Co-translational assembly of proteasome subunits in NOT1-containing assemblysomes

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Abstract

The assembly of large multimeric complexes in the crowded cytoplasm is challenging. Here we reveal a mechanism that ensures accurate production of the yeast proteasome, involving ribosome pausing and co-translational assembly of Rpt1 and Rpt2. Interaction of nascent Rpt1 and Rpt2 then lifts ribosome pausing. We show that the N-terminal disordered domain of Rpt1 is required to ensure efficient ribosome pausing and association of nascent Rpt1 protein complexes into heavy particles, wherein the nascent protein complexes escape ribosome quality control. Immunofluorescence and in situ hybridization studies indicate that Rpt1- and Rpt2-encoding messenger RNAs co-localize in these particles that contain, and are dependent on, Not1, the scaffold of the Ccr4-Not complex. We refer to these particles as Not1-containing assemblysomes, as they are smaller than and distinct from other RNA granules such as stress granules and GW- or P-bodies. Synthesis of Rpt1 with ribosome pausing and Not1-containing assemblysome induction is conserved from yeast to human cells.

Reference


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Supplemental figure legends

Figure S1. Quality control of the ribosome profiling data for the wild type duplicates. We obtained between 1 and 2 million mapped reads in the duplicated samples. a. CDS coverage was compared between replicates after normalizing for library size, showing a high correlation. b. Sample quality was assessed according to footprint length with an average fragment size of 28 nucleotides as expected. c. Footprints displayed the expected 3-nucleotide periodicity. d. Peaks of ribosome pauses were evaluated genome-wide using a custom algorithm (see materials and methods). We plotted the distribution of the peaks according to their significance (-log10 p-value). Ribosome pausing on RPT1 with codon D241 at the ribosome P site and on RPT2 with codon D165 in the ribosome P site of the ribosome, indicated on the plot, are amongst the 33 most relevant ribosome pause sites genome-wide. 4 other peaks were significant for RPT1 and are indicated.

Figure S2. Analysis of various RNCs reveals differences in particle formation, RNC stability and ribosome stalling. a. Extracts from copper induced cells expressing Flag-Rrp41-120 K12 or Flag-Rps31-120 K12 or Rpt11-120 K12 at the indicated time points after being treated with CHX were analyzed by Western blotting with antibodies to Flag and Rpl35. One representative experiment is shown. Quantification of Flag to Rpl35 signal using biological duplicates (n=2) is shown on the right. b. Extracts from copper induced cells expressing the Rpt2-RNC or the ΔN-Rpt2-RNC as indicated and treated with CHX to preserve polysomes were analyzed by sucrose gradient sedimentation. The indicated fractions were analyzed by Western blotting with antibodies to Flag and Rpl35 (left panels). The Ponceau staining is shown below the blots. The samples were probed with antibodies to V5 and on the right are shown comparisons of the signal with antibodies to Flag and V5 using comparable exposures. c. Extracts from copper induced cells expressing HA-Rpt21-166 were analyzed by sucrose gradient sedimentation. Free (F, lanes 1,2), Monosome (M, lanes 3,4) light polysome (P1, lanes 5,6) and heavy polysome (P2, lanes 7,8) fractions were analyzed by Western blotting with antibodies to HA and Rpl35. d. Extracts from copper induced cells expressing the Rpt1-RNC or Rpt2-RNC as indicated were analyzed by Western blotting with antibodies to Flag or V5. e. Total extracts from cells expressing the Rpt2-RNC and ΔN-Rpt2-RNC after copper induction were
prepared by post-alkaline lysis and equal cell-equivalent amounts (lanes 1 and 4, 6 \( \mu l \); lanes 2 and 5, 9 \( \mu l \) and lanes 3 and 6, 12 \( \mu l \)) were analyzed by Western blotting with antibodies to Flag and Rpl35 as indicated. The Ponceau stain of the gel is shown below. f. Extracts from copper-induced cells expressing the HA-\(\Delta N\)-Rpt1 and HA-Rpt1 were prepared by post-alkaline lysis. Three different cell-equivalent amounts were analyzed by Western blotting with antibodies to HA and Rpl35.

**Figure S3. Very little plasmid-encoded Rpt1 or Rpt2 co-purify with endogenous Rpn11.** Cells expressing Rpn11-ProtA were transformed with plasmids expressing the Rpt1- or Rpt2-RNC and HA-Rpt2 or HA-Rpt1, or with empty vectors (-) with the same marker genes *URA3* or *LEU2* as indicated. After 10 min copper induction extracts were prepared and incubated with IgG-sepharose beads. Rpn11 was eluted by treatment of the beads with TEV protease. The input (TE) and TEV eluate (Eluate) were analyzed by Western blotting. One blot was probed with successively anti-Rpt2 (a), followed by anti-HA (b) and finally anti-Flag (c). Hence the signal in panel c cumulates the signals in panels a and b. One blot was probed only with anti-Rpt1 (d). The positions of the detected Flag-tagged RNCs, HA-tagged proteins or tagged Rpn11 are indicated on the right, while MW markers are indicated on the left.

In panel a, the polyclonal antibodies to Rpt2 will recognize endogenous Rpt2, HA-Rpt2 and the Rpt2-RNC. In this panel the signal for HA-Rpt2 in the TE is overshadowed by a signal from Rpn11-ProtA. In panel b, we still see the signals from panel a, but we additionally see the signals from the HA antibodies. This reveals the position of HA-Rpt2 in the first TE lane not clearly seen in panel a and the low levels of HA-Rpt2 in the first Eluate lane that could not be seen with Rpt2 antibodies in panel a. HA-Rpt1 migrates nearly at the same level as Rpn11-ProtA and hence cannot be easily distinguished in the second TE lane, but a very small amount of HA-Rpt1 can be guessed in the second Eluate lane. Finally in panel c, we still see the signals from panels a and b, but we additionally see low amounts of Rpt2-RNC (Flag-tagged, see Fig. 1d) in the second Eluate lane that could not be seen with antibodies to Rpt2 (in the second Eluate lane of panel a) and of the Rpt1-RNC (Flag-tagged, see Fig. 1d) in the first Eluate lane that could not be seen with antibodies to Rpt1 (in the first Eluate lane of panel d). Although in these experiments the signals of HA-Rpt1 and HA-Rpt2 could not be easily be compared to the signals of the endogenous subunits
because of interference from the signal arising from Rpn11-ProtA, this could be assessed in cells that do not express tagged Rpn11 (blots available in the supplementary Data Set1). HA-Rpt2 was expressed at levels similar to endogenous Rpt2, whereas HA-Rpt1 was much less expressed than the endogenous Rpt1.

Figure S4. Analysis of the interaction between various Rpt1 and Rpt2 derivatives. a. Extracts from cells induced by copper and expressing Flag-Rpt1 and HA-Rpt2 or Flag-Rpt2 and HA-Rpt2 were incubated with antibodies to Flag (IP-Flag), HA (IP-HA) or Myc (IP-Myc) as a negative control. The total extract and immunoprecipitates were analyzed by Western blotting with antibodies to HA, Flag and Not1. b. The indicated strains were grown exponentially and serially diluted. 4 μl of each dilution were spotted on rich medium (YPD), or on rich medium containing hygromycin B (HYG, 0.1 mg/ml) or azetidine-2 carboxylic acid (AZC, 0.5 mg/ml) as indicated. The cells were left to grow for 3 days at 30°C. c. Extracts from cells induced by copper and expressing the Rpt1-RNC and HA-Rpt2 75-166 treated with CHX to preserve polysomes were analyzed by sucrose gradient sedimentation. The indicated fractions were analyzed by Western blotting with antibodies to HA and Flag. d. Extracts from cells induced by copper and expressing the Rpt1-RNC or ProtA-Rpt1-DP and HA-Rpt2 75-166 were incubated with anti-Flag IgG beads overnight. The flow through was collected (FT) and then after washing Flag peptide was added to elute the RNC-associated proteins (IP Flag). Total extract (TE), IP Flag and FT were analyzed by Western blotting with antibodies to Flag and HA. Biological duplicates were analyzed.

Figure S5. Ribosome pausing during production of Rpt1 is conserved. a. The patterns of ribosome footprints aligned on the P site of the ribosome for PSMC2 encoding human Rpt1 was extracted from published data 27. The P site codon, 135, where footprints accumulate, is indicated. The conserved N-terminal helix of Rpt1 that interacts with an N-terminal helix of Rpt2 in the mature proteasome (aa 45-71) is depicted in red. b. Extracts from LNCaP cells untreated or treated with arsenite were separated on a sucrose gradient. RNA from the total extract or from the indicated fractions was prepared and analyzed for the levels of PMSC2 or CDK16. Normalization was to the levels of EIF4E2 mRNA. Error bars represent standard
deviation of biological duplicates (n=2) measured in technical triplicates (Supplementary Table 1). c. Extracts from A549 lung cancer cells treated or not with arsenite were incubated with EDTA for 1 h and fractionated on sucrose gradients. The total extract (TE) and indicated fractions from the polysome profiles shown on the right were analyzed by Western blotting with anti-CNOT1 antibodies. This experiment was performed only once.

Figure S6. Characterization of NCA in mammalian cells. a. NCA are formed in response to different stress conditions. LNCaP cells were treated with oxidative stress inducer arsenite (100 μM), endoplasmic reticulum (ER) stress inducer thapsigargin (1 μM), proteasome inhibitor MG-132 (10 μM) and UV radiation for 1 h. Cells were fixed and subjected to IF using the indicated antibodies. b. CHX treatment didn’t affect NCA. LNCaP cells were treated with 100 μM arsenite and 10 μM cycloheximide (CHX) for 1 h. Cells were fixed and subjected to IF using the indicated antibodies. c. YB-1 is used as a stress granule marker to differentiate CNOT1 containing particles. Untreated or arsenite (100 μM for 1 h) treated cells were processed for IF as described above using the indicated antibodies. d. NCA are distinct from P-bodies and GW bodies. LNCaP cells treated with 100 μM arsenite for 1 h were fixed and subjected to IF using anti-DCP1A and anti-DDX6 antibodies (P body markers) and anti-GW182 antibodies (GW bodies). The slides were co-stained with anti-CNOT1 antibodies. Note that NCA stay distinct from P bodies and GW bodies. e. NCA are present in different cell lines. 22Rv1 (upper panel) and V16D (bottom panel) prostate cancer cells were treated with 100 μM arsenite for 1 h and subjected to IF using the indicated antibodies. Note that NCA are detected in both cell lines. f. In situ hybridization using mismatch probes targeting Rpt1 and Rpt2. g. LNCaP cells treated with 100 μM arsenite for 1 h were fixed and subjected to in situ hybridization using 56-FAM-labeled oligos targeting Rpt1- or Rpt2-encoding mRNAs. Following hybridization, cells were immunostained with anti-CNOT1 antibodies. h. LNCaP cells treated with 100 μM arsenite for 1 h were subjected to in situ hybridization using differently labeled oligos targeting Rpt1- or Rpt2-encoding mRNAs to look for co-localization. i. LNCaP cells transfected with siControl or siCNOT1 siRNAs were treated with 100 μM arsenite for 1 h and subjected to IF using the indicated antibodies. j. LNCaP cells transfected with siCNOT1 siRNAs were
treated with 100 μM arsenite for 1 h. The cells were fixed and subjected to in situ hybridization using differently labeled oligos targeting Rpt1- or Rpt2-encoding mRNAs to look for co-localization. Note that Rpt1 and Rpt2 co-localization is significantly lost in the absence of CNOT1. Scale bar 10 μm. Each experiment was conducted independently at least 3 times. 10-15 images were captured for each condition.

Figure S7. Alignment of the amino acid sequences of yeast Rpt1 to Rpt6 and human Rpt1. The Red box indicates the position of the codons orthologous/paralogous to the DP pause site at codon 165 of Rpt1. The Green box indicates the position of the codons orthologous/paralogous to the DP pause site at codon 241 of Rpt2. The blue box indicates the codons orthologous/paralogous to the D at position 135 of Rpt1.

Figure S8. Not4 and Not5 contribute to Rpt1 RNC stability. a. Arsenite (100 μM for 1 h) treated cells were processed for IF as described above using antibodies to CNOT6 or CNOT1 as indicated. b. LNCaP cells untreated (UT) or treated with 0.5 mM arsenite (ARS) for 1 h were fixed and subjected to in situ hybridization using 56-FAM-labeled oligos targeting Rpt1- or Rpt2-encoding mRNAs. Following hybridization, cells were immunostained with anti-CNOT6 antibodies. Each experiment was conducted independently at least 3 times. 10-15 images were captured for each condition. c. Extracts from not4Δ or not5Δ cells induced by copper and expressing the Rpt1-RNC at the indicated times after being treated with CHX were analyzed by Western blotting with antibodies to Flag and Rpl35.