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Quentin Defenouillere, Yanhua Yao, John Mouaikel, Abdelkader Namane, Aurélie Galopier, et al.. Cdc48-associated complex bound to 60S particles is required for the clearance of aberrant translation products. *Proceedings of the National Academy of Sciences of the United States of America*, 2013, 110 (13), pp.5046–5051. 10.1073/pnas.1221724110 . pasteur-01404018

HAL Id: pasteur-01404018

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Submitted on 5 Jan 2017

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A Cdc48-associated complex bound to 60S particles is required for the clearance of aberrant translation products

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Submitted to Proceedings of the National Academy of Sciences of the United States of America

Ribosome stalling on eukaryotic mRNAs triggers co-translational RNA and protein degradation through conserved mechanisms. For example, mRNAs lacking a stop codon are degraded by the exosome in association with the SKI complex, whereas the co-responding aberrant nascent polypeptides are ubiquitinated by the E3 ligases Ltn1 and Not4 and become proteasome substrates. How translation arrest is linked with polypeptide degradation is still unclear. Genetic screens with SKI and LTN1 mutants allowed us to identify Tae2 and Rqc1, two novel factors that we found associated, together with Ltn1 and Cdc48, to 60S ribosomal subunits. Tae2, Rqc1 and Cdc48 were all required for degradation of polypeptides synthesized from non-stop mRNAs (NSPD). Both Ltn1 and Rqc1 were essential for the recruitment of Cdc48 to 60S particles. Polysome gradient analyses of mutant strains revealed novel intermediates of this pathway, showing that the polyubiquitination of Non-Stop peptides is a progressive process. We propose that ubiquitination of the nascent peptide starts on the 80S and continues on the 60S, on which Cdc48 is recruited to escort the substrate for proteasomal degradation.

mRNA degradation | quality control | *Saccharomyces cerevisiae* | translation | ubiquitin

Introduction

Cytoplasmic quality control mechanisms prevent the accumulation of potentially toxic translation products by recognition and elimination of non-conforming mRNAs and their associated nascent peptides stalled on ribosomes. Three quality control pathways are known, each targeting a specific type of aberrant mRNAs: NonSense Mediated mRNA Decay (NMD) (1), NonStop mRNA Decay (NSD) (2, 3) and NoGo mRNA Decay (NGD) (4), eliminating transcripts with a premature termination codon, lacking a STOP codon or carrying a element causing translational arrest, respectively.

NSD constitutes an important quality control process especially in case of [PSI]⁺ state, a physiological context that promotes stop codon read-through, thus generating extended proteins (5). Furthermore, NSD substrates can also result from transcription termination at cryptic polyadenylation (poly(A)) sites located in the coding region of the mRNA. A global map of poly(A) sites recently generated by direct RNA sequencing revealed that many cryptic poly(A) sites exist in yeast as well as in the human genome (6).

It has been shown that ribosome stalling and NGD can be induced by the synthesis of highly positively charged amino acid sequences, such as a poly-arginine or a poly-lysine stretches. The positive charges of these basic tracts would electrostatically interact with the negative charges within the exit tunnel (7), resulting in ribosome stalling and translation arrest. Such a polybasic tract can arise from the translation of a poly(A) tail into polylysine, which occurs during translation of a *Non-Stop* mRNA (8). Hence,

the boundary between these two quality control pathways remains unclear (9). NSD and NGD would both be activated by a stalled ribosome with an empty A site recognized by Dom34/Hbs1, homologs of the eRF1/eRF3 heterodimer, which triggers ribosome dissociation and peptidyl-tRNA release (10). In *Saccharomyces cerevisiae*, Ski7, another homolog of the release factor eRF3, can also trigger NSD (2, 3). In the case of NGD, a yet unknown endonuclease cleaves the aberrant mRNA. The 5' cleavage product is degraded upon binding of the SKI complex and the exosome whereas the 3' fragment is degraded by Xrn1 (4). It was recently proposed that, in the case of NSD, an endonucleolytic cleavage also occurs, upstream of the ribosome stalled at the poly(A) tail (11). In addition, in both NGD and NSD, the nascent peptide is targeted to the proteasome and rapidly degraded. In the case of ribosome stalling induced by the synthesis of consecutive basic amino acid residues, the E3 ubiquitin ligase Not4 was shown to be required for the rapid degradation of the nascent peptide (12), whereas the E3 ligase Ltn1 was found to be required for the ubiquitination and proteasomal degradation of the aberrant peptide synthesized from a *Non-Stop* mRNA (13).

Therefore, during NGD and NSD, the degradation processes of the aberrant mRNA and of the associated nascent peptide are coupled. The absence of the SKI complex combined with the deletion of *LTN1* is synthetic slow growth (13) showing that a combination of mutations in both *Non-Stop* mRNA decay and aberrant nascent peptide degradation is detrimental to the cell.

In many cases, such as in ERAD (Endoplasmic-Reticulum-Associated protein Degradation), the AAA-ATPase Cdc48 recognizes polyubiquitinated proteins and is required for their efficient targeting to the proteasome (14, 15). The binding of Cdc48 to specific targets is determined by its physical interaction with a large number of co-factors. Here we identified two novel factors, Tae2 and Rqc1, which, together with Ltn1 and Cdc48, form a complex associated to the 60S and required for the degradation of nascent peptides synthesized from aberrant *Non-Stop* mRNAs. We also show that ubiquitination of the nascent peptides starts on the stalled 80S ribosome and continues on the 60S after ribosomal dissociation.

Reserved for Publication Footnotes

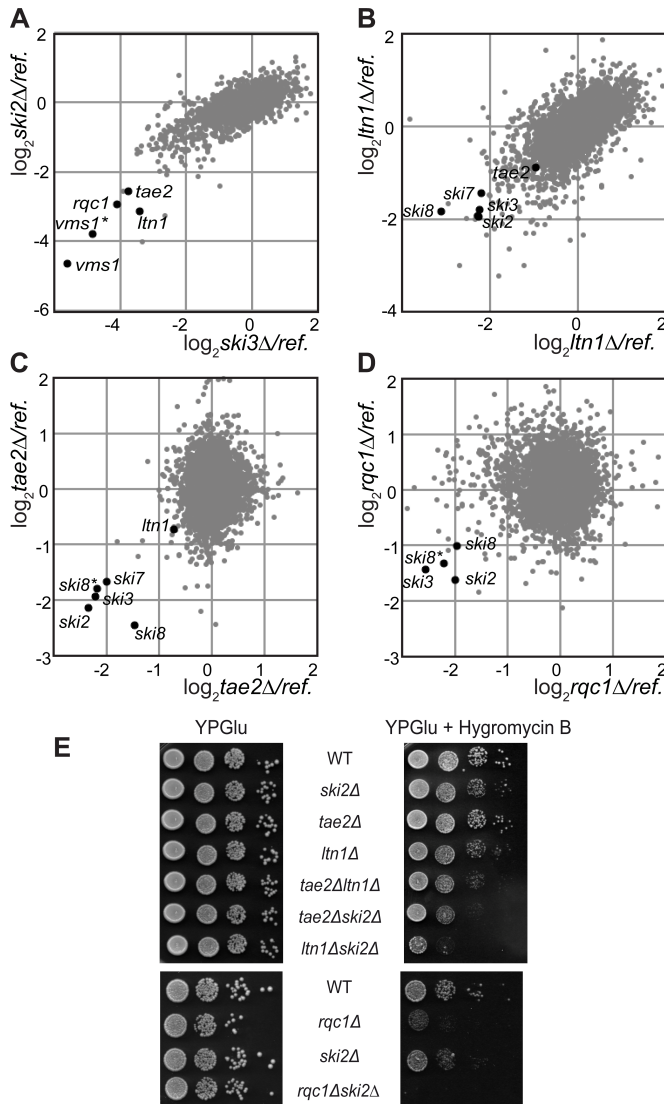


Fig. 1. Tae2, Rqc1 and Ltn1 are functionally linked to the SKI complex.(A) GIM screens with *ski2* Δ and *ski3* Δ query strains. Each dot indicates a deletion mutant strain. In each screen, we compared the relative changes in a given mutant levels in combination with the query or a reference mutation after 22 generations of a mixture of double mutants population. Microarray estimation of changes were normalised and the \log_2 of the ration query/reference was plotted. (B) GIM screens with *ltn1* Δ query strain. As Fig. 1A(C) GIM screens with *tae2* Δ query strain. As Fig. 1A(D) GIM screens with *rqc1* Δ query strain. As Fig. 1A(E) Serial dilution of wild type (BY4741), or deletion mutant strains were spotted on rich medium plates with or without hygromycin B (40 μ g/mL) and incubated for 2 days at 30°C.

Results

A new set of proteins functionally linked to the SKI complex

To identify additional factors involved in the recognition and degradation of aberrant mRNAs or associated nascent peptides, we performed genetic screens using the Genetic Interaction Mapping strategy (16) with mutants of the SKI complex (*ski2* Δ , *ski3* Δ and *ski8* Δ) and with *ltn1* Δ . GIM genome-wide genetic screens enable the identification of functional interactions, whether synthetic lethal, synthetic slow growth or epistatic, by combining a query mutation with a collection of mutant strains; either complete gene deletions (17) or DamP mutants for essential genes (18). Among the twenty mutants having the strongest growth defect when combined with gene deletions of the SKI complex, we identified four gene deletions that were common in all the

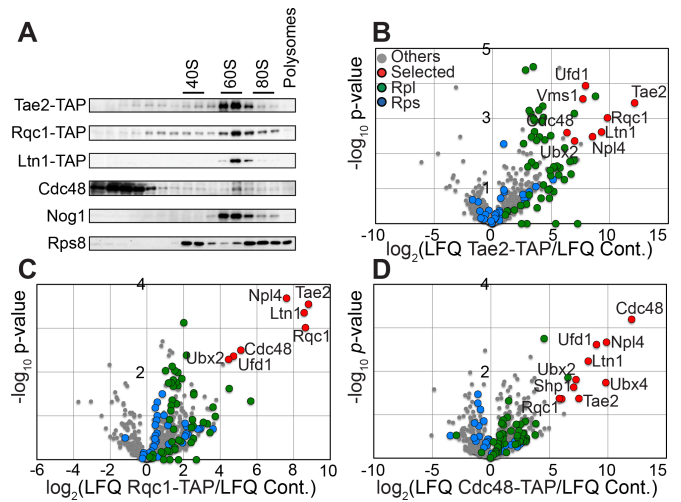


Fig. 2. Tae2, Rqc1, Ltn1 and Cdc48 are associated to 60S ribosomal particles.(A) Total cellular extracts were prepared from each strain expressing Tae2-TAP, or Rqc1-TAP or Ltn1-TAP and separated on a sucrose gradient (10/30%). Fractions of the gradient were analysed by Western blot using antibodies against TAP, Cdc48, Nog1 and Rps8. (B) Affinity purification of Tae2-TAP associated complex was subjected to LC-MS/MS identification and quantification. Tae2 associated complex was compared to BY4741 strain. Volcano plot shows the fold change (\log_2 LFQ cpx/LFQ ref.) on the x axis and the p-value distribution ($-\log_{10}$ p-value) on the y axis for the proteins identified in the TAP purification. Each circle indicates an identified protein (in red, for the most significant enriched candidates in the TAP purification; in green for RPLs; in blue for the RPSs). (C) TAP purification with Rqc1-TAP compared to BY4741 strain, As Fig. 2B. (D) TAP purification with Cdc48-TAP compared to BY4741 strain, As Fig. 2B.

screens: *ltn1* Δ , *vms1* Δ , *ypl009c* Δ (*tae2* Δ) and *ydr333c* Δ (*rqc1* Δ) (**Dataset S1**). These candidates were also selected in GIM screens performed with *ski7* Δ (**Fig. S1**). **Figure 1A** illustrates the overlap of genetic interactions between *ski2* Δ and *ski3* Δ . The genetic interactions between the SKI complex and the E3-Ubiquitin ligase Ltn1 confirmed the synthetic slow-growth phenotype reported earlier (13). Vms1 binds Cdc48 and is involved in diverse cellular functions, such as mitochondrial protein turnover (19), ER-associated protein degradation (ERAD) (20), or Cdc13 degradation (21). The deletion of the dubious ORF *YDR048C* was marked *vms1* Δ * because its deletion overlaps with *VMS1* and thus constitutes an independent mutation for this gene. *YPL009C* was named *TAE2* for Translation Associated Element 2, following the identification of this gene deletion in a screen as conferring sensitivity to cycloheximide, a translation inhibitor (22). *YDR333C* has been recently named *RQC1* for Ribosome Quality Control 1 (28). Remarkably, the GIM screen performed with *ltn1* Δ selected the four mutants of the SKI complex (*ski2* Δ , *ski3* Δ , *ski8* Δ and *ski7* Δ) (**Fig. 1B**).

In the course of this study, we further investigated the cellular role of the two factors Tae2 and Rqc1, which could explain the functional links with the SKI complex. First, we confirmed the functional interactions between *TAE2* and *RQC1* and the SKI complex by GIM screens with *tae2* Δ and *rqc1* Δ . Similarly to the *ltn1* Δ screen, the *ski2* Δ , *ski3* Δ , *ski7* Δ and *ski8* Δ mutants exhibited the strongest growth defects in association with *tae2* Δ or *rqc1* Δ , demonstrating the reciprocity and the specificity of the functional interactions between *TAE2*, *RQC1*, *LTN1* and the SKI complex (**Fig. 1C, 1D and Fig. S1**).

We next validated the functional links between the *SKI* genes and *TAE2* or *RQC1* by testing the growth of deletion mutant strains on rich medium plates, including *ltn1* Δ mutant strains as control. Surprisingly, the growth of single or double mutant strains was not significantly affected in these conditions (**Fig. 1E**).

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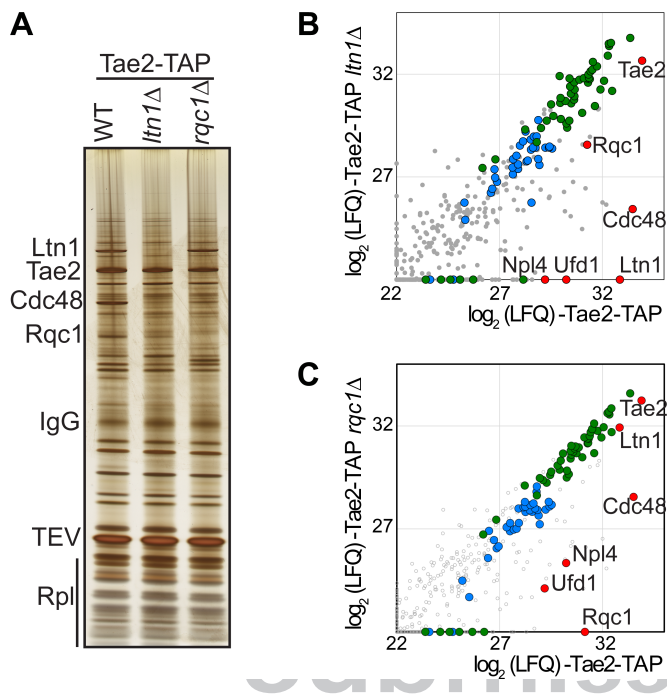


Fig. 3. Cdc48 association with Tae2 depends on Ltn1 and Rqc1. (A) Affinity purification of Tae2-TAP associated complex in WT or in the absence of Ltn1 or in the absence of Rqc1. The associated proteins were separated on a polyacrylamide gel and revealed by silver staining. (B) Affinity purification of Tae2-TAP associated complex in presence or absence of Ltn1. The graph shows the comparison between the intensities (LFQ) for each protein identified by LC-MS/MS (log₂ scale). (Colour legend as in Fig. 2). (C) Affinity purification of Tae2-TAP associated complex in presence or absence of Rqc1 as in Fig. 3B.

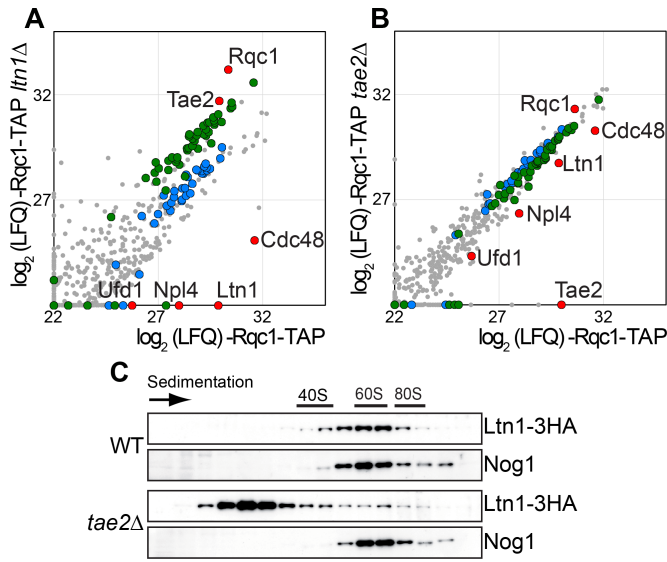


Fig. 4. Ltn1 association to 60S ribosomal particles is affected by Tae2. (A) Affinity purification of Rqc1-TAP associated complex in presence or absence of Ltn1. The graph shows the comparison of the intensities of each protein identified by LC-MS/MS (log₂ scale) as in Fig. 3B. (Legend for the dots as in Fig. 2A). (B) Affinity purification of Rqc1-TAP associated complex in presence or absence of Tae2 as in Fig. 3B. (C) Cellular extracts from the tagged strains Ltn1-3HA, and Ltn1-3HA *tae2Δ* were separated on a sucrose gradient (10/30%) as in Fig. 2. The tagged proteins were revealed by Western blot as in Fig. 2 using HA antibody.

Bengtson and Joazeiro also observed that the deletion of *LTN1*

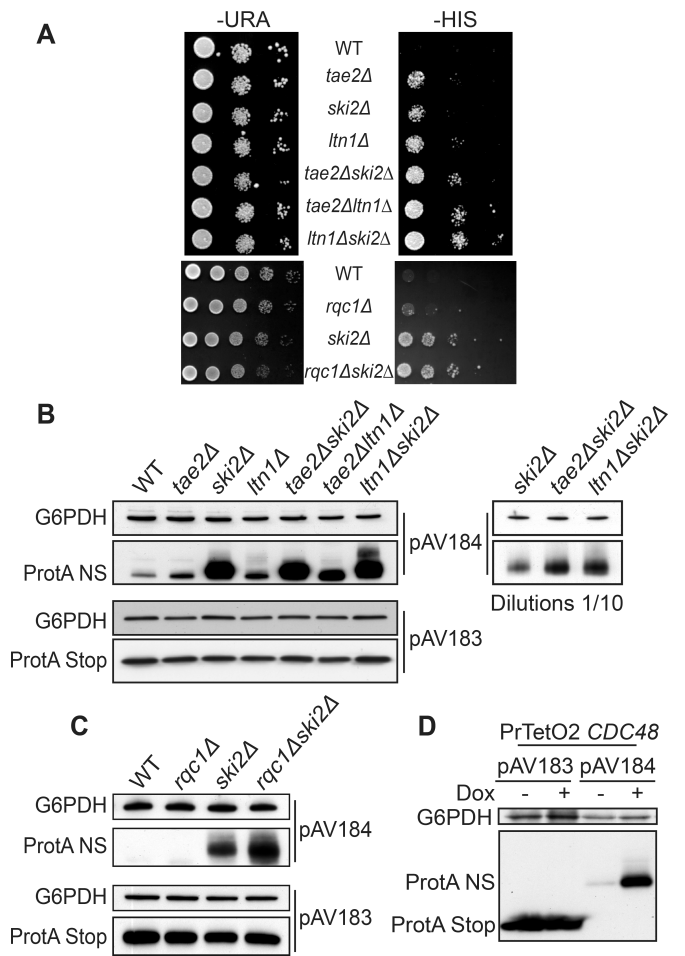


Fig. 5. The absence of Tae2, Rqc1, Ltn1 as well as Cdc48 leads to the accumulation of Non-Stop proteins. (A) The absence of Tae2 (upper panel) or Rqc1 (lower panel) enables the production of Non-Stop His3. Wild type (BY4741) or deletion mutant strains were transformed with the Non-Stop *HIS3*-containing plasmid (pAV188). Serial dilutions of the strains were spotted on SC-Ura and on SC-His and the plates were incubated for three days at 30°C. (B) The same strains as in (A) were transformed with the pAV184 plasmid containing Non-Stop protA gene under the control of *GAL10* promoter and with the pAV183 containing protA gene with a Stop codon as control. Non-diluted and 10 fold diluted total cellular extracts were separated on 10% SDS-PAGE polyacrylamide gel and the protein A was revealed by Western blot using PAP antibody. A loading control of the total protein amounts was done using anti-G6PDH antibody. (C) As Fig. 5B with the strains related to *rqc1Δ*, *ski2Δ* and *rqc1Δski2Δ*. (D) As Fig. 5B with the strain PrTetO2:*CDC48*. The depletion of Cdc48 was obtained by incubation of the strain for 12h in presence of Doxycyclin (10 μg/ml).

had no obvious phenotype on rich medium plates, but demonstrated the hypersensitivity of the *ltn1Δ* strain to hygromycin B, an antibiotic that decreases translation fidelity and causes stop codon readthrough (13). This observation prompted us to test the viability of the single and double mutant strains in presence of hygromycin B. The growth of each single mutant strain *ltn1Δ*, *ski2Δ* or *tae2Δ* was weakly affected but a stronger growth phenotype was observed for *rqc1Δ*. Interestingly, the double deletion strains were clearly more sensitive to hygromycin B when compared to the corresponding simple mutant strains. The *ski2Δ* mutant exhibited a synthetic slow growth defect in combination with *tae2Δ* and *ltn1Δ* and a synthetic lethal effect in combination with *rqc1Δ* (Fig. 1E). These growth defects correlated with those observed under competitive growth in the GIM screens, which are performed in the presence of G418 and nourseothricin (Fig. 1A-D and Fig. S1).

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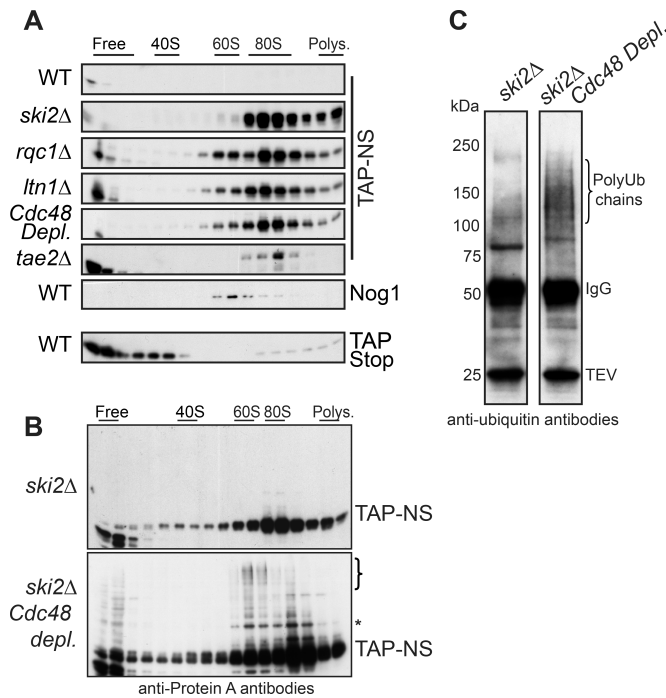


Fig. 6. Stabilized Non-Stop proteins accumulate on 80S and 60S particles. (A) Cellular extracts from wild type or deletion mutant strains bearing the TAP Non-Stop reporter were separated on sucrose gradients. The sedimentation profile of the TAP-stop protein expressed in wild type strain is shown as control. The fractions were analysed by western blot using antibodies against TAP. In addition, an antibody against Nog1 was used for the WT in order to mark the 60S position. (B) Polysome gradient of cellular extracts from *ski2Δ* single mutant and *ski2Δ Cdc48*-depleted strains that express TAP-Non-Stop construct, detected as in (A). (C) Immunoprecipitation of TAP-Non-Stop reporter in *ski2Δ* single mutant and *ski2Δ Cdc48*-depleted strain. The cellular extracts from the strains in B were purified on IgG beads, eluted with TEV protease and the associated proteins were analysed by Western blot using monoclonal anti-Ubiquitin antibodies (clone P4D1).

Tae2, Rqc1, Ltn1 and Cdc48 are bound to the same 60S ribosomal subunits

The overlap of genetic interaction patterns between *TAE2*, *RQC1* and *LTN1* suggested that these factors could be involved in similar biological processes. Since the deletion of any of these genes caused sensitivity to translation-affecting antibiotics such as hygromycin B and cycloheximide (23), and because it has been shown that Ltn1 binds 60S ribosomal particles and is involved in translational quality control (13), we suspected that Tae2 and Rqc1 could be involved in a translation-associated pathway. We therefore analysed the localization of Tae2 and Rqc1 in a polysome gradient to see if they also co-sedimented with the 60S ribosomal particles. We used strains expressing TAP-tagged versions of Tae2, Rqc1 and Ltn1 to monitor the sedimentation of each of these factors in a polysome gradient (Fig. 2A). We observed that Tae2, Rqc1 and Ltn1 were localized in the same fractions as Nog1, a pre60S-binding specific factor here used as a marker for 60S containing fractions, suggesting that all these factors are associated with 60S ribosomal subunits (24).

To see if Tae2, Rqc1 and Ltn1 were bound together to the same 60S particles, we performed Tandem Affinity Purification coupled with label-free mass spectrometry (LC-MS/MS) using strains expressing respectively the Tae2-TAP and Rqc1-TAP fusions as baits and a wild type strain (BY4741) as a negative control. Statistical analysis of label-free quantitative MS data (25) revealed significant enrichment of proteins of the large ribosomal subunit (RPL) in both Tae2-TAP and Rqc1-TAP purifications, confirming the binding of these two factors to 60S particles (Fig.

2B and 2C). In addition, we observed that Tae2, Rqc1 and Ltn1 were enriched specifically in both purifications, indicating that they are present together on the same 60S subunits. Moreover, these purifications revealed a strong enrichment of an additional component, Cdc48, and several of its known partners, Ufd1, Npl4, Vms1, Ubx2, Ubx4 and Shp1.

Affinity purifications using Cdc48-TAP as bait with the same wild type strain as a negative control showed a strong enrichment of known Cdc48 partners (Ufd1, Npl4, Ubx2 and Ubx4) and of Ltn1, Tae2 and Rqc1, which confirmed the presence of Cdc48 in complexes that also contain Tae2, Rqc1 and Ltn1 (Fig. 2D and Dataset S2). In parallel, we examined the localization of Cdc48 in a polysome gradient. We observed that, even if most of the Cdc48 hexamers were localized in lighter fractions of the gradient, a subset of Cdc48 sedimented in the 60S fractions (Fig. 2A). This subset is likely to correspond to Cdc48 physically interacting with 60S particles associated with Tae2, Rqc1 and Ltn1.

Ltn1 and Rqc1 are essential for the recruitment of Cdc48 to ribosomal subunits

Cdc48 (called p97 in mammals) is a highly conserved member of the AAA-ATPase family involved in a large number of cellular functions and forming homohexamers able to interact with diverse partners, that confer specificity toward various processes (14). One of the main functions of Cdc48 is to recognize polyubiquitinated molecules, extract them from a complex and make these substrates accessible to the proteasome for degradation (26). Therefore, the ubiquitination activity of Ltn1 could be an important step for the recruitment of the Cdc48-Ufd1-Npl4 complex to 60S particles associated with Tae2 and Rqc1.

We thus tested if the absence of Ltn1 affected the binding of Cdc48 to the Tae2 and Rqc1 associated complexes. We observed that the deletion of *LTN1* resulted in a decrease in the amount of Cdc48 and its partners Ufd1 and Npl4 from Tae2-TAP (Fig. 3A and 3B and Dataset S3) and Rqc1-TAP (Fig. 4A and Dataset S4) associated complexes. In addition, we examined the effect of the absence of Rqc1 in Tae2-TAP purifications and noticed a similar Cdc48 decrease (Fig. 3C and Dataset S3).

These data were strengthened by an independent experiment showing that the absence of Ltn1 or, to a somewhat lesser extent, Rqc1 efficiently reduced the amount of Cdc48 found in the 60S fractions of a polysome gradient (Fig. S2).

The amount of Cdc48 was also decreased to some extent from the Rqc1-TAP complex in the absence of Tae2 (Fig. 4B and Dataset S4), an observation also corroborated by the diminution of the amount of Cdc48 sedimenting with the 60S in a polysome gradient when Tae2 is absent (Fig. S2). This decrease was paralleled by a similar diminution of Ltn1 (Fig. 4B) suggesting that the decrease of Cdc48 might be indirect, resulting from the lower stability or recruitment of Ltn1 on the complex. In addition, we observed that the Ltn1-3HA fusion, which localizes with the 60S in a polysome gradient in a wild type, is shifted for a large part toward the lighter fractions in the absence of Tae2 (Fig. 4C). This confirms the importance of Tae2 for the stable association of Ltn1 with the complex.

In summary, the Cdc48-Ufd1-Npl4 complex requires both Ltn1 and Rqc1 to efficiently bind 60S particles, which suggests that ubiquitination by Ltn1 is essential for Cdc48 recruitment. Whether Rqc1 has a role in the ubiquitination process as well or if it has a more structural role for Cdc48 binding remains to be determined.

Tae2, Rqc1 and Cdc48 are involved, together with Ltn1, in Non-Stop protein decay

The new 60S associated complex described above contains Cdc48, Rqc1, Tae2 and the E3 ubiquitin Ligase Ltn1, which has been previously shown to ubiquitinate aberrant nascent peptides synthesized from *Non-Stop* mRNAs to ensure their proteasomal degradation (13). In parallel, components of the SKI complex are

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essential for the recruitment of the cytoplasmic exosome and thus aberrant mRNA clearance (2). Since Tae2 and Rqc1 are functionally linked with the SKI complex and physically associated with Cdc48 and Ltn1 on the 60S, we tested if Tae2, Rqc1 and Cdc48 were also involved in the degradation of aberrant *Non-Stop* mRNAs and the associated nascent peptides.

The *HIS3* Non-Stop reporter (pAV188) (2, 3) was used to test if the Non-Stop degradation pathway was still active in absence of Rqc1 or Tae2. In a wild type strain, *his3-Non-Stop* mRNAs are efficiently degraded; therefore cells from a parental *his3Δ100* strain do not grow on media without histidine (Fig. 5A). In contrast, mutations of the SKI complex hinder *Non-Stop* mRNA decay activity which stabilizes aberrant *Non-Stop* mRNAs, thus enabling the growth of cells transformed with pAV188 even in absence of histidine. Van Hoof and colleagues showed that a deletion of *Ltn1* also allowed pAV188 transformed cells to grow in absence of histidine, revealing that the stabilization of proteins synthesized from *Non-Stop* mRNAs can be sufficient to restore a functional level of His3 activity (27). We performed drop tests using deletion strains for either *TAE2* (Fig. 5A, upper panel) or *RQC1* (Fig. 5A, lower panel) and observed that both deletions allowed growth in absence of histidine with pAV188. In addition, combinations of *TAE2* or *RQC1* deletions with *ski2Δ* (and *ltn1Δ* in the case of Tae2) revealed a synergistic effect on growth rate on this medium. These results showed that the absence of Tae2 or Rqc1 caused an accumulation of functional His3-Non-Stop proteins, and that this accumulation was even stronger in combination with the absence of Ski2 or Ltn1.

To address whether Tae2 and Rqc1 are involved in *Non-Stop* mRNA clearance or specifically in the degradation of aberrant nascent peptides, we used the pAV184 reporter which carries a gene coding for Protein A from an mRNA that lacks a termination codon. The same gene but with a normal termination codon was used as a control (pAV183) (2). We performed Western blots and Northern blots with total extracts from mutant cells transformed with pAV184 (Non-Stop Protein A) or pAV183 (Stop Protein A). As previously shown, deletion of SKI complex components caused an accumulation of *Non-Stop* mRNAs and therefore restored expression of aberrant proteins (Fig. S3A & S3B). The *ltn1Δ* mutant exhibited an accumulation of aberrant proteins without affecting the levels of aberrant *Non-Stop* mRNAs (Fig. S3A) (13). We noticed that in the absence of Tae2, the aberrant Non-Stop Protein A was stabilized compared to the wild type (Fig. 5B upper panel) whereas Stop Protein A showed a similar abundance in all tested mutants (Fig. 5B lower panel). We showed that the absence of Tae2 did not affect the level of aberrant *Non-Stop* mRNA nor its stability (Fig. S3A and S3D). We did not see any clear effect of the *RQC1* deletion on Non-Stop Protein A, however, as for the *HIS3* Non-Stop reporter, combined deletions of *TAE2* or *RQC1* with the deletion of *SKI2* revealed a synergistic effect on aberrant proteins accumulation (Fig. 5B and 5C). We tested in parallel the effect of *CDC48* depletion, and also observed a strong accumulation of Non-Stop Protein A (Fig. 5D).

The absence of Tae2, Rqc1 or Cdc48 did not affect the steady-state levels of the *Non-Stop Protein A* mRNA (Fig. S3A, S3B and S3C), suggesting that like Ltn1, these three factors are specifically involved in the degradation of nascent peptides synthesized from *Non-Stop* mRNAs but have no role in Non-Stop mRNA decay.

Aberrant nascent peptides remain bound to the ribosome in absence of Tae2, Rqc1 or Cdc48

To understand how Tae2, Rqc1 and Cdc48 act on the recognition or the degradation of aberrant nascent peptides, we analyzed the sedimentation profile of the Non-Stop peptide in a polysome gradient in the absence of Tae2, Rqc1 or Cdc48 (in this experiment, we used a TAP-Non-Stop reporter derived from the Non-Stop Protein A reporter pAV184; see SI Methods). It has been previously shown that in mutants of the SKI complex or in the

absence of Ltn1, aberrant nascent peptides accumulated in the 80S fractions of a polysome gradient, suggesting that although Ltn1 sediments with the 60S fractions, the recognition process of aberrant proteins could start on the stalled ribosome and trigger the dissociation of the two ribosomal subunits (13). Whereas the control TAP-Stop protein was localized at the top of the gradient in the free fractions, the TAP-Non-Stop protein accumulated in the 80S fractions in the absence of Tae2, Rqc1 or Cdc48 as in the absence of Ski2 or Ltn1, (Fig. 6A). Interestingly, in the absence of Ltn1 and to a lesser extent in the absence of Rqc1 or Cdc48, we could observe a subset of aberrant proteins sedimenting with the 60S fractions in addition to the 80S fractions. This is consistent with the hypothesis that aberrant nascent peptides could remain blocked in the 60S exit tunnel even after dissociation of the stalled 80S. This subset of TAP-Non-Stop proteins localized in the 60S fractions could thus represent intermediates of degradation between ribosome dissociation and ubiquitination of the aberrant nascent peptide showed by Bengtson M.H. and Joazeiro C. (13). Yet, whether the ubiquitination of aberrant nascent peptides starts before or after ribosome dissociation remains unclear.

Cdc48 is involved in the proteasomal targeting of polyubiquitinated substrates stalled on 60S subunits

No high molecular species of the TAP-Non-Stop protein, which could correspond to ubiquitinated forms, were readily apparent in these single-mutant conditions. Given its documented function described in other pathways (14, 15), the Cdc48-Ufd1-Npl4 complex could be expected to play a role in the recognition and proteasomal targeting of polyubiquitinated nascent peptides. We therefore examined the polysome sedimentation profile of TAP-Non-Stop proteins in absence of Ski2 and Cdc48, in order to stabilize *TAP-Non-Stop* mRNAs and the polyubiquitinated forms of the TAP-Non-Stop proteins, respectively. Besides its strong accumulation in both the 80S and the 60S fractions (Fig. 6B), we observed that upon depletion of Cdc48, a subset of TAP-Non-Stop proteins migrated as high molecular weight proteins, forming a ladder of increasing size from the 80S fractions to the 60S fractions. Affinity purifications of the TAP-Non-Stop reporter and its analysis by Western blot using anti-ubiquitin antibodies revealed the presence of a smear of polyubiquitinated species upon Cdc48 depletion (Fig. 6C), suggesting that the high molecular ladder formed by the TAP-Non-Stop protein in the 60S fractions indeed corresponds to polyubiquitinated forms of this peptide.

Taken together, these results show that *i*) ubiquitination of the aberrant nascent peptide might start at the 80S level, since some TAP-Non-Stop protein shifted species of intermediate size are already visible in the 80S fractions, *ii*) high molecular polyubiquitinated forms of the aberrant peptide accumulate on 60S ribosomal particles in absence of Cdc48 and *iii*) Cdc48 is essential for proteasomal targeting of these polyubiquitinated aberrant substrates, since a depletion of Cdc48 strongly stabilized polyubiquitinated forms of the TAP-Non-Stop protein.

Discussion

Performing GIM genetic screens we identified two new factors, Tae2 and Rqc1, which similarly to Ltn1, become essential during translation stress when the SKI complex is absent (Fig. 1). Remarkably, biochemical purifications revealed that these three factors, together with Cdc48, are part of a common complex in association with the 60S ribosomal subunit (Fig. 2). We found that these factors are all required for the efficient degradation of aberrant nascent peptides synthesized from mRNA lacking a stop codon (NSPD) (Fig. 5).

During the completion of this manuscript, another study was published (28) showing the existence of the same Rqc1-Tae2-Ltn1-Cdc48 complex in association with 60S particles, coined the RQC complex for Ribosome Quality Control. This work

681 characterized the RQC complex as a trigger for degradation of
682 stalled polypeptides and as a signal for translational stress, which
683 is fully consistent with our findings.

684
685 The presence of a stalled ribosome that does not have a stop
686 codon in its A site was proposed to be the signal that triggers the
687 degradation of *Non-Stop* or *NoGo* mRNAs. Dom34/Hbs1 would
688 then be recruited instead of the canonical termination factor
689 eRF1/eRF3, peptidyl-tRNA hydrolysis would not be activated
690 and the nascent peptide, in the form of a peptidyl-tRNA, would
691 remain within the exit tunnel and the P site when Rli1 drives
692 the dissociation of the stalled ribosome. Yet, the absence of Ltn1
693 was reported to result in the stabilization of the aberrant peptide
694 on the 80S (13) suggesting that the ubiquitination process starts
695 before ribosome dissociation. It has been also shown that Ltn1 is
696 localized on the 60S, an observation strengthened by our obser-
697 vation that Ltn1, Tae2, Rqc1 and Cdc48, are present on the same
698 60S particles, supporting the existence of a hypothetical transient
699 60S-peptidyl-tRNA intermediate. Indeed, we could detect a Non-
700 Stop peptide bound to the 60S particle (Fig. 6A) and show that
701 polyubiquitinated forms of this peptide accumulate on the 60S in
702 the absence of Cdc48 (Fig. 6B and 6C). This observation, together
703 with the fact that Cdc48 does not associate with the RQC particle
704 in the absence of Ltn1 (Fig. 3B and 4A), strongly suggests that the
705 Cdc48-Ufd1-Npl4 complex recognizes polyubiquitinated forms of
706 the aberrant peptide at a late stage of the process. Considering the
707 general role of Cdc48, it might extract the polyubiquitinated pep-
708 tides from the dissociated 60S and escorts them to the proteasome
709 for degradation.

710
711 In addition, we observed that Cdc48 binding to the complex
712 is also impeded by the absence of Rqc1 (Fig. 3C), whereas Ltn1
713 binding is not affected. Whether this reflects the requirement
714 of Rqc1 for peptide ubiquitination or a more structural role
715 of this factor for Cdc48 binding remains to be determined. In
716 addition, the absence of Tae2 weakly affected Cdc48 binding to
717 the complex; this could be an indirect effect due to the decreased
718 binding efficiency of Ltn1 in this context (Fig. 4B and 4C).

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749 Finally, Cdc48 was implicated in the degradation of non-
750 functional 60S subunits (25S NRD) by promoting the dissociation
751 of stalled 80S, thus allowing the recruitment of the proteasome to
752 stalled 60S to perform ribosomal proteins proteolysis (29). These
753 results are consistent with the hypothesis that after dissociation
754 of stalled ribosomes, the 60S particles are not recycled to perform
755 new rounds or translation but targeted for proteasomal degrada-
756 tion, together with the aberrant nascent peptide.

757 **Material and methods**

758 **Yeast strains, plasmids and GIM screens**

759 The yeast strains used in this study are listed on Table. S1.
760 They were constructed by high-fidelity PCR transformation. Ser-
761 ial dilution were spotted on rich medium plates with or without
762 hygromycin B (40 µg/mL) and incubated for 2 days at 30°C.
763 Details of plasmid constructions and GIM screens (16) can be
764 found in SI Methods.

765 **Polysome gradients and Western blotting**

766 Polysome gradients analyses were conducted as described in
767 SI Methods. Collected fractions were precipitated and separated
768 on 10% acrylamide gel. The proteins were detected by hybridiza-
769 tion with the appropriate antibodies (Table. S3).

770 **Tandem Affinity Purifications mass spectrometry and data anal- 771 ysis**

772 Biochemical purifications were performed using strains express-
773 ing TAP fusion proteins. A first step purification was done
774 with magnetic beads coupled with IgG (SI Methods). An aliquote
775 of the TEV eluate was separated on acrylamide gels and silver
776 stained. The rest of the eluates was analyzed by mass spectrom-
777 etry. Briefly, after digestion, peptides were identified on a LTQ-
778 Orbitrap and Raw MS data were analyzed using the MaxQuant
779 software as described in SI Methods

780 **Acknowledgments.**

781 We thank A. Van Hoof, A. Buchberger and G. Dieci for providing
782 plasmids and antibodies against Cdc48 and Rps8 respectively. We are grateful
783 to the proteomics platform of the Pasteur Institute for the availability of the
784 Orbitrap. Q.D. and Y.Y. were supported by a fellowship from the Ministère
785 de l'enseignement supérieur et de la Recherche. This work was supported by
786 the grants from the Agence Nationale de la Recherche ANR-2011-BSV6-011-
787 02 and ANR-08-JCJC-0019-01.

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