(^+\)-stimulated GTPase activity might regulate the interaction of Nug2 with nascent 60S particles.

Nug2 is associated with nucleoplasmic pre-60S particles that also carry the Rix1–Ip1–Ip3 heterotrimer, the dynein-related AAA-ATPase Rea1, and its co-substrate Rsa4 (Extended Data Fig. 2; see also below and ref. 21). The enzymatic activity of Rea1 is required for the release of Ytm1 (ref. 22) and Rsa4 (ref. 21) and a genetic screen revealed synthetic lethality between the G1-motif mutant *nug2(K328R)* and the mutant alleles *real-DTS* and *rsat* (ref. 21) (Fig. 3a). We therefore investigated whether ATP-dependent remodelling of the Rix1 particle by the...
AAA-ATPase activity of Rea1 (ref. 21) is altered in particles containing Nug2(Lys328Arg) or Nug2(Gly369Ala). Pre-60S particles carrying Flag-tagged RpL3 were affinity purified using a tandem affinity purification (TAP)-tagging technique (Rix1–TAP) via IgG binding and tobacco etch virus (TEV) elution. The pre-60S particles were incubated in vitro to allow factor release, and then re-isolated on Flag beads via RpL3–Flag (Fig. 3b). Consistent with previous data21, incubation of the pre-60S particles with ATP in Na+–containing buffer resulted in the release of Rsa4 and Rea1, but not Nug2 (Fig. 3c). By contrast, incubation in K+-containing buffer caused the ATP-dependent release of Nug2, in addition to Rsa4 and Rea1 (Fig. 3c). Incubation with GTP in Na+– or K+-containing buffer did not induce the release of biogenesis factors (Fig. 3d). However, neither Nug2(Lys328Arg) nor Nug2(Gly369Ala) could be dissociated from pre-60S particles after ATP treatment in K+ buffer (Fig. 3e). In the case of Nug2(Lys328Arg) (defective in GTP binding), incubation with ATP in K+ buffer failed to release Rsa4, whereas pre-60S particles carrying Nug2(Gly369Ala) (defective in GTP hydrolysis) still showed Rsa4 release after ATP treatment (Fig. 3e). Mutation of one of the six ATP-binding protomers of Rea1 (AAA2; real Lys659Ala) inhibited remodelling, including Nug2 release (Extended Data Fig. 4). These findings indicate that the GTP-binding activity of Nug2 influences the remodelling activity of the Rea1 ATPase, whereas GTP hydrolysis is necessary for the final Nug2 release from the pre-60S subunit.

In vitro, Real1-dependent release of Rsa4 and Nug2 required only ATP and K+ without the addition of GTP, whereas the mutational analyses suggested that GTPase activity is necessary for Nug2 release. These findings suggest that Nug2 on the Rix1 particle might have retained bound GTP during purification (which is possible owing to its low intrinsic GTPase activity). Alternatively, ribosome-associated nucleotide diphosphate kinases can transfer the γ-phosphate from ATP to GDP to generate GTP-loaded GTPases23,24.

The pre-60S particles co-purified with Rix1 also contained small amounts of Ytm1 and Erb1 (Fig. 3c), which were previously described as nucleolar co-substrates for Real1 (ref. 22), and both were released by incubation with ATP in Na+ or K+ buffer (Fig. 3c).

To determine the step in 60S subunit biogenesis at which dissociation of Nug2 is disturbed in vivo, we affinity-purified different pre-60S particles from Nug2 wild-type and mutant cells using bait proteins that specifically enrich nucleolar, nucleoplasmic or cytoplasmic intermediates (Fig. 4a). The Nug2(K328R) mutation did not markedly alter the biochemical composition of most pre-60S particles tested. The exception was Arx1-associated particles, which showed a marked depletion of the export adaptor Nmd3 and the cytoplasmic factor Rei1 that stimulates recycling of Arx1 (ref. 25) (Fig. 4a). Nmd3 was also largely absent from Arx1 particles purified from Nug2 cells (Fig. 4b).

To test the model that Nug2 depletion allows premature recruitment of Nmd3, we used an auxin-inducible degron system26. Nug2 was expressed as a fusion protein (sAid–Nug2–sAid) with two copies of the sAid (small auxin-inducible degron) tag, which is targeted by the F-box E3 ubiquitin ligase TIR1 in the presence of auxin, inducing fast proteasomal degradation26 (Extended Data Fig. 5). Nmd3 is normally not detected on Rix1-associated particles, but was prematurely recruited to this pre-60S particle after Nug2 depletion (Fig. 4c). Concomitant with Nmd3 association, the recovery of Rea1 and Rsa4 decreased during Nug2 depletion (Fig. 4c). We conclude that Nug2 promotes the stable association of Rsa4 and Real1 with the Rix1 particles, while blocking premature recruitment of Nmd3.

To address the timing of Nug2 recruitment to pre-60S particles in comparison to Real1, Rsa4 and the Rix1–Ipi1–Ipi3 complex, we used a combination of affinity purification and immunodepletion. Affinity purification of Nug2–TAP yielded a mixture of different pre-60S particles including Rix1–Rea1 particles. Rix1–Flag immunoprecipitation was used to deplete Rix1–Rea1 particles from this mixture, leaving...
or mutant Nug2 were incubated with ATP or GTP in NaCl or KCl buffer, before
overexpression (of Nug2. C, carboxy; N, amino.

RSA4

part of the pre-ribosomes after dissociation of Has1 (ref. 27).

Nug2 particles that contained Rsa4 and several intermediate pre-60S factors including Nog1, Arx1, Nug1, Nop53, Nsa3, Rp2, Rp7 and Nsa2 (Fig. 4d). However, this Nug2 particle lacked other (further upstream) pre-60S factors such as Ytm1, Erb1 and Has1, suggesting that it corresponds to the precursor particle to which Nug2 was recruited. These data complement previous findings that Nug2 is the last ‘B-factor’ to associate with pre-ribosomes after dissociation of Has1 (ref. 27).

As outlined in Fig. 4e, we propose that a previously uncharacterized step in the reorganization of the evolving pre-60S subunit primes it for nuclear export. This involves a regulatory GTPase Nug2 that overlaps with the binding site for the essential nuclear export adaptor Nmd3. As long as intranuclear maturation is incomplete, the pre-60S subunit cannot be exported, because recruitment of this essential export factor is not possible. However, a late nucleoplasmic remodelling step, catalysed

Figure 3 | Nug2 release from pre-60S particles requires intrinsic K+-dependent GTase and Rea1 ATPase activity. a, Synthetic lethality between alleles rsa4-1 (ref. 21) or rea1-DTS and nug2(K328R) revealed by growth on 5-fluoroorotic acid (5-FOA). b-e, ATP-dependent release of Rsa4 and Nug2 from purified pre-60S particles. Scheme of the release assay (b) and experimental analyses (c–e). Affinity-purified Rix1 particles carrying wild-type or mutant Nug2 were incubated with ATP or GTP in NaCl or KCl buffer, before

matured pre-60S particles were re-isolated via RpL3–Flag affinity-purification. Final eluates were analysed by SDS–PAGE and Coomassie staining (top; indicated bands were identified by mass spectrometry) and western blotting using the indicated antibodies (bottom) (c–e). CBP, calmodulin-binding peptide. All in vitro assays were performed at least twice with highly reproducible data sets.
by the AAA-ATPase Real and its co-factor Rsa4, restructures the pre-60S particle, which could lead to both an rRNA and assembly factor rearrangement. This conformational change could also stimulate the K⁺-dependent GTPase activity of Nug2, thereby triggering its release from the matured pre-60S particles. We suggest that the Nug2 GTPase acts as molecular switch to proofread pre-ribosome maturation and regulate the acquisition of export competence. After this reorganization step, the binding site for Nmd3 becomes accessible on the pre-60S subunit, which further triggers Crm1 and RanGTP recruitment to generate nuclear export competence. Thus, our data indicate coordination between a remodel of AAA-ATPase and a conformation-sensing GTPase.

The human Nug2 orthologue GNL2 is highly expressed in proliferating cells including cancer cells, and is involved in the control of cell cycle progression. The discovery of the role of Nug2 during surveillance of ribosomal proteins? Mol. Cell 34, 315–319 (2009).

METHODS SUMMARY

Materials and methods for TAP purification, CRAC analysis, purification of ctNug2, GTPase and guanine nucleotide binding assays are described in detail in the Methods. Yeast strains and plasmids used in this study are described in Extended Data Tables 1 and 2. Adapters used for the CRAC analysis are described in Extended Data Table 3.

Online Content Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Strunk, B. S. & Karpestein, K. Powering through ribosome assembly. *RNA* 15, 2083–2104 (2009).

2. Staley, J. P. & Wooford, J. L. Jr. Assembly of ribosomes and splicosomes: complex ribonucleoprotein machines. *Curr. Opin. Cell Biol.* 21, 109–118 (2009).

3. Granneman, S. & Baserga, S. J. Ribosome biogenesis: of knobs and RNA processing. *Exp. Cell Res.* 296, 43–50 (2004).

4. Lafontaine, D. L. J. & Baserga, S. J. Ribosome biogenesis: of knobs and RNA processing. *Exp. Cell Res.* 296, 43–50 (2004).

5. Warner, J. R. & McIntosh, K. B. How common are extraribosomal functions of ribosomal proteins? *Mol. Cell* 34, 315–319 (2009).

6. Houseley, J. & Tollervey, D. The many pathways of RNA degradation. *Cell* 136, 763–776 (2009).

7. Zemp, I. & Kutay, U. Nuclear export and cytoplasmic maturation of ribosomal subunits. *FEBS Lett.* 581, 2783–2793 (2007).

8. Dez, C., Houseley, J. & Tollervey, D. Surveillance of nuclear-restricted pre-ribosomes within a subnucleolar region of Saccharomyces cerevisiae. *EMBO J.* 25, 1534–1546 (2006).

9. Tsai, R. Y. & Meng, L. Nucleostemin: a latecomer with new tricks. *Science* 326, 267–277 (2010).

10. Roper, A. K. & Kutay, U. Nuclear export and cytoplasmic maturation of ribosomal subunits. *FEBS Lett.* 581, 2783–2793 (2007).

11. Tsai, R. Y. & Meng, L. Nucleostemin: a latecomer with new tricks. *Science* 326, 267–277 (2010).

12. Sengupta, J. et al. Characterization of the nuclear export adaptor protein Nmd3 in association with the 60S ribosomal subunit. *J. Cell Biol.* 189, 1079–1086 (2010).

13. Matsuo, Y. et al. The GTP-binding protein YfgF participates in the late step of 50S ribosomal subunit assembly in *Bacillus subtilis*. *J. Bacteriol.* 281, 8110–8117 (2009).

14. Munoz, J. et al. The GTP-binding protein YfgF participates in the late step of 50S ribosomal subunit assembly in *Bacillus subtilis*. *J. Bacteriol.* 281, 8110–8117 (2009).

15. Brüning, J. et al. The GTP-binding protein YfgF participates in the late step of 50S ribosomal subunit assembly in *Bacillus subtilis*. *J. Bacteriol.* 281, 8110–8117 (2009).

16. Bourne, H. R., Sanders, D. A. & McCormick, F. The GTPase superfamily: conserved structure and molecular mechanism. *Nature* 349, 117–127 (1991).

17. Hedges, J., West, M. & Johnson, A. W. Release of the export adapter, Nmd3p, from the 60S ribosomal subunit requires Rpl10p and the cytoplasmic GTPase Lsg1p. *EMBO J.* 24, 567–575 (2005).
18. Lapik, Y. R., Misra, J. M., Lau, L. F. & Pestov, D. G. Restricting conformational flexibility of the switch II region creates a dominant-inhibitory phenotype in Obg GTPase Nog1. Mol. Cell. Biol. 27, 7735–7744 (2007).

19. Amlacher, S. et al. Insight into structure and assembly of the nuclear pore complex by utilizing the genome of a eukaryotic thermophile. Cell 146, 277–289 (2011).

20. Ash, M. R., Maher, M. J., Mitchell Guiss, J. & Jormakka, M. The cation-dependent G-proteins: in a class of their own. FEBS Lett. 586, 2218–2224 (2012).

21. Ulbrich, C. et al. Mechanochemical removal of ribosome biogenesis factors from nascent 60S ribosomal subunits. Cell 138, 911–922 (2009).

22. Baßler, J. et al. The AAA-ATPase Rea1 drives removal of biogenesis factors during multiple stages of 60S ribosome assembly. Mol. Cell 38, 712–721 (2010).

23. Kikkawa, S. et al. Conversion of GDP into GTP by nucleoside diphosphate kinase on the GTP-binding proteins. J. Biol. Chem. 265, 21536–21540 (1990).

24. Wertheimer, A. M. & Kaulenas, M. S. GDP kinase activity associated with salt-washed ribosomes. Biochem. Biophys. Res. Commun. 78, 565–571 (1977).

25. Hung, N. J. & Johnson, A. W. Nuclear recycling of the pre-60S ribosomal subunit-associated factor Arx1 depends on Rei1 in Saccharomyces cerevisiae. Mol. Cell. Biol. 26, 3718–3727 (2006).

26. Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T. & Kanemaki, M. An auxin-based degron system for the rapid depletion of proteins in nonplant cells. Nature Methods 6, 917–922 (2009).

27. Dembowski, J. A., Kuo, B. & Woolford, J. L. Jr. Has1 regulates consecutive maturation and processing steps for assembly of 60S ribosomal subunits. Nucleic Acids Res. 41, 7889–7904 (2013).

28. Chennupati, V. et al. Signals and pathways regulating nucleolar retention of novel putative nucleolar GTPase NGP-1(GLN-2). Biochemistry 50, 4521–4536 (2011).

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Author Contributions Experiments were designed and the data were interpreted by Y.M. and E.H.; all experiments except CRAC analysis were performed by Y.M.; CRAC experiments and data analyses were performed by S.G. in collaboration with D.T.; M.T. constructed rea1 mutants and performed the in vitro release assay of rea1 mutants, the ctnuG2 complementation assay and the immunodepletion assay; R.-G.M. developed the methods of the in vitro assay for nucleotide binding and GTPase activity measurement; the manuscript was written by Y.M. and E.H.; all authors discussed the results and commented on the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to E.H. (ed.hurt@bzh.uni-heidelberg.de).
METHODS

Yeast strains and genetic methods. The S. cerevisiae strains used in this study are listed in Extended Data Table 1. Gene disruption and C-terminal tagging were performed as previously described25-26.

Plasmid constructs. All recombinant DNA techniques were performed according to standard procedures using E. coli DH5α for cloning and plasmid propagation. Site-directed mutagenesis was performed by overlap-extension PCR. All cloned DNA fragments generated by PCR amplification were verified by sequencing. Plasmids used in this study are listed in Extended Data Table 2.

CRAC analysis. The CRAC experiments were performed as described27 using the Nug2- and Nmd3-3HTP (His6-TEV-ProTAP) strain. CRAC data were processed using pyCRAC (S. Webb, R. D. Hector, G. Kudla and S.G., manuscript submitted). Cells were ultraviolet-irradiated in the Megatron UV chamber1 at a dose of 1.6J cm⁻² and processed as described27,28. The CDNs from the Nug2 CRAC data were cloned into pCR4-TOPO (Invitrogen), and inserts were sequenced by Sanger sequencing. The CDNs originating from Nmd3 CRAC experiments were sequenced on the Illumina MiSeq system (single-end 50b), according to manufacturer’s procedures. The MiSeq CRAC data were processed using the pyCRAC software suite (S. Webb, R. D. Hector, G. Kudla and S.G., manuscript submitted; https://bitbucket.org/sgrann/pycrak)). To remove potential PCR duplicates, the Nmd3 MiSeq data set was collapsed using pyFastDuplicateRemover. Reads subsequently mapped to the yeast reference sequence (version 2008) using noaligno (https://www.novocraft.com). Plots of reads aligned to the 35S reference sequence were generated using pyPileup and GNuplot. Adapters using this experiment are listed in Extended Data Table 3.

Expression and purification of ctNug2. The gene encoding C. thermophilum Nug2 (UniProtKB/TrEMBL accession: G0SBX1_CHATD) was cloned from cDNA by standard procedures as recently described29. Subsequently, the ctNug2 was inserted into yeast or E. coli expression plasmids (see below). Because the C-terminal extension of ctNug2 (511-627 amino acids) is not conserved (Extended Data Fig. 1), ctNug2 from 1 to 510 amino acids was cloned into pET21 vector for the in vitro experiments. ctNug2 was expressed using PET-ctNug2-510-His6, plasmid in E. coli Rosetta-DE3 cells. Transformed cells were grown at 23 °C in LB medium until they reached an absorbance at 600 nm (A600nm) of 0.6, isopropyl-β-D-thioglactoside (IPTG) was added to a final concentration of 0.1 mM. The cells were grown for an additional 3 h and then collected by centrifugation and stored frozen at −80 °C. Frozen pellets were resuspended in buffer KCl300 (50 mM Tris, pH 8.0, 200 mM KCl, 5% glycerol, 0.01% NP-40 and 2 mM β-mercaptoethanol) with protease-inhibitor cocktail, and were broken by sonication (BANDELIN sonopuls 3200 with TITANTELLER TT13) on ice. Sonication was performed under these conditions: amplitude: 50%, 3 s on, 8 s off, processed for 10 min. The lysate was centrifuged at 39,000g for 30 min at 4 °C. The supernatant fraction was applied to a SP-sepharose column, and was washed with buffer KCl300–ctNug2-His6, was eluted by buffer KCl300 containing 300 mM KCl. Next, the eluate fraction was applied to a Ni²⁺-NTA column, and the column was washed with buffer KCl300–ctNug2-His6, was eluted with buffer KCl300 containing 250 mM imidazole, before it was finally dialyzed against buffer KCl300. Measurement of GTPase activity by single-turnover reactions. The GTPase activity experiments were performed as previously described30: ctNug2, ctNug2K339R or ctNug2G380A (1 μM) were incubated with a final concentration of 0.1 μM GTP containing 750 nCi of [γ-32P]-labelled GTP in buffer KCl300 (50 mM Tris, pH 8.0, 300 mM KCl, 10 mM MgCl2, 2 mM EDTA and 1 mM DTT). MANT-GTP or MANT-GDP are analogues of natural GTP or GDP, where either the ribose 2’-hydroxy or the 3’-hydroxy group has been esterified by the fluorescein methylisatoic acid with an excitation/emission = 355/448 nm. The fluorescence quantum yield of MANT fluorophore is very low in water and increases significantly in non-polar solvents or after binding to most proteins. This highly environmental sensitive fluorescence of MANT makes MANT-ATPase-dependent role of the atypical kinase Rio2 on the evolving pre-40S ribosomal subunit. Nature Struct. Mol. Biol. 19, 1316–1323 (2012).

Bradatsch, B. et al. Structure of the pre-60S ribosomal subunit with nuclear export factor Xpo1 bound at the exit tunnel. Nature Struct. Mol. Biol. 15, 1244–1241 (2008).

Rigaut, G. et al. A generic protein purification method for protein complex characterization and proteome exploration. Nature Biotechnol. 17, 1030–1032 (1999).

Baillier, J. et al. Identification of a 60S preribosomal particle that is closely linked to active nuclear export. Mol. Cell, 517–529 (2001).

Hurt, E. et al. A novel in vivo assay reveals inhibition of ribosomal nuclear export in cytosine- and nucleosporin mutants. J. Cell. Biol. 164, 389–401 (1999).

SenGupta, D. et al. A three-hybrid system to detect RNA-protein interactions in vivo. Proc. Natl Acad. Sci. USA 93, 8496–8501 (1996).

Saveanu, C. et al. Sequential protein association with nascent 60S ribosomal particles. Mol. Cell. Biol. 23, 4449–4460 (2003).

de la Cruz, J., Sanz-Martinez, E. & Remacha, M. The essential WD-repeat protein Rsa4p is required for rRNA processing and intra-nuclear transport of 60S ribosomal subunits. Nucleic Acids Res. 33, 5728–5739 (2005).

Gwizdek, A. et al. Ubiquitin-associated domain of Mdbp synchronizes recruitment of the mRNA export machinery with transcription. Proc. Natl Acad. Sci. USA 103, 16376–16381 (2006).

Frey, S., Pool, M. & Seedorf, M. Scl160p, an RNA-binding, polysome-associated protein, localizes to the endoplasmic reticulum of Saccharomyces cerevisiae in a microtubule-dependent manner. J. Biol. Chem. 276, 15905–15912 (2001).

Vilaridel, J. & Warner, J. R. Ribosomal protein L32 of Saccharomyces cerevisiae influences both the splicing of its own transcript and the processing of rRNA. Mol. Cell Biol. 17, 1955–1965 (1997).

Lebretton, A., Saveanu, C., Decourty, L., Jacquier, A. & Fromont-Racine, M. N2a-2 is an unstable, conserved factor required for the maturation of 28 S pre-rRNA. J. Biol. Chem. 281, 27099–27108 (2006).

Bussetti, C., Hashemi, Y., Arora, S., Franke, J. & Johnson, A. W. Integrity of the P-site is probed during maturation of the 60S ribosomal subunit. J. Cell Biol. 197, 747–759 (2012).

Lebretton, A. et al. A functional network involved in the recycling of nucleocytoplasmic pre-60S particles. J. Cell Biol. 173, 349–360 (2006).

Thomas, B. J. & Rothstein, R. Elevated recombination rates in transcriptionally active DNA. Cell 56, 619–630 (1989).

Baillier, J., Kallas, M. & Hurt, E. The NUG1 GTPase reveals a 60S RNA-binding domain that is essential for association with 60S pre-ribosomal particles. J. Biol. Chem. 281, 24737–24744 (2006).
Extended Data Figure 1 | Multiple sequence alignment of various Nug2 orthologues. Multiple sequence alignment of Yqf (bacterial homologue of Nug2; Bacillus cereus), ctNug2, DmNug2 (Drosophila melanogaster), DrNug2 (Danio rerio), HsNug2 (Homo sapiens), KlNug2 (Kluyveromyces lactis), MmNug2 (Mus musculus), ScNug2 (S. cerevisiae), SpNug2 (S. pombe), XINug2 (Xenopus laevis) and YlNug2 (Yarrowia lipolytica), using T-Coffee multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/tcoffee) and Jalview. Indicated above the alignment are the different Nug2 domains including the N, G, and C domains and the C-terminal extension. Moreover, the DAR, G1, G3, G4 motifs, point mutation sites (in red) and truncated site of ctNUG2-510 amino acids (truncation of the non-conserved C-terminal extension; red line) are indicated.
Extended Data Figure 2 | Nug2 and Nmd3 are not found on the same pre-60S particles. Indicated different TAP-tagged bait proteins were affinity purified from yeast wild-type cells. The final eluates were analysed by SDS-PAGE and Coomassie staining (top), and by western blotting using the indicated antibody (bottom). Asterisks mark the position of each bait protein. Rea1 has been identified by mass spectrometry. All affinity purifications and western analyses were performed at least twice, yielding highly reproducible data sets.
**Extended Data Figure 3 | ctNug2 can complement the lethal phenotype of a nug2 Δ null mutant.** Serial dilutions of the yeast Nug2 shuffle strain (MATa, ade2, ade3, his3, ura3, leu2, trp1, nug2-kanMX4, pHT4467-NUG2) transformed with empty plasmid, yeast ScNUG2, ctNUG2 or ctNUG2-510 (truncation of the non-conserved C-terminal extension; see Extended Data Fig. 1) under the control of the constitutive ADH1 promoter in a single-copy-number (YCplac111) or multi-copy-number (pRS425) plasmid (see Supplementary Table 2) were spotted on SDC−Leu (loading control) and SDC plates containing 5-FOA at indicated temperatures for 6 days. Note that ctNug2 only partially complements the nug2 null mutant.
Extended Data Figure 4 | Mutations in ATP-binding or MIDAS domain of Rea1 inhibit the release of Rsa4 and Nug2 from the pre-60S particle.
a, b, Wild-type REA1 and the rea1 mutants mapping in the ATP-binding site of the AAA2 domain (Lys659Ala) or in the MIDAS domain (DAA) were N-terminally tagged with eGFP and expressed in a REA1 shuffle strain (a) or overexpressed under the control of the inducible GAL1-10 promoter in REA1 wild-type strain DS1-2b (b). Transformants were spotted in tenfold serial dilution steps on the indicated plates and incubated at 30 °C for 3 days. Both of the rea1 mutant alleles do not complement the rea1 null strain (a, SDC + 5-FOA) and cause a dominant-negative phenotype after overexpression by replacing endogenous Rea1 (b, galactose). c, Overnight pre-cultures were grown in SRC2 Leu to prevent plasmid loss, followed by shifting cells (A600nm = 0.75) to galactose medium (YPG) for 7 h. Rix1 particles, which were affinity purified from a Rix1–TAP, RpL3–Flag strain containing either endogenous wild-type or overexpressed wild-type eGFP–Rea1, eGFP–Rea1(DAA) and eGFP–Rea1(Lys659Ala), were incubated with or without 4 mM ATP in KCl buffer, before the different in vitro matured pre-60S particles were re-isolated by affinity-purification via the RpL3–Flag on Flag beads. Subsequently, the in vitro matured pre-60S particles (eluates) were analysed by SDS–PAGE and Coomassie staining. Relevant bands are indicated on the right. Note that in the case of the rea1 mutants, the release of Nug2, Rsa4 but also Rea1 and the Rix1 complex is significantly inhibited. All in vitro assays were performed at least twice, yielding highly reproducible data sets.
Extended Data Figure 5 | Nug2 depletion assay using the auxin-inducible degron system. **a**, Growth of Nug2 auxin degron strains (sAid–Nug2–sAid) in the Padh-OsTIR1 background on YPD plates with or without 500 μM auxin (IAA). The cell growth of sAid–Nug2–sAid strain was inhibited by the addition of auxin. **b**, Western blotting of sAid–Nug2–sAid after auxin treatment. The depletion of sAid–Nug2–sAid occurred within about 30 min of auxin addition.
Extended Data Table 1 | Yeast strains used in this study

| Name                        | Genotype                                                                 | Source   |
|-----------------------------|--------------------------------------------------------------------------|----------|
| W303                        | *Mata, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100*          | ref. 46  |
| DS1-2b                      | *MATa, his3Δ200, leu2Δ1, trp1Δ63, ura3-52*                               | ref. 21  |
| Nug2-H1pA                   | W303, *Mata, NUG2-H1pA::His3MX6*                                         | This work|
| Nmd3-H1pA                   | W303, *Mata, NMD3-H1pA::His3MX6*                                         | This work|
| L40 coat                    | *Mata, his3-200, ura3-52, leu2-3, 112, trp1-1, ade2*, LYS2::(lexA::ops)-HIS3, LexA-M52 coat (TRP1)* |           |
| Nug2 Shuffle strain         | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, pRS416-NUG2*          | ref. 35  |
| Nug2 Shuffle strain (for ctNug2 complementation) | *Mata, ade2, ade3, his3, ura3, let2, trp1, mug2::kanMX4, pHT4467-NUG2*   | ref. 35  |
| Nmd3 Shuffle strain         | *Mata, his3, trp1, let2, ura3, LYS2, ADE2, ADE3, NMD3::His3MX6, pRS316 (URA3) Nmd3, pRS416-NUG2* | ref. 14  |
| Nug2 Shuffle strain, Ssf1-TAP | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, SSF1-TAP::TRP1, pRS416-NUG2* | This work|
| Nug2 Shuffle strain, Nsa1-TAP | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, NSA1-TAP::TRP1, pRS416-NUG2* | This work|
| Nug2 Shuffle strain, Rix1-TAP | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, RIX1-TAP::TRP1, pRS416-NUG2* | This work|
| Nug2 Shuffle strain, Arx1-TAP | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, ARX1-TAP::TRP1, pRS416-NUG2* | This work|
| Nug2 Shuffle strain, Lsg1-TAP | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, LSG1-TAP::TRP1, pRS416-NUG2* | This work|
| Nug2 Shuffle strain, Rix1-TAP, L3-Flag | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, RIX1-TAP::TRP1, pRS416-NUG2* | This work|
| Rix1-TAP, L3-Flag           | *Mata, RIX1-TAP::TRP1, RPL3-FLAG::natNT2*                               | ref. 21  |
| Real1 shuffle strain        | W303 *Mata real::kanMX6 YCG-YLR106c*                                     | This work|
| Nug2 Rs4 double shuffle strain | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, rsa4::His3MX4, pRS416-NUG2, pRS416-RSA4* | This work|
| Nug2 Real1 double shuffle strain | *Mata, his3, ura3, leu2, trp1, lys2, mug2::kanMX4, real1::kanMX4, pRS416-NUG2, pRS316-REAL1* | This work|
### Extended Data Table 2 | Plasmids used in this study

| Name                      | Features                                      | Source |
|---------------------------|-----------------------------------------------|--------|
| pACTII-NUG2               | 2μ, LEU2, P_{ADH1}, T_{ADH1}, G4AD-NUG2       | Ref. 21|
| p3A-MS2-1                 | 2μ, URA3, ADE2, P_{ADH1}, MS sites, T_{ADH1}  | Ref. 47|
| p3A-MS2-255H25            | 2μ, URA3, ADE2, P_{ADH1}, MS-255H25 sites, T_{ADH1} | This study|
| p3A-MS2-255H38            | 2μ, URA3, ADE2, P_{ADH1}, MS-255H38, T_{ADH1}  | This study|
| p3A-MS2-255H69-71         | 2μ, URA3, ADE2, P_{ADH1}, MS-255H69-71, T_{ADH1} | This study|
| p3A-MS2-255H89            | 2μ, URA3, ADE2, P_{ADH1}, MS-255H89, T_{ADH1}  | This study|
| p3A-MS2-255H90-92         | 2μ, URA3, ADE2, P_{ADH1}, MS-255H90-92, T_{ADH1} | This study|
| pCM185-NUG2               | CEN, TRP1, P_{adh1}-NUG2                      | This study|
| pCM185-nug2K328R          | CEN, TRP1, P_{adh1}-nug2K328R                 | This study|
| pCM185-nug2G369A          | CEN, TRP1, P_{adh1}-nug2G369A                 | This study|
| pCM190-NUG2               | 2μ, URA3, P_{adh1}-NUG2                       | This study|
| pCM190-nug2K328R          | 2μ, URA3, P_{adh1}-nug2K328R                  | This study|
| pCM190-nug2G369A          | 2μ, URA3, P_{adh1}-nug2G369A                  | This study|
| pRS313-NUG2               | CEN, HIS3, NUG2                               | This study|
| pRS313-nug2K328R          | CEN, HIS3, nsg2K328R                          | This study|
| pRS313-nug2G369A          | CEN, HIS3, nsg2G369A                          | This study|
| pRS313-NUG2-TAP           | CEN, LEU2, NUG2-TAP                           | Ref. 35 |
| pRS315-nug2K328R-TAP      | CEN, LEU2, nsg2K328R-TAP                      | This study|
| pRS314-NMD3-TAP           | CEN, TRP1, NMD3-TAP                           | This study|
| YCplac11-GFP-REA1         | CEN, LEU2, GFP-REA1                           | Ref. 21 & This Study |
| YCplac11-GFP-real1DA        | CEN, LEU2, GFP-real1DA                       | Ref. 21 & This Study |
| YCplac11-GFP-REA1K659A     | CEN, LEU2, GFP-real1K659A                     | This study|
| YCplac11-GFP-REA1K659A     | CEN, LEU2, GFP-real1K659A                     | Ref. 21 & This Study|
| YCplac11-GFP-REA1K659A     | CEN, LEU2, GFP-real1K659A                     | Ref. 21 & This Study|
| YCplac11-PADH-NUG2        | CEN, LEU2, P_{ADH1}-NUG2                      | This study|
| YCplac11-PADH-nug2        | CEN, LEU2, P_{ADH1}-nug2                      | This study|
| YCplac11-PADH-nug2-510    | CEN, LEU2, P_{ADH1}-nug2-510 (1-510aa)        | This study|
| pRS425-PADH-Flag-nug2-510 | 2μ, LEU2, P_{ADH1}-nug2-510 (1-510aa)         | This study|
| pRS314-RFP-NOP1 - RPL25-GFP | CEN, TRP1, mRFP-NOP1, RPL25-eGFP             | Ref. 21 |
| pRS314-RFP-NOP1 - RPS3-eGFP | CEN, TRP1, mRFP-NOP1, RPS3-eGFP              | Ref. 21 |
| pRS314-rsa4-1             | CEN, TRP1, rsa4-1                             | Ref. 21 |
| YCplac22-real1DTS         | CEN, TRP1, real1-DTS                          | Ref. 21 & This Study |
| pRS313-sAid-NUG2-sAid, P_{ADH1}-OsTIR1 | CEN, HIS3, sAid-NUG2-sAid, P_{ADH1}-OsTIR1-mycG | This study|
| pET-ctNug2-510-His6       | E. coli expression vector, Amp, ctNUG-His6 (1-510aa) | This study|
| pET-ctNug2K339R-510-His6  | E. coli expression vector, Amp, ctNUG2K339R-His6 (1-510aa) | This study|
| pET-ctNug2-G380A-510-His6 | E. coli expression vector, Amp, ctNUG2G380A-His6 (1-510aa) | This study|
### Extended Data Table 3 | Adapters used for the CRAC experiments

| Name         | Sequence                                                                 |
|--------------|--------------------------------------------------------------------------|
| **5' linkers:** |                                                                           |
| Nug2 CRAC    | 5'-invddT-GTTCArGrArGrUrCrUrCrArGrArGrUrCrGrArGrUrC-OH-3'               |
| Nmd3 CRAC 1  | 5'-invddT-ACACGrArGrCrUrCrUrCrGrArUrCrUrArGrC-OH-3'                     |
| Nmd3 CRAC 2  | 5'-invddT-ACACGrArGrCrUrCrUrCrGrArUrCrUrArGrC-OH-3'                     |
| **3' linker:**   |                                                                           |
| miRCat       | 5'-AppTGGAAATTCTCGGGTGCCAAGdC-3'                                         |
| **PCR oligos used:** |                                                       |
| P5_forward   | 5'-AATGATACGGCACCGAGATCTACTCTCTTTCCCTACACGACGCTTTCGATCT-3'               |
| Pairedendmircat | 5'-CAAGCAGAAAGACGGCATACGAGATCGGTCTCGGCAATTCTG-                       |
| Reverse      | GCCTTGCCACCCGAGAATCC-3'                                                 |

5'-invddT denotes an inverted dideoxy thymidine; N denotes random nucleotide sequences.