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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 cotranslational 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 mRNAs colocalize in these particles that contain and depend upon Not1, the scaffold of the Ccr4-Not complex. We refer to these particles as Not1-Containing Assemblysomes (NCA), as they are smaller and distinct from other RNA granules such as stress granules, GW- or P-bodies. Synthesis of Rpt1 with ribosome pausing and NCA induction is conserved from yeast to human cells.

Introduction

Multi-subunit protein complexes mediate diverse cellular functions and have been the object of studies for many years. Though the functional and structural organization of many multi-subunit complexes have been well established, our understanding of how they are assembled *in vivo* is still at its infancy.

The proteasome is a multi-subunit protease, and a major contributor to degradation of short-lived proteins in eukaryotic cells. It consists of a 20S core particle (CP) that carries the catalytic activities and of one or two 19S regulatory particles (RP) bound on one or two ends of the CP. RP is composed of a base and a lid, and it has multiple roles such as the recognition of substrates, their deubiquitination, unfolding, and delivery to the CP. RP base contains a hexameric ring of ATPases, called Rpt1 to Rpt6 that are critical for RP function (for review see [1]).

The function of the proteasome and the process by which it is assembled have been extensively investigated[2-4]. Several chaperones that efficiently and accurately mediate proteasome assembly have been identified[5]. In particular, the assembly of RP is tightly orchestrated by specific chaperones. There are three RP assembly intermediates, with specific base subunits and dedicated chaperones (Nas6, Rpn14, Adc17 and Hsm3)[6-8]. Genetic studies have indicated that the formation of the intermediary tetrameric base complex, consisting of Rpt1-Rpt2-Rpn1 with the Hsm3 chaperone, is critical for RP assembly [6,7,9,10].

Despite the large number of studies on proteasome assembly and the characterization of intermediates and chaperones, we are far from understanding how the proteasome is formed *in vivo*, and how this process is regulated. As for all protein complexes, in principle subunits can assemble after synthesis is completed, on their own or with the help of chaperones. In addition, they can assemble co-translationally, with one subunit associating with its partner protein as it is being synthesized at the ribosome, as soon as the interaction domain has been fully produced and folded [11]; this type of assembly is thought to be widespread [12,13].

One subunit of the Ccr4-Not complex, Not4, was reported to be important for the functional integrity and assembly of the proteasome [14]. The Ccr4-Not complex is a conserved multi-protein complex that regulates gene expression at all stages [15,16]. It is built upon a central scaffold protein, Not1, on which several different functional modules assemble [17]. A recent study has indicated that Ccr4-Not contributes to co-

translational assembly of the SAGA histone acetyltransferase [18]. This reported role of Ccr4-Not complex and the observed importance of Not4 for proteasome assembly led us to investigate whether proteasome assembly might be regulated during translation and if this was connected to Ccr4-Not.

Here we show that an essential step in eukaryotic proteasome base assembly is the co-translational association of the base subunits Rpt1 and Rpt2, and we provide evidence that it occurs within a new type of bodies that we name Not1-Containing Assemblysomes (NCA).

Results

Ribosome pausing during translation of *RPT1* and *RPT2*

We performed a ribosome profiling experiment in yeast to obtain information on translation of proteasome mRNAs (**Supplementary Fig. 1a-c**). We noted that 2 proteasome mRNAs, *RPT1* and *RPT2* encoding 2 base subunits, showed an accumulation of ribosome footprints at a specific codon (**Fig. 1a**). These *RPT1* and *RPT2* footprint peaks were amongst the most significant genome-wide (**Supplementary Fig. 1d;** algorithm to identify significant peaks in ribosome profiling data described in Online Methods). This led us to focus on the position of the footprint peaks on *RPT1* and *RPT2*. For both mRNAs, the peak corresponded to a CCA proline codon in the A site of the ribosomes providing the footprint. In both cases, an aspartate codon was in the P site of the ribosomes providing the footprint peak. Previous work has indicated that proline codons play a major role in stalling translation [19] and that ribosome pausing occurs at DP codon pairs particularly under limiting eIF5A [20]. Hence, our observations were consistent with substantial ribosome pausing during translation of *RPT1* and *RPT2*. Interestingly, for both mRNAs, the P site codon on which ribosomes were pausing was located just prior to the sequences encoding the AAA-ATPase domains of both proteins (**Fig. 1a**).

Previous studies have indicated that Rpt1 and Rpt2 exhibit weak affinity for each other when synthesized separately and mixed; additionally, they are almost completely insoluble when bacterially expressed [21]. Moreover, they do not interact by the yeast two-hybrid method [22]. Thus, we considered that *RPT1* and *RPT2* translation might occur with pausing of ribosomes to allow co-translational assembly of the 2 proteins. Between 25 and 38 amino acids of a newly synthesized protein are known to

occupy the ribosome tunnel [23]. Hence, the nascent peptide exposed from ribosomes paused on *RPT2* mRNA at codon 165 (**Fig. 1a**) should include the α helix formed by amino acids 75-100; in the mature proteasome, this Rpt2 helix interacts with a helix of Rpt1 (amino acids 49-75) [24] (**Fig. 1b**). That same α helix of Rpt1 is exposed from ribosomes paused on codon 241 of *RPT1* mRNA (**Fig. 1a**). Thus, pausing of ribosomes on *RPT1* and on *RPT2* could indeed allow co-translational assembly of those 2 proteasome subunits via their N-terminal α helices (**Fig. 1c**).

Expression of stalled ribosome-associated nascent Rpt1 and Rpt2 chains

We created plasmids expressing different derivatives of Rpt1 and Rpt2 (**Fig. 1d**) to test the idea that ribosome pausing during translation of *RPT1* and *RPT2* allows the co-translational assembly of their encoded N-terminal domains. The first plasmid allowed expression from the *CUP1* promoter of a ribosome-associated nascent chain Rpt1 (Rpt1-RNC) similar to the one expected to occur *in vivo* upon ribosome pausing. This was achieved by inserting a stretch of 12 lysine codons (K12) after codon 135 of *RPT1*, as previous work has indicated that stretches of lysines will provoke ribosome stalling [25]. We chose codon 135 because it corresponded to the first visible peak of ribosome pausing on the endogenous mRNA (see **Fig. 1a**) and translation stalled at codon 135 would provide a ribosome-associated nascent Rpt1 peptide whose α helix (amino acids 49-75) interacting with Rpt2 should be exposed from the ribosome tunnel. We made a similar plasmid for *RPT2* (Rpt2-RNC), in which we inserted a stretch of 12 lysine codons after codon 165, the major ribosome pause site according to the ribosome profiling (**Fig. 1a**). Translation stalled at codon 165 would provide a ribosome-associated nascent Rpt2 peptide, whose Rpt1-interacting helix (amino acids 75-100) should be exposed from the ribosome tunnel.

The Rpt1- and Rpt2-RNCs were detectable as proteins of slightly more than 35 kDa (**Fig. 2a**), larger than the expected sizes if translation stalled exactly at the first lysine of the K12 (approximately 19 kDa for Rpt1 and 22 kDa for Rpt2). This was also larger than expected if translation did not stall and continued to a stop codon in any reading frame (48 codons in the same reading frame, 82 or 18 in the other reading frames). The apparent larger size of the nascent Rpt1 and Rpt2 peptides could be due to post-translational modifications.

Paused ribosomes producing Rpt1 associate in dense bodies

Both the Rpt1 and Rpt2 nascent proteins were stable (**Fig. 2a**). Yet ribosome stalling is expected to provoke ribosome-associated quality control mechanisms that recycle ribosomes and efficiently degrade nascent protein and mRNA [26]. Consistent with this expectation, several RNCs of the same length expressed from the same plasmids were not stable (see examples on **Supplementary Fig. 2a**). The stability of Rpt1 and Rpt2 RNCs might be a feature favorable and necessary for co-translational association of the nascent peptides.

To confirm that our constructs were producing RNCs, we analyzed their sedimentation in sucrose gradients. For Rpt1-RNC, we detected the nascent Rpt1 peptide in monosome and polysome fractions (**Fig. 2b**, left panel, fractions labeled M and P1, respectively). This suggested that the nascent Rpt1 was associated with ribosomes in an Rpt1-RNC. To confirm this, we treated extracts from cells expressing the Rpt1-RNC with EDTA to separate 40S and 60S ribosome subunits prior to sucrose gradient fractionation. After EDTA treatment, the nascent peptide was partly detected in free fractions (denoted by F), but it was still also in heavy fractions of the sucrose gradient (**Fig. 2b**, right panel, +EDTA, fraction P1). Ribosomal protein Rpl35 was used to follow ribosome content of the fractions and was undetectable in fraction P1 after EDTA treatment. Hence, the nascent Rpt1 peptide was associated in heavy bodies different from polysomes after EDTA treatment. We also prepared extracts in presence of cycloheximide (CHX) to better preserve polysomes and then treated extracts with RNase A prior to sucrose gradient sedimentation: the Rpt1-RNC was still detected in heavy fractions (**Fig. 2c**, fraction P2) where Rpl35 was mostly no longer detected.

We then analyzed the sedimentation profile of the Rpt2-RNC. Like the Rpt1-RNC, it was detected throughout the sucrose gradient including in the heavy fractions, suggesting that it was ribosome-associated (**Supplementary Fig. 2b**, left panels). It was also detected in free fractions of the sucrose gradient (**Supplementary Fig. 2b**, left panel, fraction denoted by F), indicating that some amount of the peptide was not ribosome-associated. Nevertheless the sedimentation pattern of the Rpt2-RNC was different to that of a similar Rpt2 $_{1\text{-}166}$ fragment expressed from a plasmid without K12 and with instead a stop codon (HA-Rpt2 $_{1\text{-}166}$, **Fig. 1d**), which was not at all detected in polysome fractions (**Supplementary Fig. 2c**).

An in-frame V5 epitope is present in our constructs between the K12 sequence and a stop codon (**Fig. 1d**). Since we did not know where ribosomes stalled relative to the K12 sequence, we tested whether the V5 epitope was present in the Rpt1- and Rpt2-RNCs. This was the case for some of the Rpt2-RNC but not for the Rpt1-RNC (**Supplementary Fig. 2d**). It was mostly the Rpt2-RNC in the free fractions of the sucrose gradient that was detectable with antibodies to V5 (**Supplementary Fig. 2b**, right upper panels, compare Flag and V5 signals), suggesting that this Rpt2 might have been produced by translation elongation to the stop codon followed by peptide release.

N-terminal domains of Rpt1 and Rpt2 are important for efficient ribosome pausing and formation of heavy soluble particles

Rpt1 and Rpt2, like all 6 proteasome base subunits, have an N-terminal domain predicted to be highly disordered [27]. We tested the importance of the N-terminal domain of Rpt1 for the formation of the Rpt1-RNC heavy particles by deleting the first 48 amino acids of Rpt1 (ΔN-Rpt1-RNC, **Fig. 1d**). This ΔN-Rpt1-RNC construct was well expressed, but was mostly detected in free (F) and monosome (M) fractions after sedimentation on a sucrose gradient, and less in polysomes (P1 or P2) compared to the Rpt1-RNC (compare **Fig. 2d** to **Fig. 2c,** left panel and see quantification in **Fig. 2e**). Some ΔN-Rpt1-RNC in the total extract was detected by Western blotting with antibodies to V5. It was mostly the ΔN-Rpt1-RNC in the free fractions after sucrose gradient sedimentation (**Fig. 2d**) that might have been produced by translation elongation to the stop codon. These findings suggest that for the Rpt1-RNC the N-terminal domain of Rpt1 contributed to efficient ribosome pausing and incorporation in heavy particles.

We similarly tested an Rpt2-RNC lacking the first 48 amino acids of Rpt2 (ΔN-Rpt2-RNC, **Fig. 1d**). The amount of the ΔN-Rpt2-RNC in soluble extracts was reduced compared to the Rpt2-RNC (**Supplementary Fig. 2b,** compare TE lanes in left panels), despite being expressed at higher levels (**Supplementary Fig. 2e**). This indicates that for the Rpt2-RNC, the N-terminal domain of Rpt2 was necessary for solubility. The sedimentation pattern of the ΔN-Rpt2-RNC was similar to that of the Rpt2-RNC (**Supplementary Fig. 2b**, right panels).

We created plasmids that expressed either the full-length Rpt1 or the N-terminally deleted Rpt1 under the *CUP1* promoter (HA-Rpt1 and HA-ΔN-Rpt1, **Fig. 1d**) and tested whether they could complement the deletion of the endogenous gene in a plasmid shuffle assay. The Rpt1 derivatives were similarly expressed (**Supplementary Fig. 2f**) but only the full-length HA-Rpt1 could complement the null mutant (**Fig. 2f**). Hence, the N-terminal domain of Rpt1 is important for Rpt1 function.

Rpt1 and Rpt2 interact with their nascent partner stalled at the ribosome

If Rpt1 and Rpt2 assemble co-translationally, we expect that the full-length proteins should associate when co-expressed as nascent proteins stalled at the ribosome. To test this prediction, we co-expressed full-length Rpt1 (HA-Rpt1, **Fig. 1d**) with the Rpt2-RNC and full-length Rpt2 with the Rpt1-RNC (HA-Rpt2, **Fig. 1d**). We fractionated total extracts on sucrose gradients and detected the full-length proteins and the RNCs in the heavy fractions of the sucrose gradient (**Fig. 3a**). We pooled the polysome fractions and immunoprecipitated the full-length proteins or the RNCs, finding that the partner RNC or full-length proteins coimmunoprecipitated (**Fig. 3b**).

To investigate how the endogenous proteasome subunits could affect the association of the plasmid-encoded subunits, we isolated Rpn11 (a component of the 19S lid) from cells expressing full-length HA-Rpt1 and the Rpt2-RNC or full-length HA-Rpt2 and the Rpt1-RNC from episomes, or carrying empty plasmids as a control. After copper induction, Rpt2- and Rpt1-RNCs were monitored with antibodies and found to be expressed at levels similar to the endogenous proteins (**Supplementary Fig. 3a** second TE lane and **Supplementary Fig. 3d** first TE lane, respectively). Yet, only very low levels of the RNCs were detected in the Rpn11 purifications (**Supplementary Fig. 3**, first and second Eluate lanes), whereas the same amount of endogenous Rpt1 and Rpt2 co-purified in all cases (**Supplementary Fig. 3**, compare all Eluate lanes). Similarly, very little HA-Rpt1 (**Supplementary Fig. 3b**, second Eluate lane) or HA-Rpt2 (**Supplementary Fig. 3a** and **Supplementary Fig. 3b** first Eluate lane) co-purified with Rpn11. Hence, it seems unlikely that the endogenous subunits could be scaffolding the interaction of the episomally expressed proteasome subunits under the conditions of these experiments, with short (10 minute) copper

induction (**Fig. 3**), probably because most endogenous proteins are stably incorporated into proteasome complexes.

Full-length Rpt1 and Rpt2 in Not1-containing heavy particles

The results presented above show that stable Rpt1- and Rpt2-RNCs were present in heavy bodies, allowing recruitment of the partner subunit to the stalled nascent peptide. We tested whether the scaffold of the Ccr4-Not complex, Not1, was a component of these bodies, since previous studies have indicated that the Ccr4-Not complex is important for proteasome assembly [14]. We prepared extracts from cells expressing Rpt2-RNC and full-length HA-Rpt1, treated them with EDTA and separated on a sucrose gradient. The heavy fractions P1 and P2 were pooled and, as expected, contained full-length HA-Rpt1 and Rpt2-RNC that co-immunoprecipitated (**Fig. 3c**). Not1 was detected in these pooled heavy fractions and it also co-immunoprecipitated with Rpt2-RNC and full-length HA-Rpt1 (**Fig. 3c**).

We next questioned whether newly produced full-length HA-Rpt1 and/or HA-Rpt2, synthesized with ribosome pausing and producing transiently RNCs, were detectable in heavy particles, in the absence of co-expressed artificial RNC. We separated extracts from cells expressing the full-length HA-Rpt1 or HA-Rpt2 on sucrose gradients, with or without prior treatment of extracts with EDTA. HA-Rpt1 and HA-Rpt2 were both detected in the heavy fractions of the sucrose gradient before EDTA treatment, and also after EDTA treatment though to a lesser extent, as was Not1 (**Fig. 4a,b**). Other proteins induced from the same *CUP1* promoter, including other proteasome subunits such as the CP subunit HA-Pup2, were not detected in heavy fractions of the sucrose gradient, and their production was not accompanied by a similar induction of Not1 in these heavy fractions (**Fig. 4c**).

Although both HA-Rpt1 and HA-Rpt2 expressed without co-induction of their partner were detected in dense fractions of the sucrose gradient, both proteins turned over rapidly (**Fig. 5a,b**).

Codons DP at the pause site are critical for Rpt1- Rpt2 interaction

To determine whether co-induced full-length Rpt1 and Rpt2 would associate, we cloned Flag-tagged versions of each protein under the *CUP1* promoter (Flag-Rpt1 and Flag-Rpt2, **Fig. 1d**) and co-expressed it with the HA-tagged version of the partner

subunit. We observed efficient co-immunoprecipitation of Flag-Rpt1 with HA-Rpt2 and vice-versa (**Supplementary Fig. 4a**) and Not1 also co-immunoprecipitated (**Supplementary Fig. 4a**).

We next tested the stability of full-length HA-Rpt2 induced in cells in which Protein A-tagged Rpt1 (ProtA-Rpt1-DP, **Fig. 1d**) was expressed from a plasmid from Rpt1's own promoter, to complement the deletion of the endogenous *RPT1* gene (**Fig. 5c**). HA-Rpt2 was more stable in those cells than in cells expressing endogenous Rpt1 from the genome (**Fig. 5b**). This allowed us to use this set up to test the importance of the codons at the pause site of *RPT1* for interaction of Rpt1 with Rpt2.

We mutated *RPT1* Asp241 (GAT) and Pro242 (CCA) codons at the major ribosome pausing site to Ala codons (GCG and GCG) in the plasmid expressing Protein A-tagged Rpt1 (ProtA-Rpt1-AA, **Fig. 1d**). Residues Asp241 and Pro242 are located in a flexible linker that connects Rpt1 OB and AAA domains (**Fig. 1a**). Both side chains are solvent exposed and located on the side opposite to the Rpt2 interaction surface. Thus, a DP to AA mutation should neither affect Rpt1 protein structure nor its direct interaction with Rpt2. Consistently, codon-changed Rpt1 was able to complement the deletion of the *RPT1* gene, even in the context of an *RPN4* deletion, and when tested on medium with translation inhibitors and amino acid analogs on which proteasome mutants did not grow (**Supplementary Fig. 4b**).

The codon-changed ProtA-Rpt1 was expressed similarly to the wild type Rpt1, except that a shorter form of the protein, consistently detected for wild type ProtA-Rpt1-DP by Western blotting, was not detectable in the AA mutant (**Fig. 5d**). This observation was consistent with the shorter form of ProtA-Rpt1 being the nascent protein expressed from ribosomes paused at the DP site, and with the codon change to AA having abolished the ribosome pausing. We next tested the stability of HA-Rpt2 in presence of wild type (DP) or AA ProtA-Rpt1, and found that codon-changed Rpt1 was less effective in stabilizing Rpt2 (**Fig. 5e**). The difference in stability of HA-Rpt2 expressed with ProtA-Rpt1-DP compared ProtA-Rpt1-AA was mild, but reproducible and significant. These results are consistent with ribosome pausing at the Rpt1 DP codons contributing to productive association of Rpt1 and Rpt2.

Interaction of the Rpt1 and Rpt2 N-terminal domains alleviates ribosome pausing

Previous studies have proposed that the Hsm3 chaperone scaffolds the association of Rpt1 and Rpt2, since it associates with the C-terminal domain of Rpt1 [10,28] and can also interact with Rpt2 [28], most likely with its AAA-ATPase domain [21]. Instead our data suggest that co-translational association of nascent Rpt1 and Rpt2 underlies their productive association. To confirm that ribosome-associated nascent Rpt1 can interact with Rpt2 independently of Hsm3, we created a plasmid expressing an Rpt2 N-terminal fragment that included the Rpt1-interacting helix, but lacked the Hsm3-interacting domain (HA- Rpt2 $_{75\text{-}166}$, **Fig. 1d**). HA-Rpt2 $_{75\text{-}166}$ was not detectable when expressed alone (**Fig. 6a**, left panel). However it became detectable and stable upon co-expression of the Rpt1-RNC (**Fig. 6a**, right panel) and was detected in ribosome-containing fractions of a sucrose gradient (**Supplementary Fig. 4c**). It also co-purified with the Rpt1-RNC (**Supplementary Fig. 4d**), indicating that it had been recruited to the Rpt1-RNC. These findings support our model that co-translational association of the N-terminal domains of Rpt1 and Rpt2 occurs.

Emerging from our model that translation of Rpt1 and Rpt2 occurs with ribosome pausing, thus allowing the N-terminal α helices of Rpt1 and Rpt2 to interact, is the idea that association of the helices might in turn signal the paused ribosomes to continue translation elongation. We tested this idea with our artificial constructs and analyzed whether the interaction of HA-Rpt2 $_{75\text{-}166}$ with the Rpt1-RNC shown above (**Supplementary Fig. 4d**) altered the stalling efficacy of the K12 sequence. Indeed, the V5 epitope present after K12 in Rpt1-RNC was not detectable when the nascent Rpt1 was expressed alone, but it became detectable upon co-expression of HA-Rpt2 $_{75\text{-}166}$ (**Fig. 6b**). Rpt1-RNC was mainly detected in the ribosome-containing fractions with similar sedimentation patterns, whether or not HA-Rpt2 was co-expressed (**Fig. 6c**).

Evidence for Not1-containing assemblysomes

To explore and visualize the Not1-containing dense bodies induced upon Rpt1 or Rpt2 synthesis, we considered that human cells would allow better resolution in immunolocalization experiments. The structural organization of the proteasome base ATPase subunits is well conserved between yeast and mammalian cells [27]. Moreover, we noted evidence for ribosome pausing on *PSMC2* encoding human Rpt1 from published ribosome profiles [29] at a position that should expose the conserved Rpt2-

interacting helix of Rpt1 (**Supplementary Fig. 5a**). We also noted that CNOT1, the ortholog of yeast Not1, was recruited to heavy bodies under conditions of arsenite stress where Rpt1 and Rpt2 co-translational assembly was promoted (**Supplementary Fig. 5b,c**).

We followed CNOT1 localization in the human LNCaP prostate cancer cells, before and after arsenite treatment, by immunofluorescence (IF). We observed some punctate localization of CNOT1 in cells before arsenite stress, in addition to weak diffuse staining in the cytoplasm (**Fig. 7a**). Staining with a classical stress granule marker G3BP1 [30] (red) showed diffuse staining in the cytoplasm. Upon arsenite stress, the smaller distinctive bodies of CNOT1 increased in number and were brighter. Stress granules with G3BP1 became evident but CNOT1 was mostly absent from those granules (**Fig. 7a**). Similar CNOT1 particles were induced by a number of different stresses and by proteasome inhibitor MG132 (**Supplementary Fig. 6a**). The CNOT1 particles were not sensitive to cycloheximide treatment which completely prevented the formation of stress granules upon arsenite stress as evidenced by G3BP1 staining (**Supplementary Fig. 6b**). The localization of CNOT1 after arsenite treatment was compared to that of another stress granule marker YB-1 (**S6c**), or to that of GW182 (marker for GW-bodies), DDX6 or Dcp1a (markers for P-bodies) (**Supplementary Fig. 6d**) but CNOT particles were mostly distinct from these mRNP complexes. Similar CNOT1 particles were induced by arsenite in a number of different cell lines (**Supplementary Fig. 6e**).

We used single molecular RNA FISH to determine whether Rpt1- and Rpt2-encoding mRNAs co-localized with the CNOT1 particles. Indeed, after arsenite treatment we observed co-localization of CNOT1 with both Rpt1-encoding and Rpt2-encoding mRNAs (**Fig. 7b** and **Supplementary Fig. 6f,g**). Moreover, the Rpt1- and Rpt2-encoding mRNAs themselves co-localized (**Fig. 7c**). This co-localization was also observed under pre-treatment of cells with CHX (**Supplementary Fig. 6h**). Finally, to know whether CNOT1 was necessary for the co-localization of the Rpt1- and Rpt2-encoding mRNAs, we knocked down CNOT1 prior to arsenite treatment. Arsenite still induced the formation of stress granules, but the mRNAs encoding Rpt1 and Rpt2 now mostly did not co-localize (**Fig. 7c** and **Supplementary Fig. 6i,j**).

Thus, small CNOT1 particles containing Rpt1- and Rpt2-encoding mRNAs are induced upon proteotoxic stress in mammalian cells, and they are neither stress granules nor GW- or P-bodies. Instead, they are consistent with the Not1-containing

bodies of Rpt1 and Rpt2 co-translational assembly that we observed in yeast. For this reason, we have chosen to name them Not1-Containing-Assemblysomes (NCA).

Discussion

Rpt1 and Rpt2 assemble co-translationally

In this work, yeast ribosome profiling data strongly suggest that the translation of proteasome *RPT1* and *RPT2* mRNAs occurs with ribosome pausing to allow helices in the exposed nascent peptides to associate co-translationally (see model on **Fig. 8**). We support this model by showing that ribosome pausing is conserved in human cells, in which we also show that the mRNAs encoding Rpt1 and Rpt2 co-localize. We further suggest that interaction of nascent peptides is a signal for translation elongation to proceed after ribosome pausing.

The idea that co-translation assembly of proteins could be widespread was initially proposed in 2011 [12]. Recently, the Bukau laboratory, using ribosome profiling, has suggested that co-translational subunit engagement with partner proteins is prevalent [13]. They also provide evidence that this process often occurs unidirectionally, with one fully synthesized subunit engaging with its nascent partner subunit. Here we reveal a different mechanism that can ensure the productive assembly of proteins in the crowded eukaryotic cytoplasm. This mechanism entails co-localization of the mRNAs encoding partner subunits in Not1-containing granules, ribosome pausing to expose interaction domains of partner subunits outside of the ribosome tunnel, association of the interaction domains and translation elongation. This mechanism might be useful in particular for proteins that do not readily associate if produced separately, as is the case for Rpt1 and Rpt2.

Ribosome pausing needs more than just a pause site

The ribosome pausing identified in this work occurs at DP motifs of *RPT1* and *RPT2*. By aligning the mRNAs encoding the yeast proteasome base subunits and the mRNA encoding human Rpt1, and their amino acid sequences, we noted that the DP motif at position 165 of Rpt2 is conserved in Rpt1, Rpt4, Rpt6 and in human Rpt1 (**Supplementary Fig. 7**). Yet, a significant pause was detectable in our data only at the paralogous codon in Rpt6 (position 131). Not all DP codons show ribosome footprint accumulation, and it is not clear whether this is because not all sites

correspond to ribosome pausing or whether footprint accumulation requires more than just ribosome pausing, such as formation of heavy particles with the corresponding RNCs. We also noted that the same K12 stalling sequence introduced in different mRNAs did not show the same efficacy of ribosome stalling. This supports the idea that actual ribosome pausing requires more than just a pause site. Moreover, we showed that productive interaction of the exposed nascent peptide with its partner allowed translation elongation through the K12 sequence. Hence, the availability and ease of a protein to associate with its partner nascent peptide might also define whether pausing can be detected. While we reached these conclusions using RNCs stalled with an artificial K12 sequence, we expect that ribosome pausing at DP codons or other pause sites of endogenous mRNAs is likely to be similarly regulated.

Not1-containing assemblysomes

We refer to the particles in which Rpt1 and Rpt2 mRNAs co-localize as Not1-Containing Assemblysomes (NCA) as we propose that they enforce co-translational assembly of Rpt1 and Rpt2. Besides co-localizing mRNAs encoding partner subunits, NCA might also limit new translation initiation on paused RNCs, thereby avoiding ribosome collision and ribosome quality control [31], giving time for the partner subunits to associate. They might also concentrate factors necessary for folding, pausing and thereafter translation elongation. We expect that the composition and properties of NCA might evolve during the different steps of the process.

Translation of *RPT1* or *RPT2* mRNAs will produce full-length proteins even without a productive co-translational interaction of the nascent peptides. However, the protein produced without partner and released into NCA is unstable. Hence, NCA might also ensure that proteins that have not associated with their partner are degraded and do not aggregate in the cytoplasm (see model on **Fig. 8**).

It will be interesting to determine whether Not1 plays any roles beyond co-localization of mRNAs in NCA. Not1 might contribute to repress translation initiation in the NCA, since it is the scaffold of the Ccr4-Not complex, which can contribute to translation repression when tethered to mRNAs (for review, see [15]). At present we do not know whether Not1 in NCA is functioning as part of a Ccr4-Not complex. We have started addressing the role of other Ccr4-Not subunits in NCA and so far have found no evidence for a role of the deadenylase Ccr4 or its ortholog CNOT6. In yeast Ccr4 deletion had no impact on expression or stability of Rpt1-RNC, and in LNCaP

cells treated with arsenite, we observed little staining with antibodies to CNOT6, and no evidence for co-localization of CNOT6 with the Rpt1- and Rpt2-encoding mRNAs (**Supplementary Fig. 8a,b**). In contrast, Rpt1-RNCs were unstable in yeast cells lacking Not4 or Not5 (**Supplementary Fig. 8c**), which in the case of Not4, is consistent with its described importance for proteasome assembly and integrity [14,32].

Our knowledge about the presence and role of Ccr4-Not components in different granules is still limited. In yeast, the Not proteins have been detected in P-bodies in a decapping mutant [33] and mutation of the Not1-interaction domain of the Dhh1 ATPase was shown to prevent P-body disassembly [34]. In HeLa cells, CNOT2 depletion alters Ccr4-Not complex integrity and disrupts P-bodies, but it is not a structural component of P-bodies [35], whereas in male gonocytes CNOT3 can be recruited to P-bodies by Nanos2 [36]. Ccr4 and Caf1 have also been detected in P-bodies in mammalian cells [37,38] and also in specific mutants and/or conditions in yeast, though in yeast they did not seem to significantly impact P-body formation [39].

Many RNA functions are undertaken in membrane-less organelles that have variable sizes, composition and function. The exchange of components between the cytoplasm and such organelles can be direct, dynamic and reversible. They are often referred to as RNA granules and they are formed by well-controlled transitions from disperse soluble RNA and protein to a condensed state [40-42]. NCAs are likely to be such granules. The next challenge will be to characterize their protein and RNA content, their physical-chemical nature and define their interchangeability with other mRNPs and RNA granules. An important question will be whether Not1 particles are specific to proteasome or apply to other protein complexes, and how the specificity is determined. Not1 is important for the co-translational assembly of the largest subunit of RNA polymerase II with its chaperone [43], and for subunits of the SAGA histone acetyltransferase [18]. Hence it seems likely that Not1 will be relevant for co-translational assembly of other proteins.

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Author Contributions

OOP did the ribosome profiling, FB wrote the algorithm for peak detection in the ribosome profiling, JC analyzed human ribosome profiling data and worked on representation of the peak data, SC did the yeast ribosome profiling analysis, SS did the immunofluorescence in human cells, RR did immunofluorescence experiments in yeast and tested conditions for copper induction, ZV, MZ and MAC did many of the experiments in Fig.2 to Fig.6 and supplementary figures, ML and JI did many of the initial experiments that have been the basis of the model, and JI constructed many of the plasmids and did some of the experiments in the supplementary figures, CPC and VGP did the structural analysis and contributed to the conception of the work. OOP, SS, ZV, MZ and MAC participated in writing the manuscript and conception of the work.

Competing Financial Interests Statement

The authors declare no competing interests.

References

1 Bar-Nun, S. & Glickman, M. H. Proteasomal AAA-ATPases: structure and function. *Biochim Biophys Acta* **1823**, 67-82, doi:10.1016/j.bbamcr.2011.07.009 (2012).

2 Nath, S. R. & Lieberman, A. P. The Ubiquitination, Disaggregation and Proteasomal Degradation Machineries in Polyglutamine Disease. *Front Mol Neurosci* **10**, 78, doi:10.3389/fnmol.2017.00078 (2017).
3 Budenholzer, L., Cheng, C. L., Li, Y. & Hochstrasser, M. Proteasome Structure and Assembly. *J Mol Biol*, doi:10.1016/j.jmb.2017.05.027 (2017).
4 Im, E. & Chung, K. C. Precise assembly and regulation of 26S proteasome and correlation between proteasome dysfunction and neurodegenerative diseases. *BMB Rep* **49**, 459-473 (2016).
5 Finley, D., Ulrich, H. D., Sommer, T. & Kaiser, P. The ubiquitin-proteasome system of Saccharomyces cerevisiae. *Genetics* **192**, 319-360, doi:10.1534/genetics.112.140467 (2012).
6 Funakoshi, M., Tomko, R. J., Jr., Kobayashi, H. & Hochstrasser, M. Multiple assembly chaperones govern biogenesis of the proteasome regulatory particle base. *Cell* **137**, 887-899 (2009).
7 Roelofs, J. *et al.* Chaperone-mediated pathway of proteasome regulatory particle assembly. *Nature* **459**, 861-865 (2009).
8 Hanssum, A. *et al.* An inducible chaperone adapts proteasome assembly to stress. *Mol Cell* **55**, 566-577, doi:10.1016/j.molcel.2014.06.017 (2014).
9 Le Tallec, B., Barrault, M. B., Guerois, R., Carre, T. & Peyroche, A. Hsm3/S5b participates in the assembly pathway of the 19S regulatory particle of the proteasome. *Mol Cell* **33**, 389-399, doi:10.1016/j.molcel.2009.01.010 (2009).
10 Saeki, Y., Toh, E. A., Kudo, T., Kawamura, H. & Tanaka, K. Multiple proteasome-interacting proteins assist the assembly of the yeast 19S regulatory particle. *Cell* **137**, 900-913 (2009).
11 Kramer, G., Boehringer, D., Ban, N. & Bukau, B. The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins. *Nat Struct Mol Biol* **16**, 589-597, doi:10.1038/nsmb.1614 (2009).
12 Duncan, C. D. & Mata, J. Widespread cotranslational formation of protein complexes. *PLoS Genet* **7**, e1002398, doi:10.1371/journal.pgen.1002398 (2011).
13 Shiber, A. *et al.* Cotranslational assembly of protein complexes in eukaryotes revealed by ribosome profiling. *Nature* **561**, 268-272, doi:10.1038/s41586-018-0462-y (2018).
14 Panasenko, O. O. & Collart, M. A. Not4 E3 ligase contributes to proteasome assembly and functional integrity in part through Ecm29. *Molecular and cellular biology* **31**, 1610-1623, doi:10.1128/MCB.01210-10 (2011).
15 Collart, M. A. The Ccr4-Not complex is a key regulator of eukaryotic gene expression. *Wiley Interdiscip Rev RNA*, doi:10.1002/wrna.1332 (2016).
16 Collart, M. A. & Panasenko, O. O. The Ccr4--not complex. *Gene* **492**, 42-53, doi:10.1016/j.gene.2011.09.033 (2012).
17 Maillet, L., Tu, C., Hong, Y. K., Shuster, E. O. & Collart, M. A. The essential function of Not1 lies within the Ccr4-Not complex. *J Mol Biol* **303**, 131-143 (2000).
18 Kassem, S., Villanyi, Z. & Collart, M. A. Not5-dependent co-translational assembly of Ada2 and Spt20 is essential for functional integrity of SAGA. *Nucleic Acids Res* **45**, 1186-1199, doi:10.1093/nar/gkw1059 (2017).
19 Artieri, C. G. & Fraser, H. B. Accounting for biases in riboprofiling data indicates a major role for proline in stalling translation. *Genome research* **24**, 2011-2021, doi:10.1101/gr.175893.114 (2014).

20 Schuller, A. P., Wu, C. C., Dever, T. E., Buskirk, A. R. & Green, R. eIF5A Functions Globally in Translation Elongation and Termination. *Mol Cell* **66**, 194-205 e195, doi:10.1016/j.molcel.2017.03.003 (2017).
21 Barrault, M. B. *et al.* Dual functions of the Hsm3 protein in chaperoning and scaffolding regulatory particle subunits during the proteasome assembly. *Proc Natl Acad Sci U S A* **109**, E1001-1010, doi:10.1073/pnas.1116538109 (2012).
22 Fu, H., Reis, N., Lee, Y., Glickman, M. H. & Vierstra, R. D. Subunit interaction maps for the regulatory particle of the 26S proteasome and the COP9 signalosome. *EMBO J* **20**, 7096-7107, doi:10.1093/emboj/20.24.7096 (2001).
23 Zhang, Y., Wolfle, T. & Rospert, S. Interaction of nascent chains with the ribosomal tunnel proteins Rpl4, Rpl17, and Rpl39 of Saccharomyces cerevisiae. *J Biol Chem* **288**, 33697-33707, doi:10.1074/jbc.M113.508283 (2013).
24 Aufderheide, A. *et al.* Structural characterization of the interaction of Ubp6 with the 26S proteasome. *Proc Natl Acad Sci U S A* **112**, 8626-8631, doi:10.1073/pnas.1510449112 (2015).
25 Matsuda, R., Ikeuchi, K., Nomura, S. & Inada, T. Protein quality control systems associated with no-go and nonstop mRNA surveillance in yeast. *Genes Cells* **19**, 1-12, doi:10.1111/gtc.12106 (2014).
26 Inada, T. Quality control systems for aberrant mRNAs induced by aberrant translation elongation and termination. *Biochim Biophys Acta* **1829**, 634-642, doi:10.1016/j.bbagrm.2013.02.004 (2013).
27 Inobe, T. & Genmei, R. N-Terminal Coiled-Coil Structure of ATPase Subunits of 26S Proteasome Is Crucial for Proteasome Function. *PLoS One* **10**, e0134056, doi:10.1371/journal.pone.0134056 (2015).
28 Takagi, K. *et al.* Structural basis for specific recognition of Rpt1p, an ATPase subunit of 26 S proteasome, by proteasome-dedicated chaperone Hsm3p. *J Biol Chem* **287**, 12172-12182, doi:10.1074/jbc.M112.345876 (2012).
29 Ingolia, N. T., Ghaemmaghami, S., Newman, J. R. & Weissman, J. S. Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science* **324**, 218-223, doi:10.1126/science.1168978 (2009).
30 Kedersha, N. & Anderson, P. Mammalian stress granules and processing bodies. *Methods Enzymol* **431**, 61-81, doi:10.1016/S0076-6879(07)31005-7 (2007).
31 Simms, C. L., Yan, L. L. & Zaher, H. S. Ribosome Collision Is Critical for Quality Control during No-Go Decay. *Mol Cell* **68**, 361-373 e365, doi:10.1016/j.molcel.2017.08.019 (2017).
32 Chen, H., Sirupangi, T., Wu, Z. H., Johnson, D. L. & Laribee, R. N. The conserved RNA recognition motif and C3H1 domain of the Not4 ubiquitin ligase regulate in vivo ligase function. *Sci Rep* **8**, 8163, doi:10.1038/s41598-018-26576-1 (2018).
33 Muhlrad, D. & Parker, R. The yeast EDC1 mRNA undergoes deadenylation-independent decapping stimulated by Not2p, Not4p, and Not5p. *Embo J* **24**, 1033-1045 (2005).
34 Mugler, C. F. *et al.* ATPase activity of the DEAD-box protein Dhh1 controls processing body formation. *Elife* **5**, doi:10.7554/eLife.18746 (2016).

35 Ito, K. *et al.* CNOT2 depletion disrupts and inhibits the CCR4-NOT deadenylase complex and induces apoptotic cell death. *Genes Cells* **16**, 368-379, doi:10.1111/j.1365-2443.2011.01492.x (2011).
36 Suzuki, A., Igarashi, K., Aisaki, K., Kanno, J. & Saga, Y. NANOS2 interacts with the CCR4-NOT deadenylation complex and leads to suppression of specific RNAs. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 3594-3599, doi:10.1073/pnas.0908664107 (2010).
37 Cougot, N., Babajko, S. & Seraphin, B. Cytoplasmic foci are sites of mRNA decay in human cells. *J Cell Biol* **165**, 31-40, doi:10.1083/jcb.200309008 (2004).
38 Andrei, M. A. *et al.* A role for eIF4E and eIF4E-transporter in targeting mRNPs to mammalian processing bodies. *RNA* **11**, 717-727, doi:10.1261/rna.2340405 (2005).
39 Teixeira, D. & Parker, R. Analysis of P-body assembly in Saccharomyces cerevisiae. *Mol Biol Cell* **18**, 2274-2287 (2007).
40 Weber, S. C. & Brangwynne, C. P. Getting RNA and protein in phase. *Cell* **149**, 1188-1191, doi:10.1016/j.cell.2012.05.022 (2012).
41 Hyman, A. A., Weber, C. A. & Julicher, F. Liquid-liquid phase separation in biology. *Annu Rev Cell Dev Biol* **30**, 39-58, doi:10.1146/annurev-cellbio-100913-013325 (2014).
42 Jarvelin, A. I., Noerenberg, M., Davis, I. & Castello, A. The new (dis)order in RNA regulation. *Cell Commun Signal* **14**, 9, doi:10.1186/s12964-016-0132-3 (2016).
43 Villanyi, Z. *et al.* The Not5 subunit of the ccr4-not complex connects transcription and translation. *PLoS Genet* **10**, e1004569, doi:10.1371/journal.pgen.1004569 (2014).

Figure legends

Figure 1. Peaks of ribosome footprints indicative of ribosome pausing correspond to structural features of Rpt1 and Rpt2. a. The patterns of ribosome footprints aligned by their P sites are shown for *RPT1* and *RPT2* mRNAs, with a cartoon representation of the protein domain architecture below, showing N-terminal, coiled coil (CC), oligonucleotide/oligosaccharide-binding (OB), AAA-ATPase and C-terminal domains; amino acid positions that delineate these domains are indicated on top. The α helices from Rpt1 and Rpt2 involved in those subunits' interaction with each other (PDB 5A5B [24]) are depicted in red and green below the footprints, with amino acid numbers marked. The size of the vertical lines is proportional to the number of times a footprint was found at that location. We indicate below the footprint profiles the codons at the P sites (D241 for *RPT1* and D165 for *RPT2*) and A sites (P242 for *RPT1* and P166 for *RPT2*) of the ribosomes paused at the major pause

sites and we indicate the D135 codon for *RPT1* where a minor accumulation of footprints is detected. **b.** Partial view of the structure of the proteasome regulatory particle (RP) (PDB 5A5B [24]), showing the Rpt1 and Rpt2 subunits The helices of Rpt1 and Rpt2 that interact are shown in red and green, respectively. **c.** Model of co-translational assembly of protein domains. **d.** Cartoon representation of the different Rpt1 and Rpt2 constructs used in this work.

Figure 2. Interaction of ribosome-associated Rpt1 with N-terminal fragments of Rpt2. a. Western blot analysis, using antibodies to Flag or Rpl35, of extracts from cells expressing RNC constructs. Cells were induced 10 min with copper then treated with cycloheximide (CHX) for the indicated times. **b.** Western blot analysis (top) and polysome profiles (bottom) of sucrose gradient sedimentation fractions of Rpt1-RNC in extracts from copper-induced cells, treated or untreated with EDTA. TE, total extracts. Fractions are: F, free; M, monosomes; P1, light polysome). **c.** Experiment as in panel **b** but extracts were prepared with CHX (0.1 mg/ml) to preserve polysomes, treated or not with RNase A. P2, heavy polysomes. **d.** Experiment as in panel **c** for cells expressing ΔN-Rpt1-RNC and extracts prepared with CHX without RNase A. **e.** Quantification from biological duplicates of peptide in fractions F, P1 or P2 compared to M for the data from extracts not treated with RNase in panels **c** and **d**. Error bars represent the range of the 2 values; source data are available in **Supplementary Table 1**. **f.** Serial dilution spot assay to evaluate cells ability to grow after losing their *URA3* plasmid. Cells were spotted on plates containing 5-fluoro-orotic acid (FOA) or on YPD as control. *rpt1Δ* cells carrying a *URA3* plasmid expressing Protein A-tagged Rpt1 (*RPT1*p-ProtA-*RPT1*) were transformed with plasmids expressing either wild type Rpt1 (*CUP1*p-HA-*RPT1*) or N-terminally deleted Rpt1 (*CUP1*p-HA-*ΔN-RPT1*) from the copper promoter. All experiments were repeated at least 3 times with similar results. Data with biological replicates are provided in **Supplementary Data Set 1**.

Figure 3. Nascent Rpt1 forms heavy bodies. a. Western blot analysis of the sedimentation pattern in sucrose gradients of co-expressed HA-Rpt1 and the Rpt2-RNC or co-expressed HA-Rpt2 and Rpt1-RNC. Total extracts (TE) and the indicated fractions were analyzed with HA and Flag antibodies. The positions of 40S, 60S, 80S and polysomes are indicated. **b.** Western blot analysis of immunoprecipitates, with

HA or Flag antibodies. Polysome fractions were combined (input) and proteins were immunoprecipitated without (IP 0) or with anti-HA (IP HA) or anti-Flag (IP Flag) antibodies. **c.** Western blot analysis of immunoprecipitates, with antibodies to HA, Flag and Not1. Total extracts from cells co-expressing HA-Rpt1 with the Rpt2-RNC were treated with EDTA and separated on a sucrose gradient. The heavy fraction P2 was incubated with protein G beads covered by antibodies to HA, Flag or Myc as a negative control. This experiment was performed more than 3 times with similar results. Uncropped images and biological replicates are provided in **Supplementary Data Set 1**.

Figure 4. Newly produced Rpt1 and Rpt2 are present in heavy bodies. a,b. Western blot analyses of cell extracts after fractionation in sucrose gradients with or without prior EDTA treatment. Extracts were prepared from cells induced with copper for 10 min and expressing HA-tagged Rpt1 alone (**a**) or HA-Rpt2 alone (**b**) The polysome profiles are shown at the bottom. Both experiments were performed with biological duplicates (data shown in **Supplementary Data Set1**). **c.** Experiment as in panels **a** and **b**, except that cells expressed HA-tagged Rpt2 or HA-tagged Pup2 as indicated. The Ponceau staining of the gel is shown at the bottom as loading control. The polysome profiles are shown on the right. The experiment was done in biological triplicate (data shown in **Supplementary Data Set 1**).

Figure 5. The DP codons at the ribosome pause site of *RPT1* are favorable for interaction of Rpt1 and Rpt2. **a,b.** Western blot analyses of extracts from copper-induced cells expressing HA-Rpt1 or HA-Rpt2. Cell extracts were prepared at the indicated times after addition of CHX and probed with HA and Rpl35 antibodies. **c.** Same as in panels **a** and **b** with extracts from *rpt1Δ* cells expressing from an episome ProtA-Rpt1 from the *RPT1* promoter and HA-Rpt2 from the *CUP1* promoter. **d.** Western blot analyses of extracts from *rpt1Δ* cells expressing from an episome Protein A-tagged Rpt1 from the *RPT1* promoter (ProtA-Rpt1-DP) or codon-changed (DP to AA) Protein A-tagged Rpt1 (ProtA-Rpt1-AA). Blots were probed with PAP antibodies. A high (left) and a low (right) exposure are shown. Uncropped images of the blots shown in **a-d** are displayed in **Supplementary Data Set 1**. **e.** Left, experiment as in panel **c** except with *rpt1Δ* cells expressing either ProtA-Rpt1 and

HA-Rpt2 (lanes 1-3) or ProtA-Rpt1-AA and HA-Rpt2 (lanes 4-6) as indicated. Right, quantification of HA to Rpl35 signal from biological duplicates; error bars represent the range of the 2 values; source data are in **Supplementary Table 1**. The expression of HA-Rpt2 was significantly different when cells expressed ProtA-Rpt1-AA compared to ProtA-Rpt1-DP (t-test, p-value= 0.013). The data shown are representative of more than 3 independent experiments; the blots for the quantified duplicates and a biological triplicate are provided in **Supplementary Data Set1**.

Figure 6. Interaction of the Rpt1 and Rpt2 N-terminal domains alleviates ribosome pausing. a. Western blot analyses of extracts prepared from cells carrying a plasmid driving expression of HA-Rpt2 $_{75\text{-}166}$ from the *CUP1* promoter, alone or together with the Rpt1-RNC. Cells were induced with copper for 10 min, and then treated with CHX for the indicated times (o/n, overnight). Blots were probed with antibodies to HA, Flag or Rpl35. **b.** Western blot analyses of extracts prepared by post-alkaline lysis from copper-induced cells expressing the Rpt1-RNC alone or together with HA-Rpt2 $_{75\text{-}166}$. Blots were probed with antibodies to HA, Flag or V5. The experiment was performed more than 5 times with similar results **c.** Western blot analyses of extracts from the same cells as in panel **b,** separated on a sucrose gradient. Fractions (TE, total extract; F, free; M, monosomes; P1, light polysomes; P2, heavy polysomes) were probed with antibodies to Flag, V5 and Rpl35. Uncropped blots and biological replicates are shown in **Supplementary Data Set 1**.

Figure 7. Rpt1- and Rpt2- encoding mRNAs co-localize with each other and with CNOT1 in small bodies distinct from stress granules. LNCaP cells, untreated or treated with arsenite (100 μM) for 1 h, were fixed and subjected to immunostaining or *in situ* hybridization. Slides were counterstained with DRAQ5 to detect nuclei. Inserts show the enlargements. Scale bars, 10 μm. **a.** LNCaP cells were subjected to immunostatining with antibodies to CNOT1 and the stress granule marker protein G3BP1. **b.** LNCaP cells were 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. **c.** LNCaP cells were transfected with siControl or CNOT1 siRNAs, then treated with arsenite. Fixed cells were subjected to *in situ* hybridization using differently labeled oligos targeting Rpt1- or Rpt2-encoding

mRNAs. Note that Rpt1 and Rpt2 co-localize in CNOT1 proficient cells but not in CNOT1 deficient cells. Each experiment was conducted independently at least 3 times. 10-15 images were captured for each condition.

Figure 8. Model for co-translational assembly of Rpt1 and Rpt2 in Not1-Containing Assemblysomes (NCA). In response to proteotoxic stress, proteasome mRNAs are induced and translation is initiated. Top, for *RPT1* and *RPT2* mRNAs, ribosomes pause and the RNCs are assembled into Not1-containing particles where they are stable. The stability of the RNCs depends upon their disordered N-terminal domains and allows Rpt1-RNC and Rpt2-RNC particles to fuse (bottom right). The co-localization of the Rpt1- and Rpt2-encoding mRNAs requires Not1. The N-terminal domains of Rpt1 and Rpt2 can assemble co-translationally, and translation can proceed, leading to productive interaction of Rpt1 and Rpt2 and ultimately formation of proteasome. If the nascent Rpt1- and Rpt2 do not assemble, synthesis of Rpt1 and/or Rpt2 will occur separately (bottom left), and the proteins will not productively associate and instead will be degraded.

Online Methods

Yeast strains, plasmids, oligonucleotides and antibodies.

All strains, plasmids and oligonucleotides used in this work are listed in **Supplementary Table 2**. Antibodies were either commercial or previously described [44] and are also listed in **Supplementary Table 2**. Proteasome and other proteins were expressed from the plasmids under control of the inducible *CUP1* promoter (350 nt). Full-length Rpt1 and Rpt2, or truncated versions, contain an N-terminal HA_7 tag as do N-terminal fragments of Rpt2. All clones were obtained by the drag and drop procedure [45]. Truncated stalled proteins contain a triple Flag tag at the N-terminus. All the encoding sequences are cloned with a $(AAAAAG)_6$ stalling sequence at the 3', followed by an XhoI site, and 45 codons including a V5-His6 sequence before a stop codon. The plasmid expressing Protein A-tagged Rpt1 was recovered from MY6277 and sequenced. *RPT1* with the $241_{GAT}242_{CCA}$ codons was changed to $241_{GCG}242_{GCG}$ by PCR amplification and cloned in the same plasmid backbone. Cells transformed

with plasmids containing *CUP1* promoter-driven genes were grown to exponential phase and induced for 10 min with 0.1 mM $CuSO_4$ (Cu). Cycloheximide (CHX) was used at a concentration of 100 µg/ml. Media were standard.

Immunoprecipitation and affinity purification

100 OD_{600} units of cells were broken with 0.3 ml of glass beads in 0.4 ml of lysis buffer (20 mM Hepes pH 7.5, 20 mM KCl, 10 mM $MgCl_2$, 0.1 % Triton X-100, 1 mM DTT, 1 mM PMSF and a protease inhibitor cocktail) for 15 min at 4°C. After clarification, 0.8 ml of the supernatant containing 4 mg of total protein were incubated with 1 µg anti-Flag, anti HA or anti Myc antibodies and 30 µl of protein G magnetic beads (Dynabeads, Thermo Fisher Scientific) for 5 h. The beads were washed three times with 0.8 ml of lysis buffer then incubated for 10 min at 65°C with SDS sample buffer before SDS-PAGE and western blotting.

Rpt1-RNC purification

To purify the Rpt1-RNC we used the FLAG(R) Immunoprecipitation Kit (SIGMA, FLAGIPT1-1KT) and released Rpt1-RNC from the IgG beads with the Flag peptide according to the manufacturer's instructions.

Rpn11 purification

To purify Rpn11 and associated proteins [46], 1 liter of cells grown to OD_{600} of 3.0 in the media selective for the plasmids were collected in 2 ml lysis buffer (50mM Tris-Cl pH 8.0, 5 mM $MgCl_2$, 1 mM EDTA a protease inhibitor cocktail and 1 mM ATP) and frozen by drops in liquid nitrogen. Cells were then broken in liquid nitrogen using the Retsch CryoMill 400 to form a powder. 10 ml of cell powder was thawed by addition of lysis buffer to 50 ml with 0.5 ml of 100 mM ATP at 4°C. After clarification at 4000 rpm for 10 min at 4°C, the lysate was further clarified by ultracentrifugation in a Beckmann Ti70 at 40000 rpm for 30 min. The clarified lysate was filtered through 125 mM Whatman filters (N° 10311644, GE healthcare) and the protein concentration evaluated by the Bradford assay. 0.15 ml of IgG beads (IgG sepharose fast flow, GE Healthcare) per 100 mg of total protein were washed with water then with lysis buffer. 10 ml of extract (10 mg/ml) were added to the beads and incubated for 2 h at 4°C. The beads and extract were loaded on a column, and the column dried by gravity. The column was washed with 50 bead volumes of buffer 2

(50 mM Tris-Cl pH 7.5, 100 mM NaCl, 5mM $MgCl_2$, 1 mM EDTA, 1 mM ATP) and 15 bead volumes of TEV buffer (50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, and 1 mM DTT), and dried by gravity flow. 1 bead volume of TEV buffer and 100 units of AcTEV™ protease (Life technologies at Thermo Fischer, Cat. No. 12575015) per 100 µl of buffer were incubated with the beads in the column for 1 h at 30°C, with mixing every 10 min. The eluate was recovered by gravity flow. The beads were washed twice with 1 bead volume of TEV buffer and the eluates were combined. Glycerol was added to a final concentration of 10% and the eluates were concentrated using microcon tubes (Amicon Ultra-2 Centrifugal Filter Unit (EMD Millipore; Cat. No. UFC205024) to a final volume of 100 µl.

Polysome fractionation.

Extracts were fractionated in sucrose gradients as described before [47]. CHX was added when indicated at 0.1 mg/ml. For immunoprecipitation, polysome fractions were combined and incubated with anti HA, anti Flag or anti Myc antibodies as described [47]. When indicated, polysomes were disrupted by treatment of extracts with 25 mM EDTA or 10 µg/ml RNase A for 5 min at room temperature prior to sucrose gradient fractionation.

Western blot quantification

Western blots were quantified based on signals originating from biologically duplicated experiments with the Fiji software [48].

Ribosome profiling.

Ribosome profiling was performed for the wild type cells (MY3415) in biological duplicates as described before [49]. Briefly, cells were grown to exponential phase, total extracts prepared in presence of CHX (0.1 mg/ml) were treated with RNase I, and monosomes were isolated after sucrose gradient separation. Libraries were made from the ribosome protected fragments and subjected to deep sequencing. The positions of the P-sites of the ribosomes were deduced from the deep sequencing data in the following way. Adapters were trimmed from the 3' end of the reads using cut_adapt utility and discarding all reads without valid adapter. To improve further the alignment, one nucleotide was removed from the 5' end of each read (if this

nucleotide is left, it is aligned erroneously in the majority of reads, as it frequently represents an untemplated addition during reverse transcription [50]). Trimmed reads were aligned to the April 2011 (sacCer3) *S. cerevisiae* genome assembly from UCSC [51]. The alignment was performed by Tophat aligner using the gene annotations from the *Saccharomyces* Genome Database (www.yeastgenome.org) [52] in '—non-novel-juncs' mode. Optionally, filtering of the ribosomal RNA can be performed before the alignment step, which speeds up the processing, without affecting the alignments for the protein coding genes. Detection of ribosome pausing was performed in the following way: 1) The P-sites were obtained from the reads by shifting the reads by a fixed amount for each read length (shift by 11, 12, 13, 12 nucleotides for RPF reads of length 27, 28, 29, 30, respectively). The amount of offset was determined by observing the position of the peak at the start of ORF for the given nucleotide length. 2) Counts were combined for each *codon* in the reading frame, thus allowing for the reduction of systematic difference between the nucleotides in the codon, as well as the observed three-nucleotide periodicity, and making the algorithm less sensitive to errors in position of 1 nucleotide. 3) For each gene, the distribution of the counts for all the codons was individually modeled by a negative binomial distribution. 4) For each codon the ratio of its count to the average over the gene and the P-value according to the fitted negative binomial distributions were obtained. These two numbers allow the quantification of the level of pausing and its statistical reliability. False discovery correction for multiple testing is then applied in order to obtain the FDR corrected Q-values. In order to validate the performance of our algorithm against more deeply sequenced ribosome profiling data and to ensure that detected peaks were not simply due to heterogeneous coverage resulting from fragment sequence bias [53-55], we downloaded data from two publicly available datasets which included matched RNA-seq and ribosome profiling samples (GEO accessions GSE63789 and GSE53268). We ran our algorithm on both the wild-type RNA-seq and ribosome profiling samples from each dataset. In all cases, the algorithm detected many more statistically significant (adjusted p-value < 0.01) peaks in the ribosome profiling data than in the matched RNA-seq data (772 vs. 3 and 2250 vs. 2, respectively), giving us confidence that the detected pausing events were unique to ribosome footprints.

Mammalian cell lines, polysome profiling and RNA analysis

Cell lines used in this study were LNCaP (from ATCC), 22Rv1 (from ATCC) and V16D prostate cancer cells (from the Vancouver Prostate Centre). The cells were tested to be mycoplasma free and were authenticated. A549 cells derived from human lung adenocarcinoma and bearing an oncogenic mutated KRAS [56] were used for the polysome profiling followed by CNOT1 analysis and have not been authenticated. For polysome profiling cells were grown until 70% confluency in two 15 cm diameter Petri dishes for each sample, treated or not with arsenite, scraped and lysed in lysis buffer (10 mM HEPES, 100 mM KCl, 5mM $MgCl_2$, 100 mg/ml cycloheximide and 2% of Triton X-100 (pH 7.4)). For CNOT1 analysis A549 cell extracts were analyzed after fractionation on 12 ml 7–47% sucrose gradients as in [43]. For RNA analyses, LNCaP prostate cancer cell extracts were used and RNasin Plus (Promega) at 0.2 unit/µl was added in extracts prior to sucrose gradient fractionation. RNA was isolated from extracts, monosomes and heavy polysome fractions by the Trizol reagent (Invitrogen) following the recommendations of the manufacturer. Total RNA was precipitated upon addition of 3 µl of linear acrylamide (Fermentas). Pellets were resuspended in H_2O and were DNaseI treated (RQ1 RNase-free DNase, Promega), then RNA concentration was measured by nanodrop. For qPCR analysis 500 ng of total RNA obtained from monosomes, polysomes or from total extracts were reverse transcribed with M-MLV RT and oligo(dT) primers in a total volume of 25 µl. After synthesis, cDNAs were diluted to a final volume of 250 µl and 5 µl were used for qPCR using gene-specific primers as described in [43]. Gene-specific primer sequences are listed in **Supplementary Table 2.** Relative mRNA abundances were determined by the Pfaffl method [57] and normalized to wild-type RNA levels. *EIF4A2* mRNA was used as a loading control.

Immunofluorescence and *in situ* hybridization

For imaging, LNCaP, 22Rv1 and V16D cells were seeded at 20-25% confluence in 6 cm culture dishes containing round cover glasses (Fisher Scientific, 12CIR-1D). LNCaP cells were treated with siControl (Control siRNA-A; sc-37007 from Santa Cruz) or siCNOT1 (CNOT1 (h)-PR; sc-93370-PR from Santa Cruz) siRNAs for 3 days. Cells were then treated with vehicle alone or exposed to arsenite (100 µM arsenite) for 1 h. Immunofluorescence (IF) was carried out as described previously [58]. For the localization of Rpt1- and Rpt2-encoding mRNAs in NCA, *in situ*

hybridization was performed as described previously [58] with 56-FAM- or 5TEX615-oligos described in **Supplementary Table 2**. The cells were then counterstained with CNOT1 antibodies as described. Cells processed as above were immersed in DRAQ5 (10 µM, Biostatus) for nuclear staining, mounted with FluorSave and viewed using Nikon Eclipse Ti-E inverted confocal microscope at 40X and 100X oil-immersion objectives. Images were captured using EZ-C1 software and were further processed using ImageJ software.

Validation of peak-calling algorithm

To validate the specificity of our algorithm for identification of peaks in ribosome footprinting data, we ran it against published data that had higher coverage than ours, good quality measures, and a matched total RNA-seq sample. In the matched ribosome footprinting data, 2250 codons had stalling even at a high stringency cut off (logQ < -2) while only 2 codons of the total RNA-seq data passed the same cut off. This validated that the algorithm was identifying patterns of accumulation that specifically occur in ribosome footprints, rather than heterogenous transcript coverage due to sequence or fragment biases, which could also be present in the RNA-seq data.

Statistics and Reproducibility

All experiments presented in the manuscript were performed at least in biological triplicates with similar results with the exception of **Supplementary Fig. 5b** (biological duplicates) and **Supplementary Fig. 5c** (single experiment). To analyze the significance of the difference of expression of HA-Rpt2 between cells expressing ProtA-Rpt1-DP and ProtA-Rpt1-AA, the 2h and o/n time points were pooled and a linear model was fitted (R^2=0.84) with expression as dependent variable and two binary explanatory variables indicating condition (AA/DP) and time (2h/o/n), respectively. The condition coefficient estimated a statistically significant 29.4 % expression increase (95% CI [9.49,49.3]) in DP relative to AA (two-sided t-test, p-value= 0.013), when controlling for the time variable (coefficient: -27.165% expression loss - 95% CI [-47.1,-7.3] - from leaving overnight, p value= 0.017).

Sequence alignments

Amino acid and coding DNA sequences for yeast *RPT1-6* were downloaded from the *Saccharomyces* Genome Database (https://www.yeastgenome.org/), and amino acid

and coding DNA sequences for human PSMC2 (coding for *RPT1*) were downloaded from the NCBI Gene RefSeq database (https://www.ncbi.nlm.nih.gov/gene/). Multiple sequence alignments were performed using Clustal Omega with default parameters (https://www.ebi.ac.uk/Tools/msa/clustalo/).

Data Availability Statement

The ribosome profiling data is available under SRA accession code SRP134678. The working realization of the algorithm for ribosome peak detection is available on https://github.com/fedxa/RiboPeaks. The quantification data shown in Figures 2e, 5e, supplementary Figure 2a and 5b are available in **Supplementary Table 1**. Uncropped images and replicate experiments for Figures 2, 3, 4, 5 and 6 and Supplementary Figures 2, 3, 4, 5c and 8c are available in **Supplementary Data Set 1**. Any other data is available upon request.

References

44 Frey, S., Pool, M. & Seedorf, M. Scp160p, 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, doi:10.1074/jbc.M009430200 (2001).

45 Jansen, G., Wu, C., Schade, B., Thomas, D. Y. & Whiteway, M. Drag&Drop cloning in yeast. *Gene* **344**, 43-51 (2005).

46 Leggett, D. S., Glickman, M. H. & Finley, D. Purification of proteasomes, proteasome subcomplexes, and proteasome-associated proteins from budding yeast. *Methods Mol Biol* **301**, 57-70 (2005).

47 Panasenko, O. O. & Collart, M. A. Presence of Not5 and ubiquitinated Rps7A in polysome fractions depends upon the Not4 E3 ligase. *Molecular microbiology* **83**, 640-653, doi:10.1111/j.1365-2958.2011.07957.x (2012).

48 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682, doi:10.1038/nmeth.2019 (2012).

49 Ingolia, N. T. Genome-wide translational profiling by ribosome footprinting. *Methods Enzymol* **470**, 119-142, doi:10.1016/S0076-6879(10)70006-9 (2010).

50 Ingolia, N. T., Brar, G. A., Rouskin, S., McGeachy, A. M. & Weissman, J. S. The ribosome profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-protected mRNA fragments. *Nat Protoc* **7**, 1534-1550, doi:10.1038/nprot.2012.086 (2012).

51 Rosenbloom, K. R. *et al.* The UCSC Genome Browser database: 2015 update. *Nucleic Acids Res* **43**, D670-681, doi:10.1093/nar/gku1177 (2015).

52 Cherry, J. M. *et al.* Saccharomyces Genome Database: the genomics resource of budding yeast. *Nucleic Acids Res* **40**, D700-705, doi:10.1093/nar/gkr1029 (2012).

53 Hansen, K. D., Brenner, S. E. & Dudoit, S. Biases in Illumina transcriptome sequencing caused by random hexamer priming. *Nucleic Acids Res* **38**, e131, doi:10.1093/nar/gkq224 (2010).

54 Lahens, N. F. *et al.* IVT-seq reveals extreme bias in RNA sequencing. *Genome Biol* **15**, R86, doi:10.1186/gb-2014-15-6-r86 (2014).
55 Roberts, A., Trapnell, C., Donaghey, J., Rinn, J. L. & Pachter, L. Improving RNA-Seq expression estimates by correcting for fragment bias. *Genome Biol* **12**, R22, doi:10.1186/gb-2011-12-3-r22 (2011).
56 Gazdar, A. F., Girard, L., Lockwood, W. W., Lam, W. L. & Minna, J. D. Lung cancer cell lines as tools for biomedical discovery and research. *J Natl Cancer Inst* **102**, 1310-1321, doi:10.1093/jnci/djq279 (2010).
57 Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45 (2001).
58 Somasekharan, S. P. *et al.* YB-1 regulates stress granule formation and tumor progression by translationally activating G3BP1. *J Cell Biol* **208**, 913-929, doi:10.1083/jcb.201411047 (2015).