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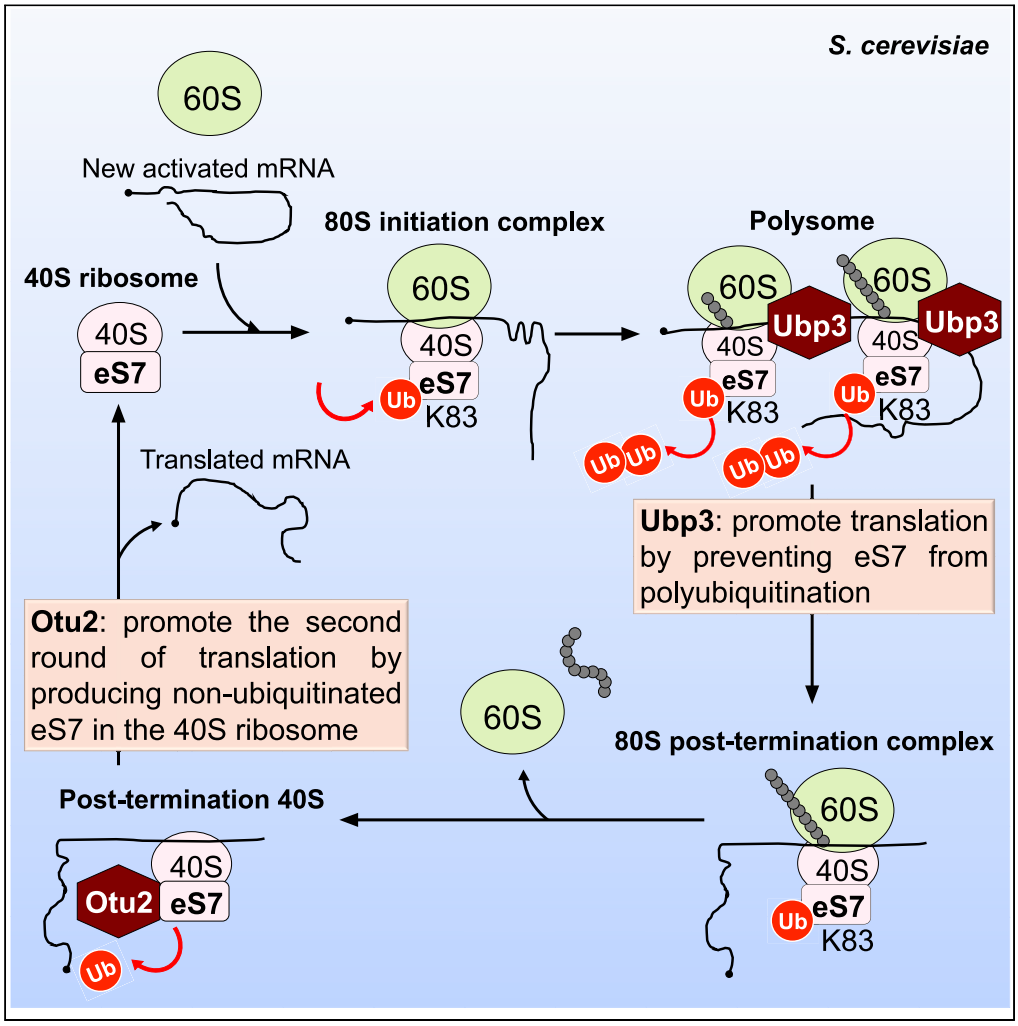

Yuka Takehara, Hideki Yashiroda, Yoshitaka Matsuo, ..., Hidetaka Kosako, Toshifumi Inada, Shigeo Murata

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HIGHLIGHTS

eS7 ubiquitination at lysine 83 is required for efficient protein translation

Ubp3 prevents eS7 from polyubiquitination in 80S ribosomes and polysomes

Otu2 deubiquitinates mono-ubiquitinated eS7 in the 40S ribosome

Otu2 facilitates dissociation of mRNAs from 40S ribosomes in ribosome recycling

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The ubiquitination-deubiquitination cycle on the ribosomal protein eS7A is crucial for efficient translation

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SUMMARY

Ubiquitination is a major post-translational modification of ribosomal proteins. The role of ubiquitination in the regulation of ribosome functions is still being elucidated. However, the importance of ribosome deubiquitination remains unclear. Here, we show that the cycle of ubiquitination and deubiquitination of the 40S ribosome subunit eS7 is important for efficient translation. eS7 ubiquitination at lysine 83 is required for efficient protein translation. We identified Otu2 and Ubp3 as the deubiquitinating enzymes for eS7. An *otu2Δubp3Δ* mutation caused a defect in protein synthesis. Ubp3 inhibited polyubiquitination of eS7 in polysomes to keep eS7 in a mono-ubiquitinated form, whereas Otu2 was specifically bound to the free 40S ribosome and promoted the dissociation of mRNAs from 40S ribosomes in the recycling step. Our results provide clues for understanding the molecular mechanism of the translation system via a ubiquitination-deubiquitination cycle.

INTRODUCTION

The ribosome is a crucial component in the process of translation in all living cells. The eukaryotic 80S ribosome comprises two subunits, the large 60S ribosomal subunit and the small 40S ribosomal subunit. These two subunits cyclically assemble and disassemble in the translation process. The translation process comprises four phases: initiation, elongation, termination, and recycling (Dever et al., 2016; Jackson et al., 2010). In the initiation phase, initiation factors (eIF1, eIF1A, eIF2, eIF3, and eIF5) and Met-tRNA$_i^{Met}$ are recruited to the 40S ribosome to form a 43S preinitiation complex (43S PIC). Next, the 43S PIC attaches to the capped 5′-end of mRNA with the cap-binding complex eIF4F, forming a 48S preinitiation complex (48S PIC) (Eliseev et al., 2018). Then, the joining of the 60S ribosome to the 48S PIC is mediated by eIF5B to produce an 80S initiation complex that is competent for protein synthesis (Hinnebusch, 2014). During the elongation phase, translating 80S ribosomes form polysomes on a single molecule of mRNA. In the termination phase, eukaryotic release factor 1 (eRF1) recognizes the stop codon and releases the synthesized polypeptide chain (Hellen, 2018). In the recycling phase, the 80S post-termination complex (80S PTC) is dissociated into a free 40S and 60S ribosomes, deacylated tRNA, and mRNA to initiate new rounds of translation by a two-step process of recycling. In the first step of recycling, the ATPase Rli1 (yeast)/ABCE1 (mammals) forces a split of the 80S PTC into a free 60S ribosome and a tRNA/mRNA-bound 40S ribosome (Becker et al., 2012; Young and Guydosh, 2019; Young et al., 2015). In the second step of recycling, tRNA and mRNA are released from the 40S ribosome to produce a free 40S ribosome.

Recent studies suggest that ubiquitination and deubiquitination of the ribosome play crucial roles in controlling translation. For example, ubiquitination of uS10 is required for ribosome-associated quality control (RQC), leading to the dissociation of 80S ribosomes into 40S and 60S ribosomes (Ikeuchi et al., 2019; Matsuo et al., 2017). Sequential ubiquitination of uS3 is required for 18S nonfunctional ribosomal RNA decay (Sugiyama et al., 2019). Ubiquitinated 40S ribosomal proteins are deubiquitinated to escape from lysosomal degradation (Meyer et al., 2020). Deubiquitination of uL25 is required for the 60S ribosome ribophagy upon nutrient starvation (Ossareh-Nazari et al., 2014). Even during constitutive translation, many ribosomal proteins are reportedly ubiquitinated and most likely deubiquitinated (Mayor et al., 2005, 2007; Peng et al., 2003; Tagwerker et al., 2006). However, the biological significance and mechanisms of ubiquitination and especially deubiquitination during constitutive translation remain largely unknown.

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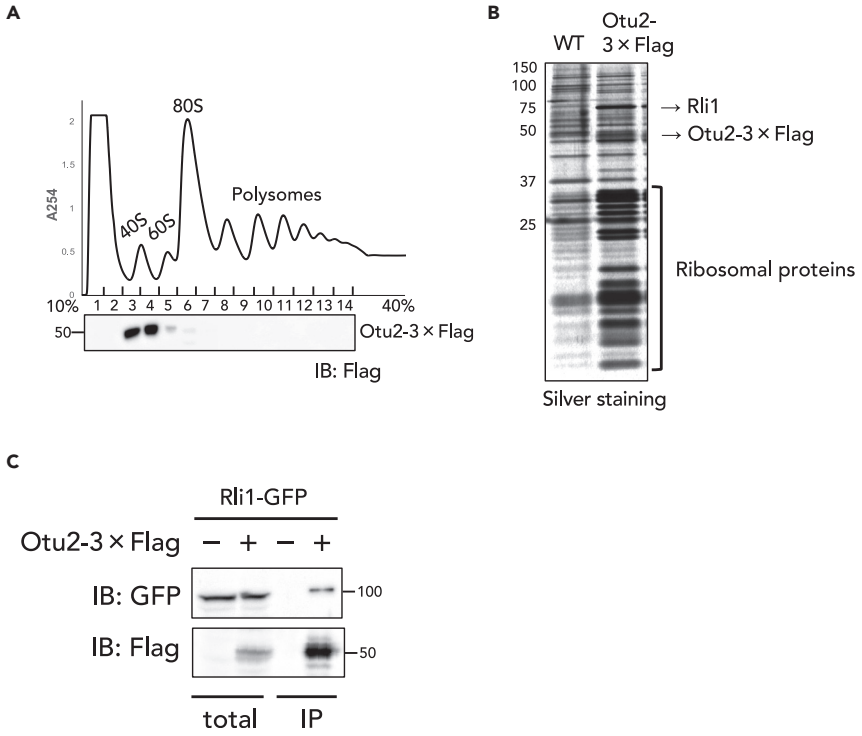


Figure 1. Otu2 is associated with 40S ribosomes

(A) Total cell extract from the cells endogenously expressing C-terminally 3×Flag-tagged Otu2 (Otu2-3×Flag) was fractionated using 10%–40% sucrose density gradient (SDG) centrifugation. Absorbance at 254 nm of each fraction was measured to quantify the contents of ribosomes. The distribution patterns of Otu2-3×Flag were investigated by immunoblotting with anti-Flag antibodies.

(B) Proteins immunoprecipitated with anti-Flag M2 beads were subjected to SDS-PAGE followed by silver staining.

(C) Western blot analysis of immunoprecipitates with Otu2-3×Flag by using anti-GFP antibodies (top panel) and anti-Flag antibodies (lower panel).

Ubiquitination is carried out by three types of enzymes: El ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin ligases (Amerik and Hochstrasser, 2004; Haq and Ramakrishna, 2017). Of these enzymes, E3 ligases are responsible for substrate recognition and mediate transfer of ubiquitin onto the lysine residues of targeted proteins. Ubiquitination can be reversed by deubiquitinating enzymes (DUBs). DUBs cleave off ubiquitin from ubiquitin-conjugated proteins. Budding yeast DUBs are classified into four superfamilies based on the architecture of their catalytic domains. Three of them, ubiquitin-specific protease (USP) family, ubiquitin C-terminal hydrolase (UCH) family, and ovarian tumor protease (OTU) family, belong to the cysteine protease family. The remaining Jab1/MPN/Mov34 (JAMM) family is a metalloprotease family. Although the functions of many E3 ligases are known, DUB functions remain elusive.

Otu2 is a potential DUB of the OTU family that was identified as a 40S ribosome-associated protein by a global mass spectrometry (Fleischer et al., 2006). However, the role of Otu2 in ribosome function remains unknown. In this study, we identified Otu2 and Ubp3 as deubiquitinating enzymes for eS7 and propose that the cycle of ubiquitination and deubiquitination of eS7 is important for efficient protein synthesis.

RESULTS

Otu2 is associated with 40S ribosomes

Otu2 was previously identified as a 40S ribosome-associated protein. To confirm the association of Otu2 with 40S ribosomes, we prepared cells endogenously expressing C-terminally 3×Flag-tagged Otu2 (Otu2-3×Flag). The cell extract was fractionated using sucrose density gradient (SDG) centrifugation. Immunoblot analysis using anti-Flag antibodies showed that Otu2 was distributed specifically in the 40S ribosome fractions, consistent with the previous report (Figure 1A).

We next analyzed Otu2-3×Flag immunoprecipitates using mass spectrometry. Multiple 40S ribosomal proteins were identified as Otu2-binding proteins as expected. Of note, we identified Rli1, a ribosome-associated factor, which was the most prominent binding protein other than 40S ribosomal proteins (Figure 1B). Rli1 recycles terminating ribosomes and controls translation initiation. The interaction of Rli1 with Otu2 was further confirmed by immunoblot analysis using strains endogenously expressing GFP-tagged Rli1 and Otu2-3×Flag (Figure 1C). These results suggest that Otu2 is associated with 40S ribosomes at least, in the recycling and initiation steps of the translation cycle, in which Rli1 plays a role.

Otu2 and Ubp3 have overlapping functions in efficient protein synthesis

Single deletion of Otu2 did not cause apparent growth defect under normal culture conditions (Figure 2A). Deubiquitinating enzymes often have overlapping functions, which mask the effect of each single deletion. Thus, we crossed the *otu2*Δ strain with all other 18 single DUB deletion strains and found that *otu2*Δ caused a growth defect when combined with *ubp3*Δ (Figure 2A), indicating that Otu2 has overlapping function with Ubp3.

The deubiquitinating activity of the Ubp3 has been shown previously (Baker et al., 1992), but whether Otu2 is a bona fide DUB has remained unexplored. Exploiting the *otu2*Δ*ubp3*Δ growth defect, we examined whether the conserved cysteine 178 in the OTU domain of Otu2 was essential for the Otu2 function. Whereas wild-type (WT) Otu2 rescued the *otu2*Δ*ubp3*Δ growth defect, expression of catalytically inactive Otu2 mutant (Otu2-C178S) did not (Figure S1A). Furthermore, overexpression of Otu2-C178S in the *ubp3*Δ strain caused growth defect (Figure S1B). These results suggest that Otu2 acts as a genuine deubiquitinating enzyme and that Otu2 and Ubp3 have a common substrate for deubiquitination. We also noticed that overexpression of Otu2-C178S had a deleterious effect even in *otu2*Δ*ubp3*Δ cells, which indicates that there are overlapping deubiquitinating enzymes other than Ubp3 (Figure S1C).

Ubp3 is also thought to interact with the ribosome, especially with 60S ribosomes and polysomes (Fleischer et al., 2006). We speculated that Otu2 and Ubp3 have overlapping functions related to the ribosome and compared the ribosome profiles of WT, *otu2*Δ, *ubp3*Δ, and *otu2*Δ*ubp3*Δ cells by SDG analysis. Although both ribosome patterns of single *otu2*Δ and *ubp3*Δ mutant cells were similar to that of WT cells, *otu2Δubp3Δ* cells decreased the amount of polysomes, suggesting that protein synthesis is defective in *otu2Δubp3Δ* cells (Figure 2B).

To confirm that Otu2 and Ubp3 are involved in the synthesis of nascent proteins, the incorporation of L-homopropargylglycine, an amino acid analog of methionine containing an alkyne moiety, was monitored (Mittal et al., 2017). Compared with the strain complemented with both the *OTU2* and *UBP3* genes, the efficiency of protein synthesis was decreased by approximately 50% by the lack of *UBP3*, although no apparent decrease was observed by the lack of *OTU2* alone (Figure 2C). The defect in protein synthesis was prominent in *otu2Δubp3Δ* cells, in which a 90% decrease in the efficiency was observed (Figure 2C). These results indicate that cooperative deubiquitination by Otu2 and Ubp3 is required for efficient protein synthesis.

Otu2 and Ubp3 deubiquitinate eS7A in the distinct ribosome complexes

To identify potential substrates for Otu2, the plasmid encoding Flag-6×His-tagged ubiquitin was expressed in WT, *otu2*Δ, *ubp3*Δ, and *otu2*Δ*ubp3*Δ strains, and the cell extracts were fractionated by SDG centrifugation. Immunoblotting analysis using anti-Flag antibodies revealed that the ubiquitinated proteins of ~34 kDa that were observed in the 80S and polysome fractions in the WT strain emerged in the 40S ribosome fractions of the *otu2*Δ strain (Figure 3A). Further accumulation of these proteins in the 40S fractions was observed in the *otu2*Δ*ubp3*Δ strain. These results suggest that the ~34 kDa ubiquitinated proteins are ribosome-associated proteins that may be deubiquitinated by Otu2 and Ubp3.

We then purified ubiquitinated proteins from 40S ribosome fractions of each strain. The ~34 kDa ubiquitinated proteins were purified most efficiently from the 40S fractions of *otu2*Δ*ubp3*Δ cells and not from those of WT and *ubp3*Δ cells (Figure 3B). Subsequent mass spectrometry analysis revealed that eS7A and eS7B were included in the corresponding bands (Figure S2A). eS7A and eS7B are 87% identical in amino acid sequence and are both 40S ribosomal proteins. To examine whether Otu2 and Ubp3 were involved in deubiquitination of eS7, hemagglutinin (HA)-tagged eS7A was expressed in the *eS7AΔ* and the *otu2Δubp3ΔeS7AΔ* strains. eS7A was detected as a major 28 kDa band, the predicted molecular weight for eS7A, and a

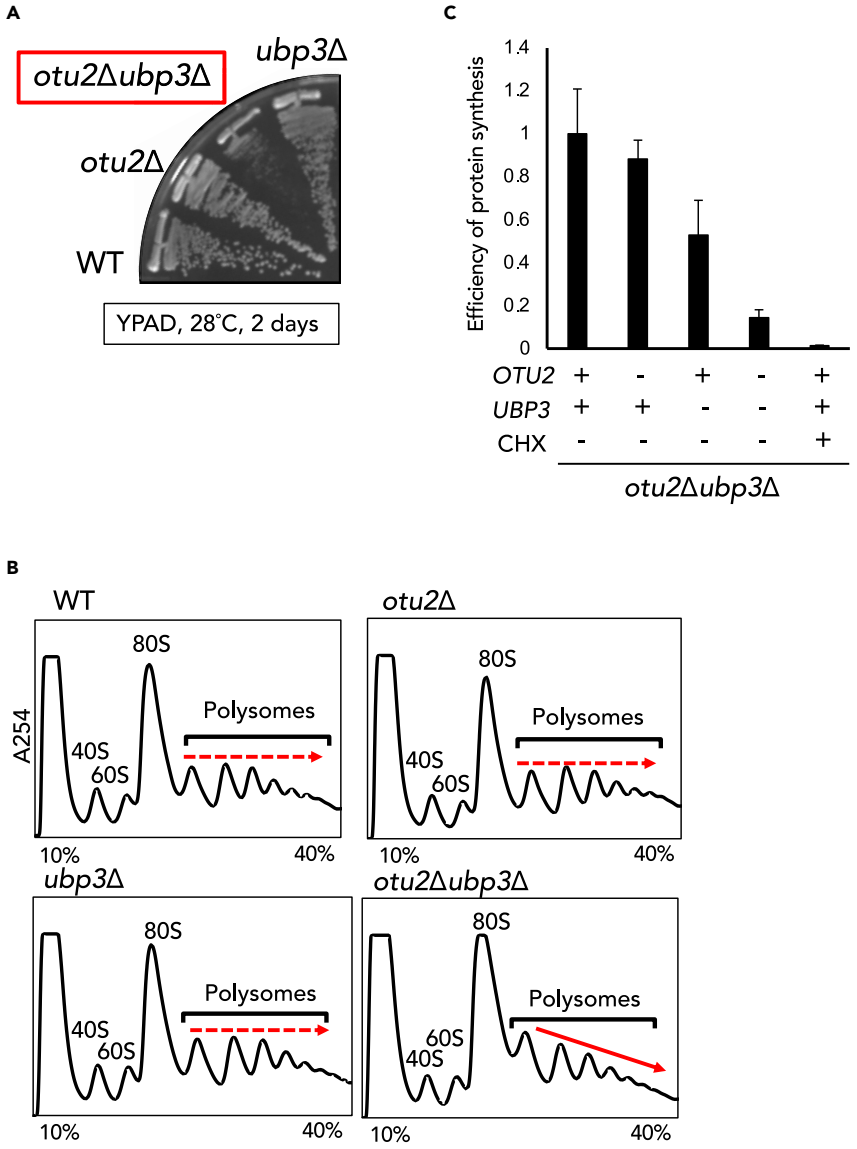


Figure 2. Otu2 and Ubp3 have overlapping functions in efficient protein synthesis

(A) Each strain was streaked on a YPAD plate and incubated for 2 days at 28°C. See also Figure S1.

(B) Total cell extracts were fractionated using 10%–40% SDG centrifugation. Absorbance at 254 nm of each fraction was measured to quantify the contents of ribosomes.

(C) Efficiency of the protein synthesis in each strain was monitored by measuring the ability to incorporate L-homopropargylglycine (HPG) into nascent proteins (n = 3, mean ± SD). HPG is an amino acid analog of methionine containing an alkyne moiety. Cycloheximide (CHX) was used as a translation inhibitor.

minor 34 kDa band by anti-HA antibodies in *eS7AΔ* cells (Figure 3C). On the other hand, the 34 kDa band became more apparent in *otu2Δubp3ΔeS7AΔ* cells. This 34 kDa band was also detected using anti-ubiquitin antibodies in *otu2Δubp3ΔeS7AΔ* cells (Figure 3C). The 34 kDa band also accumulated through the overexpression of Otu2-C178S in the *ubp3Δ* strain (Figure S2B). These results indicate that Otu2 and Ubp3 are involved in deubiquitination of eS7.

To determine the ubiquitination sites of eS7A and eS7B, we analyzed the 34 kDa ubiquitinated proteins in the *ubp3Δ* strain expressing Otu2-C178S by mass spectrometry. We identified lysines 56 and 83 as potential ubiquitination sites of eS7A and eS7B (Figures S2B–S2D). We then substituted lysines 56 and 83 with arginines in eS7A and expressed the resultant eS7A-K56R and -K83R in the *eS7AΔ* and the

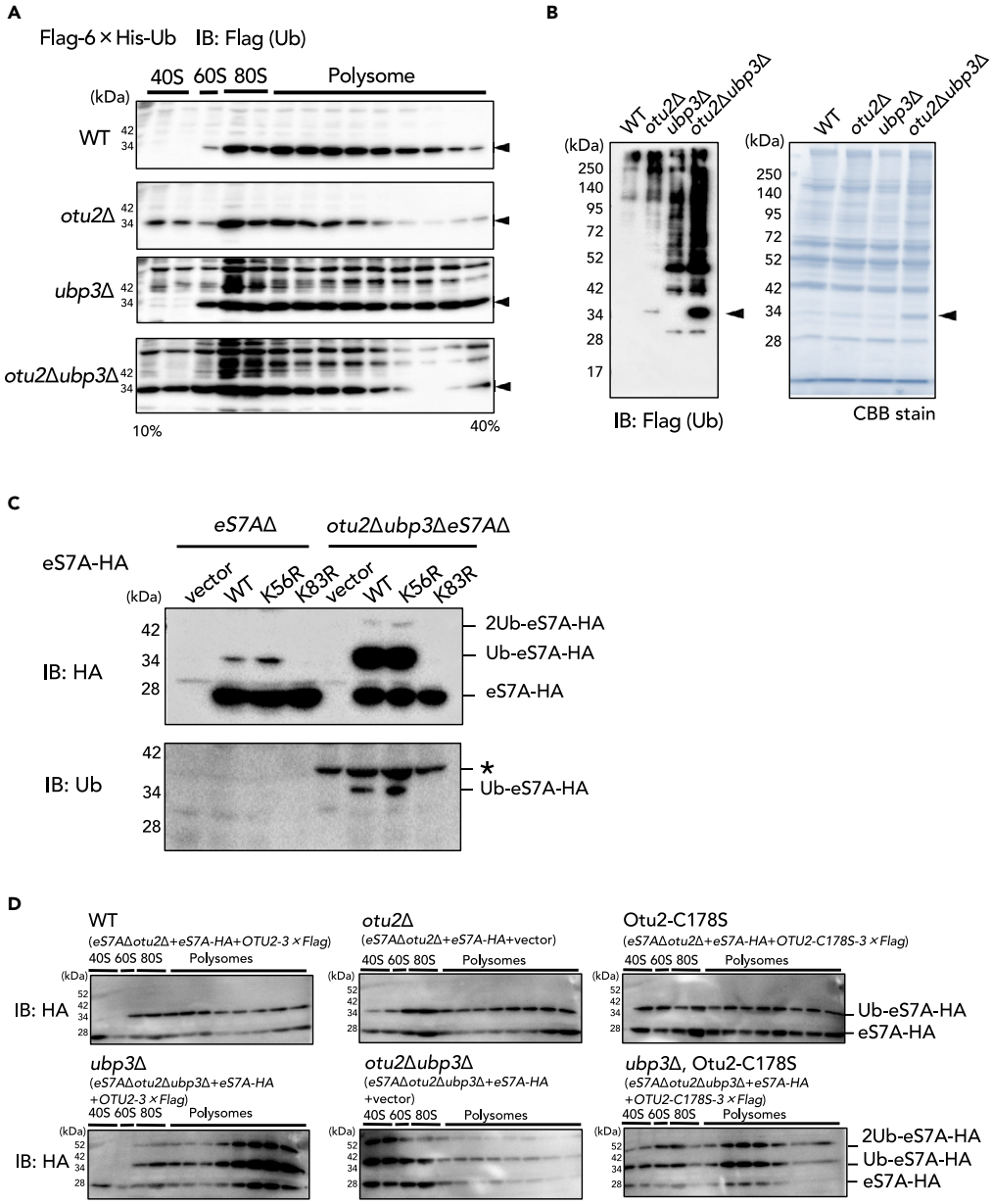


Figure 3. Otu2 and Ubp3 deubiquitinate eS7A in the distinct ribosome complexes

(A) Distribution patterns of ubiquitinated proteins. Total cell extracts from Flag-6×His-Ub-expressing cells were fractionated using 10%–40% SDG centrifugation. Each fraction was subjected to immunoblot analysis with anti-Flag antibodies.

(B) Ubiquitinated proteins purified with Ni-NTA beads from 40S ribosome fractions were subject to SDS-PAGE followed by the immunoblot analysis using anti-Flag antibodies (left panel) and Coomassie Brilliant Blue (CBB) staining (right panel). See also Figure S2.

(C) Immunoblot analysis of eS7A-HA with anti-HA (upper panel) and anti-ubiquitin (lower panel) antibodies. The asterisk indicates another potential substrate of Otu2 and Ubp3.

(D) Distribution patterns of eS7A-HA. Total cell extracts were fractionated using 10%–40% SDG centrifugation. Each fraction was subjected to immunoblot analysis with anti-HA antibodies.

otu2Δubp3ΔeS7AΔ strains. Mono- and possibly di-ubiquitinated forms of eS7A were eliminated by the K83R mutation in both strains, whereas the K56R mutation did not affect the ubiquitination status of eS7A (Figure 3C). These results indicate that K83 is the major ubiquitination site of eS7A and that Otu2 and Ubp3 deubiquitinate K83-ubiquitinated eS7.

To verify that the 34 kDa ubiquitinated proteins that accumulate in the 40S fractions in cells lacking Otu2 were ubiquitinated eS7, cell lysates fractionated by SDG centrifugation were blotted for eS7A-HA. In WT cells, mono-ubiquitinated eS7A was observed in the 80S and the polysome fractions, as reported previously (Figure 3D). In *otu2*Δ and Otu2-C178S-expressing cells, mono-ubiquitinated eS7A emerged in the 40S ribosome fraction in addition to the 80S and the polysome fractions. Single deletion of Ubp3 caused accumulation of di-ubiquitinated eS7A in the 80S and the polysome fractions (Figure 3D). These results suggest that Otu2 and Ubp3 deubiquitinate eS7 in the distinct ribosome complexes; Otu2 deubiquitinates mono-ubiquitinated eS7 in 40S ribosomes to the non-ubiquitinated form, whereas Ubp3 deubiquitinates eS7 in 80S ribosomes and polysomes to the mono-ubiquitinated form.

Meanwhile, simultaneous loss of Otu2 and Ubp3 activities resulted in the accumulation of di-ubiquitinated eS7A in the 40S ribosome fraction. This is consistent with the idea that these entities are dissociated products of 80S ribosomes in the absence of Ubp3 and that Otu2 can deubiquitinate di-ubiquitinated eS7 in free 40S ribosomes. However, we cannot exclude the possibility that eS7 is ubiquitinated in nascent 40S ribosomes, and ubiquitinated eS7 in nascent 40S ribosomes was also accumulated in *otu2*Δ cells.

Deubiquitination of eS7 by Otu2 is required for efficient translation in vitro

To examine whether Otu2 deubiquitinates eS7A directly in the 40S ribosome, recombinant His-Otu2 was added to mono- and di-ubiquitinated eS7A-3HA prepared from 40S ribosome fractions of *otu2Δubp3Δ eS7A-3HA* cells. When His-Otu2 was added at 6.25 nM or above, both mono- and di-ubiquitinated eS7A proteins disappeared. On the other hand, the addition of His-Otu2-C178S had no effect on ubiquitinated eS7A. GST-Ubp3 and its inactive form GST-Ubp3-C469A also did not change the ubiquitination status of eS7A in the 40S ribosomes (Figure 4A). These results suggest that Otu2, but not Ubp3, directly deubiquitinates eS7 in the 40S ribosome.

As shown in Figure 2C, protein synthesis was severely impaired in *otu2Δubp3Δ* cells. We then examined whether the defect in protein synthesis in *otu2Δubp3Δ* cells was relieved by recombinant His-Otu2. We added His-Otu2 and *in vitro* transcribed reporter mRNAs encoding *Renilla* luciferase to cell extracts prepared from the *otu2Δubp3ΔeS7A-3HA* strain and measured *in vitro* translation activity. Luciferase expression was clearly increased by the addition of His-Otu2 at 62.5 nM but not by the same concentration of His-Otu2-C178S (Figure 4B). We also confirmed that the increase in luciferase expression was accompanied with deubiquitination of eS7A (Figure 4C). These results suggest that deubiquitination of eS7 by Otu2 is directly required for efficient protein translation.

However, the precise mechanism by which recombinant Otu2 upregulates the synthesis of *Renilla* luciferase (Figure 4B) remains unknown. We speculate that Out2 prevents abortive translation by transiently associating with 80S ribosomes. To confirm this, we expressed β-galactosidase and GFP double-tagged with Flag at the N-terminus and V5 at the C-terminus to detect possible truncated translation products caused by abortive translation in WT and Δ*otu2* cells. However, immunoblot analysis results reveal no difference in band patterns after using anti-Flag and anti-V5 antibodies, even when cells were treated with the proteasome inhibitor bortezomib, which inhibits the degradation of unstable truncated translation products (Figure S3). These results suggest that the deletion of Otu2 does not cause abortive translation.

Ubiquitination of lysine 83 in eS7A is important for translation

Although we had revealed the importance of eS7 deubiquitination in 40S ribosomes for protein translation, the significance of eS7 ubiquitination that constantly existed in the translating 80S ribosomes and polysomes remained unknown. To investigate the biological importance of eS7 ubiquitination, the efficiency of protein synthesis was measured in the WT, *eS7AΔ*, and *eS7A-K83R* strains. The K83R mutation decreased the synthesis of nascent proteins to the same degree as the *eS7AΔ* mutation (Figure 5A). We further confirmed that the K83R mutation blocked the ubiquitination of eS7A (Figure 5B). This result indicates that ubiquitination of lysine 83 in eS7A in 80S ribosomes and polysomes during the translation process is important for translation.

Otu2 binds to recycled 40S ribosomes and the 43S preinitiation complex but not to the 48S preinitiation complex

After dissociation of 80S ribosomes into 40S and 60S at the translation termination, 40S ribosomes exist from the recycling to translation initiation steps in the translation cycle (Figures 6A). It is possible that

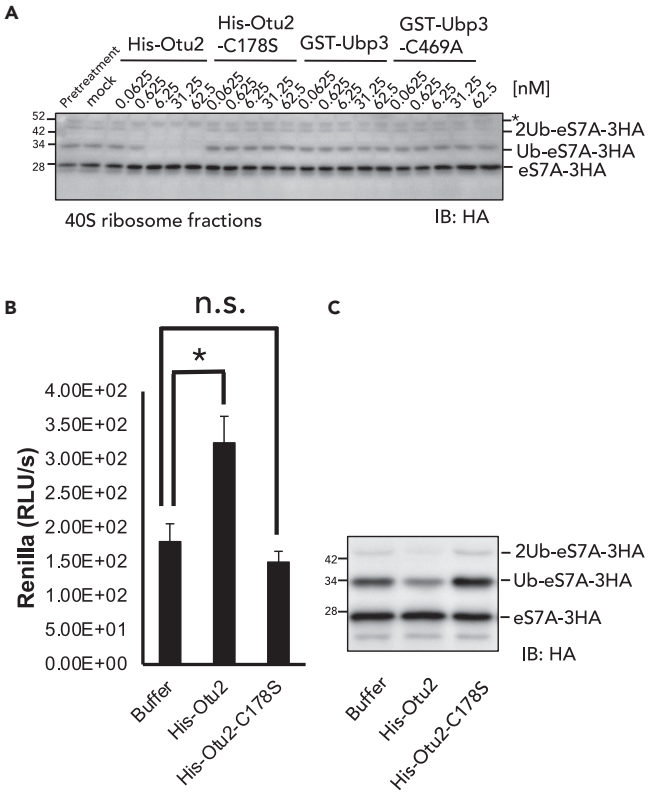


Figure 4. Deubiquitination of eS7 by Otu2 is required for efficient translation *in vitro*

(A) *In vitro* deubiquitination assay of eS7A-3HA using indicated recombinant deubiquitinating enzymes and 40S ribosome fractions prepared from *otu2Δubp3Δski2ΔeS7A-3HA* cells. The asterisk indicates a non-specific band.

(B) *In vitro* translation assay using recombinant His-Otu2 (WT and C178S), synthesized *Renilla* luciferase mRNAs, and a yeast *in vitro* translation extract obtained from the *otu2Δubp3Δski2ΔeS7A-3HA* strain ($n = 4$, mean ± SD, Student's t test, *$p < 0.05$, n.s.: not significant). Ski2 was deleted to prevent *Renilla* luciferase mRNAs from degradation.

(C) Detection of the ubiquitination status of eS7A-3HA in the *in vitro* translation extract assayed in (B) by immunoblot analysis with anti-HA antibodies. See also Figure S3.

Otu2 plays a role during ribosome recycling because it was specifically distributed in the 40S ribosome fractions (Figure 1). During the process from recycling to translation initiation, various ribosome-associated factors bind to and dissociate from 40S ribosomes in order, forming 43S- and 48S-preinitiation complexes (PIC). To more precisely determine which status of 40S ribosomes Otu2 is associated with, we examined which of the binding proteins characteristic of each status of 40S ribosomes was coimmunoprecipitated with Otu2. Rli1 and Hcr1 are recycling factors associated with the 80S PTC, the post-termination and free 40S ribosomes, and the 43S preinitiation complex (43S PIC) (Beznosková et al., 2013; Young and Guydosh, 2019). eIF3A is a subunit of the eIF3 complex, which acts from ribosome splitting through translation initiation (Valasek et al., 2003). eIF5 and eIF2 with Met-tRNA$_i^{Met}$ are recruited to the free 40S ribosome to form the 43S PIC. eIF4A and eIF4E are subunits of the cap-binding eIF4F complex in the 48S PIC (Figures 6A and 6B) (Dever et al., 2016). Of these proteins, Rli1, Hcr1, eIF3A, eIF5, eIF2α, and eIF2γ were coimmunoprecipitated with Otu2, whereas eIF4A and eIF4E were not (Figure 6C). These results suggest that Otu2 binds to post-termination and free 40S ribosomes in the recycling process and 43S PICs, but Otu2 is released from 43S PICs before 48S PICs are formed.

Otu2 plays a crucial role in dissociation of mRNAs from 40S ribosomes in ribosome recycling

Translated mRNAs are released from 40S ribosomes in the recycling process (Figure 6A). To further specify the status of Otu2-bound 40S ribosomes, we examined whether mRNAs were bound to 40S ribosomes purified with Otu2. 40S ribosomes were pulled down from 40S ribosome fractions of the strain expressing endogenously tagged uS15-3×Flag (Figure 7A). Similarly, Otu2-bound 40S ribosomes were purified from 40S ribosome fractions of the strain expressing the Otu2-3×Flag. Importantly, 48S PICs, which contain

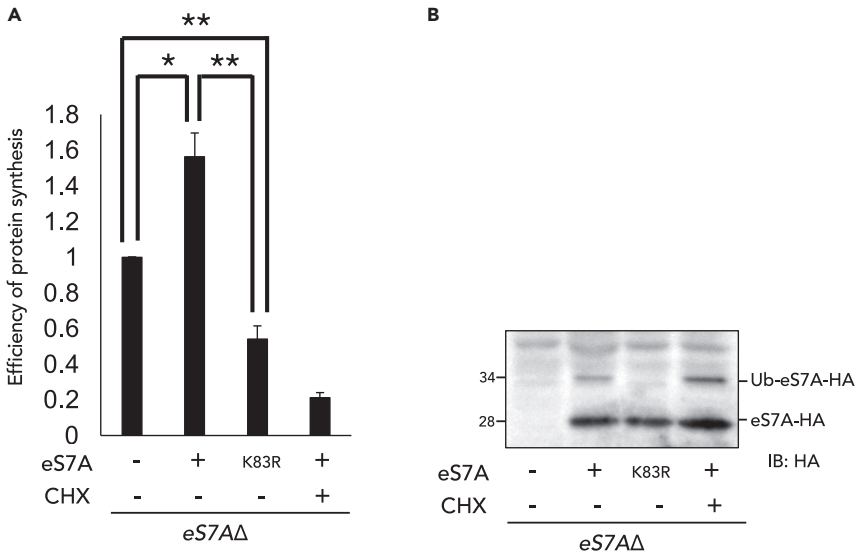


Figure 5. Ubiquitination of lysine 83 in eS7A is important for translation

(A) The *eS7A*Δ strain was transformed with pRS315 (vector), p315-7A (pRS315-*eS7A-HA*), or pUT156 (pRS315-*eS7A-K83R-HA*). Efficiency of the protein synthesis in each transformant was monitored by measuring the ability to incorporate L-homopropargylglycine (HPG) into nascent proteins as in Figure 2C ($n = 3$, mean ± SD, Student's t test, $^{**}p < 0.01$, $^{*}p < 0.05$). Cycloheximide (CHX) is used as a translation inhibitor.

(B) Detection of the ubiquitination status of eS7A-HA in each strain assayed in (A) by immunoblot analysis with anti-HA antibodies.

new activated mRNAs, should not be included in Otu2-bound 40S ribosomes based on the results in Figure 6C. Nevertheless, RT-qPCR analysis using RNAs extracted from each purified 40S ribosome revealed that mRNA levels of all the tested housekeeping genes (*ACT1*, *PGK1*, *eS27A*, and *eS27B*) were higher in 40S ribosomes purified with Otu2-3×Flag than those purified with uS15-3×Flag (Figure 7B). These results suggest that Otu2 mainly binds to 40S ribosomes before releasing mRNAs.

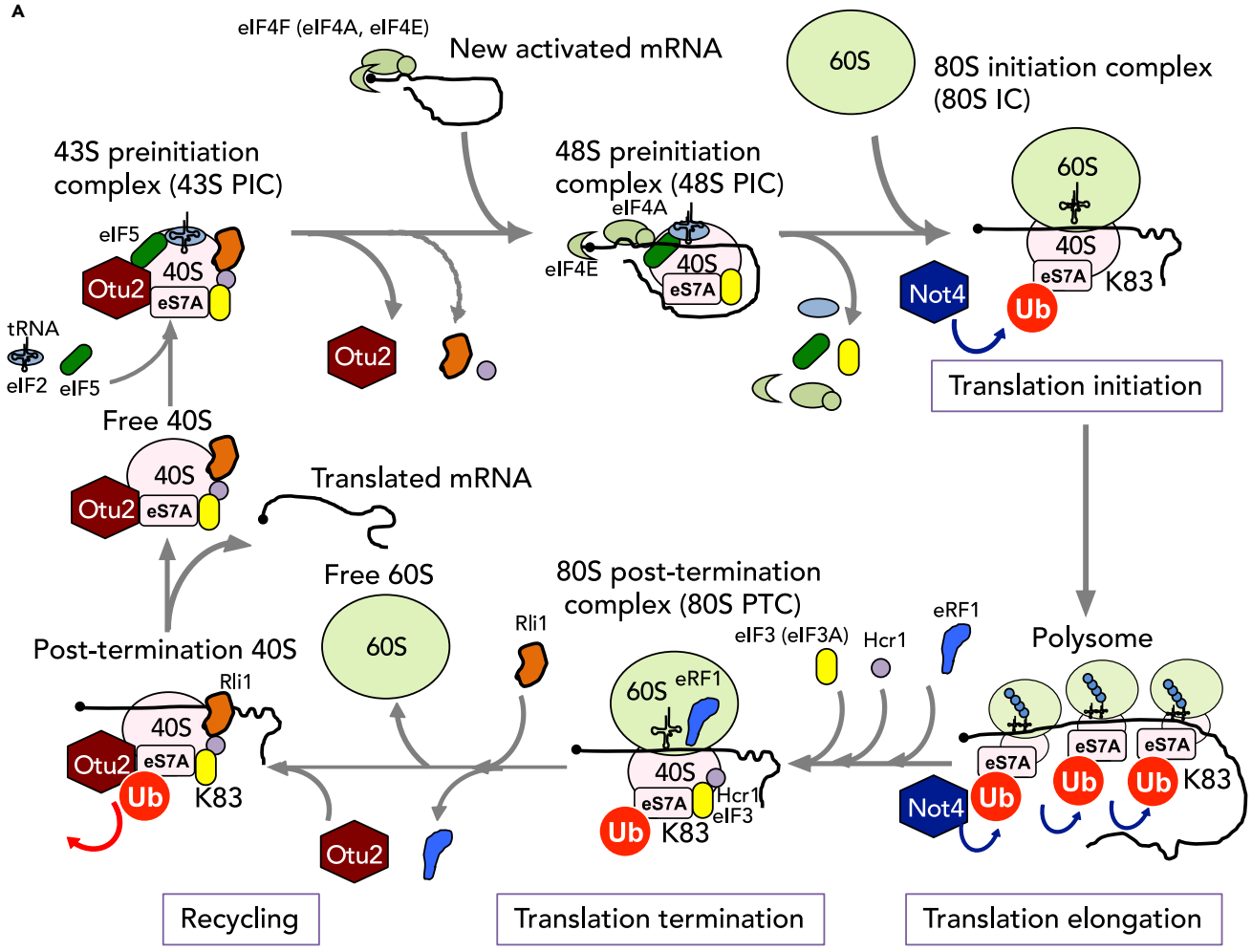
We then investigated whether Otu2 deficiency caused an increase in mRNA-bound 40S ribosomes. RNAs were extracted from 40S and 80S ribosomes purified from the WT and *otu2*Δ strains expressing uS15-3×Flag, and *PGK1* and *ACT1* mRNA levels were examined by RT-qPCR (Figure 7C). Although no or a slight increase in *PGK1* and *ACT1* mRNA levels was detected in the 80S ribosomes of *otu2*Δ cells, mRNA levels of both genes were much higher in 40S ribosomes from *otu2Δ* cells than in those from WT cells (Figure 7D). These results suggest that post-termination 40S ribosomes are accumulated by the deletion of Otu2, and deubiquitination of eS7A of post-termination 40S ribosomes by Otu2 plays a role in the dissociation of mRNAs from 40S ribosomes during ribosome recycling.

DISCUSSION

Based on the present study, we propose a model in which ubiquitination and deubiquitination of eS7 promotes the translation cycle (Figure 6A). eS7 is ubiquitinated in the 80S ribosome for efficient translation (Figure 5), whereas Ubp3 deubiquitinates eS7 to keep it as a mono-ubiquitinated form (Figure 3). After translation termination, 80S ribosomes are split into 40S and 60S ribosomes. Otu2 forms a stable complex with the 40S ribosome and deubiquitinates mono-ubiquitinated eS7 into non-ubiquitinated eS7, which triggers dissociation of translated mRNAs and serves to reinitiate a second round of translation (Figures 3 and 7). Thus, the cycle of mono-ubiquitination and deubiquitination of eS7 in the 80S/polysome and the 40S ribosome, respectively, plays an important role for efficient translation.

It has been shown that Not4 is an E3 ligase responsible for ubiquitination of eS7A. Polysomes were decreased in the Not4-deleted strain, suggesting that eS7A ubiquitination is important for efficient translation (Panasenko and Collart, 2012). Panasenko and Collart (2012) also showed simultaneous mutations of five lysine residues (K72, K76, K83, K84, and K101) to arginine in eS7A-abrogated eS7A ubiquitination by Not4. However, it has remained uncertain which lysine residue was actually ubiquitinated and responsible

A



B

	Termination	Recycling	Initiation	
	80S	40S	43S	48S
Hcr1	→	→	→	⇢
eIF3A	→	→	→	→
Rli1		→	→	⇢
eIF5 eIF2α eIF2γ			→	→
eIF4A eIF4E				→

C

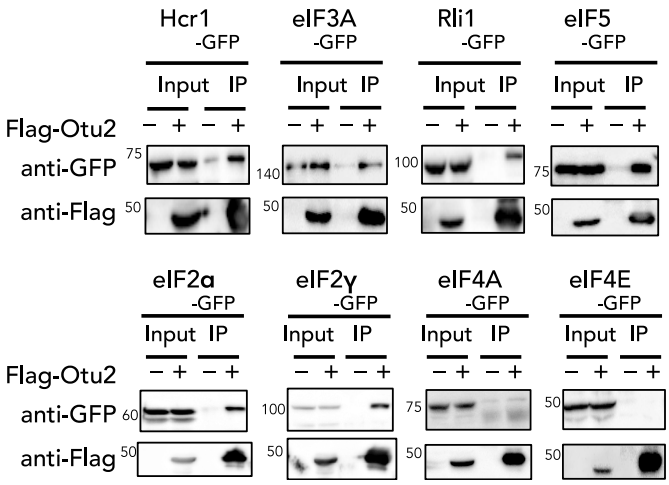


Figure 6. Otu2 binds to recycled 40S ribosomes and the 43S preinitiation complex

(A) Schematic model for the translation cycle in *Saccharomyces cerevisiae*. Only proteins and complexes, which appear in the main text, are depicted.

(B) Diagram shows the interactions between the ribosome and the ribosome-associated proteins examined in (C). The dotted lines of Hcr1 and Rli1 indicate the uncertainty of when Hcr1 and Rli1 are dissociated from the initiation complex.

(C) Immunoblot analysis of coimmunoprecipitated proteins with Flag-Otu2.

for efficient translation. We identified K83 as the major ubiquitination site of eS7 by mass spectrometry and site-directed mutagenesis (Figures 3C and S2D). Translation was remarkably repressed in the strain expressing eS7A-K83R, suggesting that ubiquitination of eS7 at K83 is critical for the normal translation cycle (Figure 5). Recently, Buschauer et al. (2020) showed that eS7 ubiquitination by Not4 is involved in the function of Not5 in sensing codon optimality by using the eS7-4KR (K72, 76, 83, 84R) mutant. eS7 ubiquitination helps Not5 associate with ribosomes (Buschauer et al., 2020). Among the four lysine residues, K83 may be essential for this association. It has also been shown that eS7 could be ubiquitinated at K84 by Not4 and that eS7 ubiquitination at K84 is equivalent to that at K83 for RQC-uncoupled no-go decay (Ikeuchi et al., 2019). The reason we did not observe eS7 ubiquitination at K84 in the eS7A-K83R mutant may have been because of the different contexts or because the K83R mutation affected K48 ubiquitination. In addition, we observed translation repression in *otu2*Δ*ubp3*Δ cells, accompanied by accumulation of poly-ubiquitinated eS7 (Figures 2 and 3). This observation indicates that the deubiquitination of eS7 is also required for efficient translation. Although both Otu2 and Ubp3 deubiquitinate eS7, Otu2 and Ubp3 play distinct roles in different ribosome complexes.

We showed that Ubp3 maintains eS7 in a mono-ubiquitinated form and prevents eS7 from poly-ubiquitination in 80S ribosomes and polysomes. eS7 is reported to undergo polyubiquitination by the E3 ligase Hel2 when K6 and K8 of eS10 are mutated to arginine to suppress translation for triggering a quality control mechanism (Ikeuchi et al., 2019). It is considered that translation is repressed due to activation of the quality control mechanism when eS7 is polyubiquitinated. Thus, it is conceivable that Ubp3 has a function to prevent activation of an incorrect quality control mechanism by keeping the state of mono-ubiquitination of eS7 during translation.

Our findings suggest that Otu2 facilitates dissociation of mRNAs from 40S ribosomes through deubiquitination of eS7 in the ribosome recycling step for a new round of translation. mRNAs were enriched in Otu2-bound 40S ribosomes, compared with whole free 40S ribosomes, and deletion of Otu2 increased 40S ribosome-associated mRNAs (Figure 7). However, several important questions remain unanswered. One of these questions is how Otu2 binds specifically to the 40S ribosome. It is unlikely that this specific binding of Otu2 to the 40S ribosome is mediated by recognition of ubiquitinated eS7s, because ubiquitinated eS7s also exist in 80S ribosomes. eS7 interacts with eL19 in the 80S ribosome (Jenner et al., 2012; Klinge et al., 2012; Wilson and Doudna Cate, 2012). Thus, Otu2 may bind to eS7 at the same site as eL19. Structural analysis of the 40S ribosome with Otu2 will be helpful to address this issue. Another question is how deubiquitination of eS7 promotes dissociation of mRNAs from 40S ribosomes. The mechanism for dissociation of tRNA and mRNA from the 40S ribosome remains uncertain (Pisarev et al., 2010; Skabkin et al., 2010; Young et al., 2018). At present, Tma64/eIF2D, Tma20/MCT-1, and Tma22/DENR are most likely to play an important role in the release of mRNAs from 40S ribosomes. It has been reported that unrecycled 40S ribosomes reinitiate translation at AUGs in the 3′ UTR or re-associate with 60S ribosomes in an AUG-independent manner in the *tma* mutants (Young et al., 2018). These unusual events might have occurred in *otu2*Δ cells and may be one of the reasons why 40S ribosomes were not accumulated in the *otu2*Δ strain (Figure 2B). Thus, it is interesting to examine whether Otu2 and mono-ubiquitin on eS7 affects the affinity and activity of Tma proteins to recycling 40S ribosomes.

Although it has been well established that ubiquitination of various ribosomal proteins is required for many critical aspects in translational control (Dougherty et al., 2020), we have demonstrated for the first time that eS7 is cyclically ubiquitinated and deubiquitinated in the normal translation process. Our results reveal another layer of regulation for ribosome functions in which a ubiquitination-deubiquitination cycle plays a key role.

Limitations of the study

In this study, we demonstrated that deubiquitination of eS7 by Otu2 was involved in mRNA release from the 40S ribosome. However, it remains unclear how deubiquitination of eS7A mediates dissociation of mRNAs from 40S ribosomes. We also demonstrated that eS7 was mono-ubiquitinated in 80S ribosomes

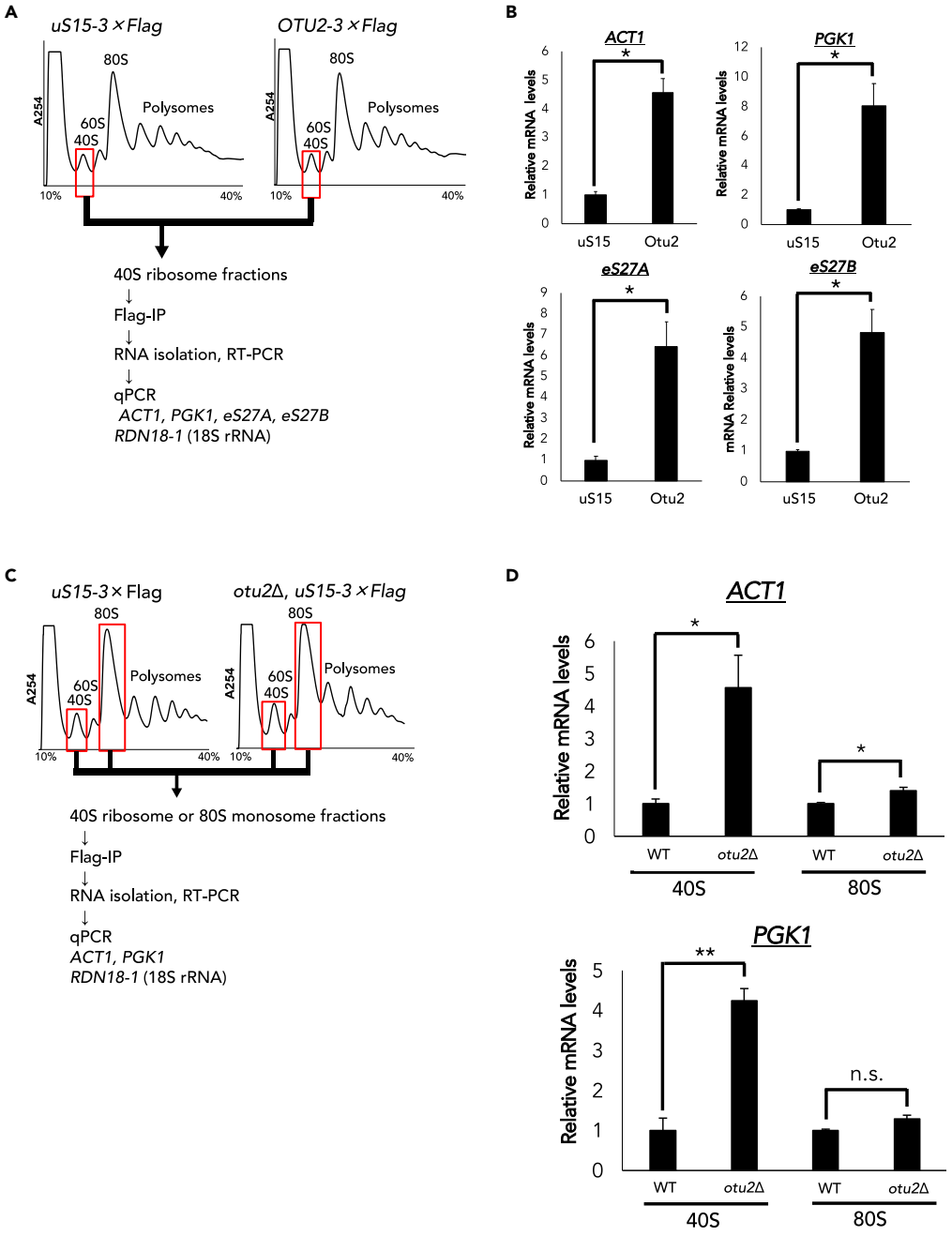


Figure 7. Otu2 plays a crucial role in dissociation of mRNAs from 40S ribosomes in ribosome recycling

(A) Experimental procedure for (B). Whole free and Otu2-bound 40S ribosomes were purified with anti-Flag antibodies from 40S ribosome fractions of the strains expressing uS15-3×Flag and Otu2-3×Flag, respectively.

(B) mRNA levels in the 40S ribosomes purified with uS15- and Otu2-3×Flag examined by RT-qPCR analysis ($n = 3$, mean ± SD, Student's t test, *$p < 0.05$).

(C) Experimental procedure for (D).

(D) mRNA levels in the 40S and 80S ribosomes purified with uS15-3×Flag from WT and *otu2Δ* cells examined by RT-qPCR analysis ($n = 3$, mean ± SD, Student's t test, **$p < 0.01$, *$p < 0.05$, n.s.: not significant). *RDN18-1* was used as the endogenous reference gene for internal controls (B and D).

and polysomes and that mono-ubiquitination of eS7 at K83 is required for efficient translation. Ubp3 prevented eS7 from polyubiquitination. However, it remains unclear how Ubp3 leaves eS7 mono-ubiquitinated and why the mono-ubiquitination of eS7 is required for efficient translation. We should also

consider the importance of mRNA co-translational decay as a regulatory mechanism for translation cycle in our future study.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Shigeo Murata (smurata@mol.f.u-tokyo.ac.jp).

Materials availability

All unique/stable reagents generated in this study are available from the Lead contact with a completed Materials Transfer Agreement.

Data and code availability

This study did not generate datasets.

METHODS

All methods can be found in the accompanying Transparent methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102145.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.T., H.Y., and S.M.; Methodology, Y.T., H.Y., Y.M., H.K., T. I., and S.M.; Investigation, Y.T., H.Y., Y.M., X.Z., A.K., T.M., and H.K.; Writing—Original Draft, Y.T., H.Y., and S.M.; Funding Acquisition, Y.M., H.K., T.I., and S.M.; Project Administration, S.M.; Resources, Y.T., H.Y., Y.M., and A.K.; Supervision, S.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

The ubiquitination-deubiquitination cycle on the ribosomal protein eS7A is crucial for efficient translation

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SUPPLEMENTAL INFORMATION

Supplemental Figures

Figure S1

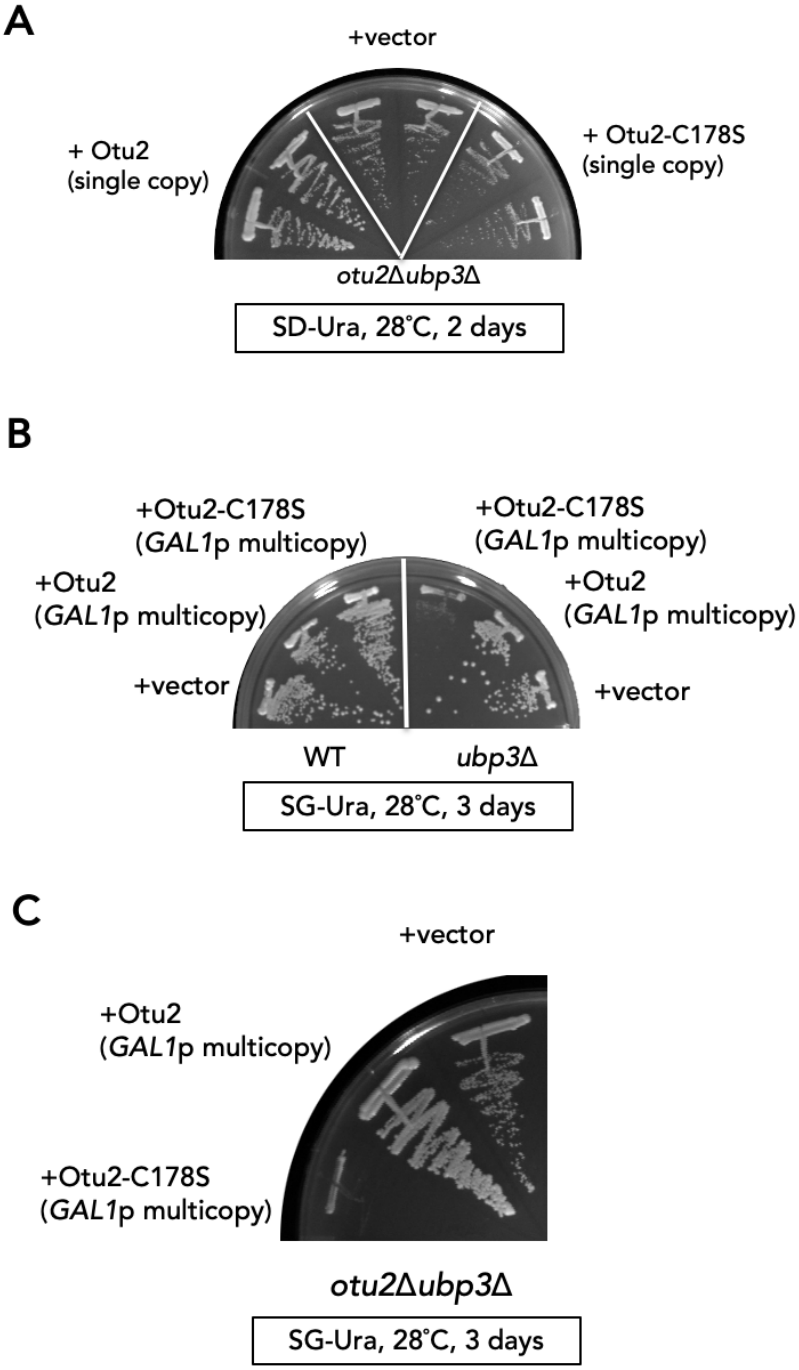


Figure S1. Otu2 and Ubp3 have overlapping functions, and the cysteine 178 is essential for Otu2 function. Otu2 may also have overlapping functions with deubiquitinating enzymes other than Ubp3. Related to Figure 2.

(A) Growth of *otu2*Δ*ubp3*Δ cells carrying a vector or a plasmid encoding wild-type (WT) Otu2 or putatively inactive Otu2 mutant (C178S) under its own promoter on an SD-Ura plate for 2 days at 28˚C. (B) WT and mutant Otu2 (C178S) under the *GAL1* promoter were expressed in the WT and *ubp3*Δ strains on SGal-Ura plate for 3 days at 28˚C. (C) Growth of *otu2*Δ*ubp3*Δ cells carrying a vector or a plasmid encoding WT Otu2 or Otu2 mutant (C178S) under the control of the *GAL1* promoter on an SGal-Ura plate for 3 days at 28˚C.

Figure S2

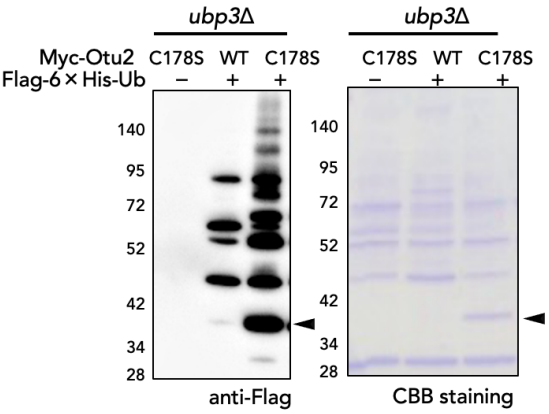
A

otu2Δubp3Δ+Flag 6×His-Ub

	Name	Peptides (95%)	% Cov
eS7B	40S ribosomal protein S7-B	9	49.5
ILV6	Acetolactate synthase small subunit, mitochondrial	13	37.9
UBI3	Ubiquitin-40S ribosomal protein S31	10	34.2
RPN11	26S proteasome regulatory subunit	7	20.3
PRE10	Proteasome component C1	5	25
IPP1	Inorganic pyrophosphatase	3	10.8
eS7A	40S ribosomal protein S7-A	10	42.6

Ribosome protein Bait protein (ubiquitin)

B



C

ubp3Δ+Flag 6×His-Ub+Myc-Otu2 C178S

	Name	Peptides (95%)	% Cov
eS7A	40S ribosomal protein S7-A	9	36.3
eS7B	40S ribosomal protein S7-B	7	36.3
UBI3	Ubiquitin-40S ribosomal protein S31	6	30.3
ILV6	Acetolactate synthase small subunit, mitochondrial	2	6.5
FBA1	Fructose-bisphosphate aldolase	1	2.8

Ribosome protein
Bait protein (ubiquitin)

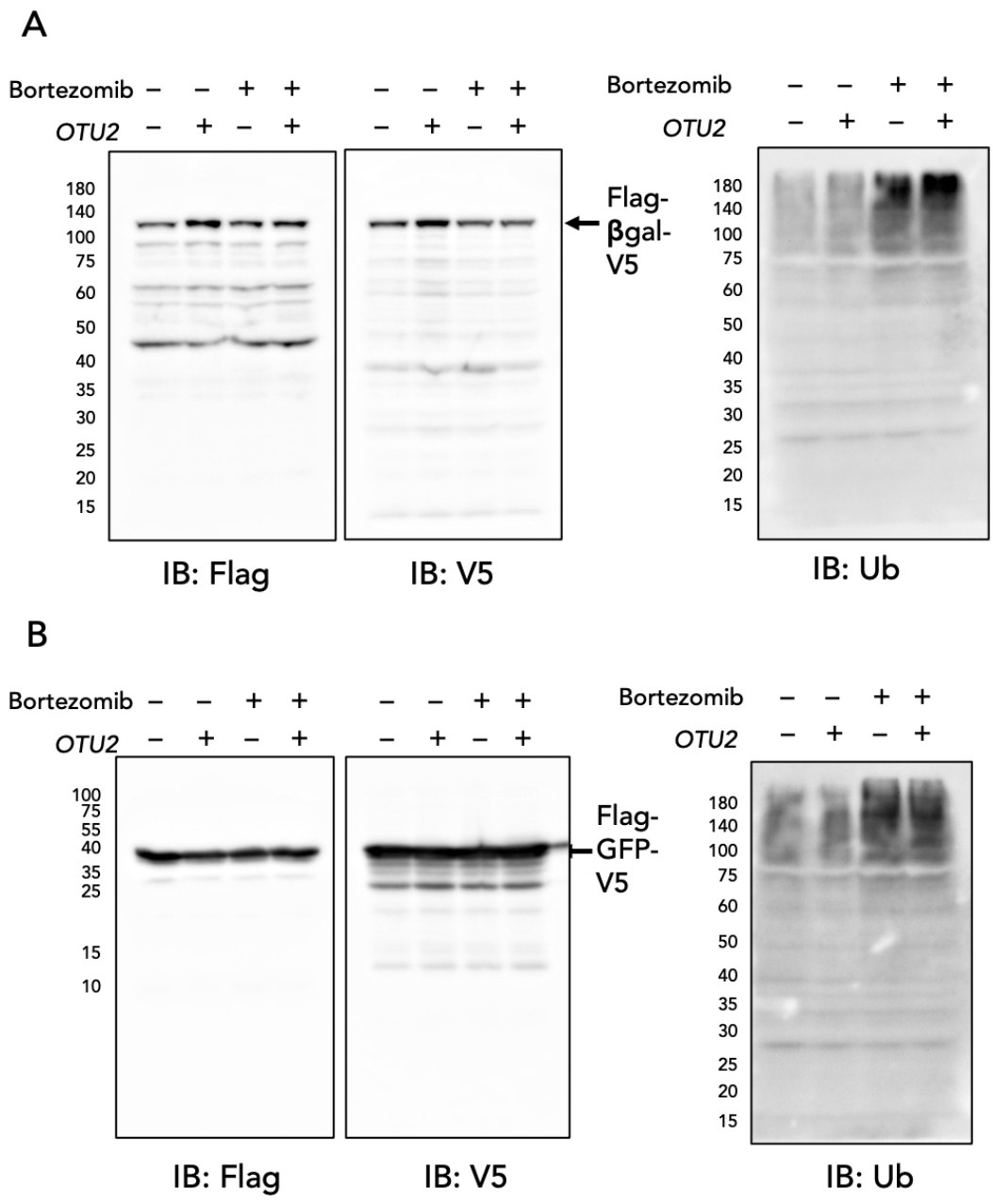
D

Master	Accession	Description	Sum PEP Score	Coverage	# Peptides	# PSMs	# Unique Peptides	# Protein Groups	# AAs
Master Protein	P48164	40S ribosomal protein S7-B	477.5080467	88.94736842	30	3047	18	1	190
Confidence	Annotated Sequence	Modifications	Qvality PEP	Qvality q-value	# Protein Groups	# Proteins	# PSMs	Master Protein Accessions	Positions in Master Proteins
High	[R].ELEKKFPDR.[H]	1xGG [K4]; 1xGlyGly [K4(99.8)]	0.0280575	0	1	1	15	P48164	P48164 [80-88]
High	[K].KALVLFVPVPALSAYHK.[V]	1xGG [K1]; 1xGlyGly [K1(100)]	0.000237409	0	1	1	2	P48164	P48164 [56-72]

Figure S2. Otu2 and Ubp3 are involved in deubiquitination of eS7. Related to Figure 3.

(A) Identification of ubiquitinated 40S ribosome proteins accumulated in *otu2*Δ*ubp3*Δ cells by mass spectrometry. (B) Ubiquitinated proteins purified with Ni-NTA beads from 40S ribosome fractions were subject to SDS-PAGE followed by the immunoblot analysis using anti-Flag antibodies (left panel), and Coomassie Brilliant Blue (CBB) staining (right panel). Flag-6×His-Ub and Myc-tagged Otu2 or Otu2-C178S were overexpressed for 16 h in media containing galactose. The protein band denoted by the arrowhead was excised from the CBB stained gel for mass spectrometry analysis. (C) Identification of ubiquitinated 40S ribosome proteins in *ubp3*Δ cells expressing Otu2-C178S by mass spectrometry analysis of the sample prepared in (B). (D) Ubiquitination sites in eS7 were also determined by the mass spectrometry analysis in (C).

Figure S3


A
Bortezomib – – + + – – + +
OTU2 – + – + – + – +
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Flag-βgal-V5
IB: Flag
IB: V5
Bortezomib – – + +
OTU2 – + – +
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IB: Ub
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Bortezomib – – + + – – + +
OTU2 – + – + – + – +
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Flag-GFP-V5
IB: Flag
IB: V5
Bortezomib – – + +
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15
IB: Ub

Figure S3. Deletion of Otu2 does not cause abortive translation. Related to Figure 4.

(A) Detection of β-galactosidase with N-terminal Flag and C-terminal V5 tags. Flag-β-galactosidase-V5 was expressed in the YUT8 (Δ*otu2*) strain transformed with pRS315 or pUT55 (pRS315-*OTU2*) using the *GAL1* promoter for 5 h at 28 °C. Cells were treated with or without the proteasome inhibitor bortezomib (100 μM) for 2 h before harvest. Flag-β-galactosidase-V5 was detected by immunoblot analysis using anti-Flag (left panel) and anti-V5 (middle panel) antibodies. Cell lysates were also subjected to immunoblot analysis with anti-Ub antibodies to detect the accumulation of ubiquitinated proteins upon treatment with bortezomib (right panel). (B) Detection of GFP with N-terminal Flag and C-terminal V5 tags. Lysates from the cells expressing Flag-GFP-V5 were subjected to immunoblot analysis as in (A).

Transparent Methods

Strains

Yeast strains

Yeast strain genotypes are given in Table S1. Yeast knock-out strains (cat #: YSC1053) and yeast GFP fusion collection (cat #: 95702) were purchased from Horizon Discovery (Cambridge, UK) and Thermo Fisher Scientific (Waltham, MA, USA), respectively. All of the double mutant strains were generated by mating, sporulation, and tetrad dissection. Unless otherwise noted, standard media and methods were used for culture, mating, sporulation, tetrad analysis, and transformation (Burke et al., 2000).

E. coli strains

The *E. coli* strain DH5**α** was used for propagating plasmids. The plasmids used in this study are listed in Table S2. Rosetta-gami 2 (DE3) cells (Merck, Darmstadt, Germany) were used for expression and purification of recombinant proteins.

Western Blotting

SDS-PAGE and Western blotting were performed according to standard protocols. For extraction of total cell lysates, cells were treated with 0.1N NaOH for 5 min at room temperature and subsequently boiled in SDS sample buffer (Kushnirov, 2000). Proteasome inhibition was performed using 100 μM bortezomib in the medium, in which L-proline was used as the sole nitrogen source and 0.003% SDS was added (Liu et al., 2007). Antibodies used in this study are all listed in Table S3.

Identification of eS7A

Flag and 6×His tagged Ub (Flag-6×His-Ub) were overexpressed for 6 h in media containing galactose (SGal-Ura). After fractionation by 10-40% sucrose density gradient centrifugation, Flag-6×His-Ub were immunoprecipitated with cOmplete His-Tag Purification Resin (Merck) from the fractions containing 40S ribosome under denaturing condition. Purified proteins were subject to SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining. The target protein band was excised from the CBB stained gel, digested with trypsin, and identified using mass spectrometry by the TOF/TOF 5800 system (SCIEX Massachusetts, USA).

Polysome analysis

We followed a method for polysome analysis described by Ikeuchi et al (Ikeuchi et al., 2019). Cycloheximide (CHX)-treated and iced cells were dissolved to lysis buffer (20 mM HEPES-KOH pH 7.5, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM DTT, 1 mM PMSF, 0.1 mg/ml CHX and 1

pill/40 ml complete EDTA free) and disrupted by multi-beads shocker (Yasui Kikai, Osaka, Japan). After centrifugation at 21,500 g, 4°C for 20 min followed by thorough centrifugation of supernatant at 21,500 g, 4°C for 30 min (TOMY MX-305, Tokyo, Japan), 37.5 A_{260} × ml (1.5 mg RNA amount) of supernatant was layered on sucrose density gradient (10-40% sucrose in 10 mM Tris-acetate, pH 7.4, 70 mM NH_4OAc and 4 mM $Mg(OAc)_2$) prepared in 14 × 89 mm SETON OPEN-TOP POLYCLEAR™ CENTRIFUGE TUBES (SETON, CA, USA) using a Gradient Master (BioComp, Fredericton, Canada). After ultracentrifugation at 29,000 rpm (10,5588 g), 4°C for 3 h in a P40S rotor and himac CP100a (Koki Holdings, Tokyo, Japan), the gradient was fractionated by a fractionator (BioComp) and 320 µl of each fraction was collected and precipitated by 10% TCA. Pellets were dissolved in 40 µl of TCA sample buffer (168 mM Tris-HCl [pH 6.8], 6.88% SDS, 22.4% v/v glycerol, 20% 2-mercaptoethanol, 0.01% bromophenol blue, 14.2 mM Tris-HCl [pH 8.8]). Samples were incubated at 95°C for 5 min to proceed to 10% SDS–PAGE followed by Western blotting.

Protein purification

Recombinant His-Otu2, His-Otu2-C178S, GST-Ubp3, and GST-Ubp3-C469A were purified from *E. coli* Rosetta-gami 2 (DE3) harboring pUT154, pUT153, pUT150, and pUT165 with Ni-conjugated agarose (Qiagen, Hilden, Germany) and Glutathione Sepharose 4B (GE healthcare, Chicago, USA). The purified proteins were dialyzed with Spectra/Por 3 Dialysis Tubing, 3.5 kDa MWCO (Spectrum) in TE buffer (20 mM HEPES-KOH pH 7.5, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 10% Glycerol, 2 mM DTT, 0.5 mM PMSF).

In vitro deubiquitination assay

Ubiquitinated 40S ribosomes were prepared from the 40S ribosome fractions of YUT216 (*otu2*Δ*ubp3*Δ*ski2*Δ *eS7A-3HA*) cells using 10-40% SDG centrifugation. Purified His-Otu2 or His-Otu2-C178S were then added to ubiquitinated 40S ribosomes, and incubated at 28°C for 90 min. To stop the deubiquitination reaction, the reaction lysates were collected and precipitated by 10% TCA. Pellets were dissolved in 40 µl of TCA sample buffer (168 mM Tris-HCl [pH 6.8], 6.88% SDS, 22.4% v/v glycerol, 20% 2-mercaptoethanol, 0.01% bromophenol blue, 14.2 mM Tris-HCl [pH8.8]). Samples were incubated at 95°C for 5 min to proceed to 10% SDS–PAGE followed by Western blotting.

In vitro translation

We performed in vitro translation assays using a modified protocol from Waters and Blobel (Waters and Blobel, 1986). Synthesized reporter mRNAs encoding renilla luciferase were produced using the mMessage mMachine Kit (Thermo Fisher Scientific). Yeast cell-free

translation extract was prepared from YUT216 (*otu2*Δ*ubp3*Δ*ski2*Δ *eS7A-3HA*) cells as described below. YUT216 cells were grown in YPD medium to OD_{600} of 1.5–2.0. Spheroplasts were prepared from harvested and washed cells using 10 mM DTT for 15 min at room temperature and 2.08 mg zymolyase per 1 g of cell pellet for 75 min in 1 M sorbitol at 30°C. Spheroplasts were then washed and lysed in a Dounce homogenizer using lysis buffer comprising 20 mM HEPES pH 7.5, 100 mM KOAc, 2 mM Mg $(OAc)_2$, 10% glycerol, 1 mM DTT, 0.5 mM PMSF and complete EDTA-free protease inhibitors (GE Healthcare). The S100 fraction of lysate supernatant was passed through a PD10 column (GE Healthcare) and used for in vitro translation. In vitro translation was performed at 17°C for 4 h with an excess of template mRNA (3.75 µg per 41.5 µl of extract) and recombinant His-Otu2 (WT and C178S). The expression of renilla luciferase was monitored using GloMAX® Discover System GM3000 (Promega).

Measuring the rate of global protein synthesis

The rate of global protein synthesis was measured using the Click-iT® HPG Alexa Fluor® 488 Protein Synthesis Assay kit (Thermo Fisher Scientific) according to the manufacturer's instruction. Before L-Homopropargylglycine (HPG) exposure, the cells were incubated for 1 h in complete medium until they attained the growth phase (OD_{600}=0.5); then, they were incubated at 28˚C for 30 minutes in complete medium that lacked methionine and was supplemented with 50 mM HPG. Negative control and cycloheximide (CHX)-treated cells were also prepared with complete medium and with complete medium that lacked methionine and was supplemented with 50 mM HPG and 100 µg/ml CHX. HPG incorporation was analyzed using a Attune NxT (Thermo Fisher Scientific). Analysis of flow cytometry data was completed by FlowJo software according to the manufacturer's instruction.

Co-immunoprecipitation

Cycloheximide (CHX)-treated and iced cells were dissolved to lysis buffer (20 mM HEPES-KOH pH 7.5, 100 mM KOAc, 2 mM $Mg(OAc)_2$, 1 mM DTT, 1 mM PMSF, 0.1 mg/ml CHX and 1 pill/10 ml complete-mini EDTA free) and disrupted by multi-beads shocker (Yasui Kikai). After centrifugation at 21,500 g, 4°C for 20 min followed by thorough centrifugation of the supernatant at 21,500 g, 4°C for 10 min (TOMY MX-305),

The anti-FLAG M2 agarose beads were added to the supernatant, which was gently rotated for 2 h at 4 °C. After washing 4 times with lysis buffer, the anti-FLAG M2 agarose beads were boiled in SDS-PAGE sample buffer at 95 °C for 5 min. Eluted proteins were analyzed by SDS-PAGE followed by immunoblotting.

Quantitative RT-PCR

40S ribosomes were purified from the 40S ribosome fractions of YUT61 (*OTU2-3×Flag*), YUT141 (*uS15-3×Flag*), and YUT140 (*uS15-3×Flag otu2*Δ) cell lysates fractionated using 10-40% SDG centrifugation. Total RNA was extracted from the purified 40S ribosomes using a High Pure RNA isolation kit (Roche). The RNA was reverse transcribed using a Super Script VILO cDNA synthesis kit (Thermo Fisher Scientific), and the relative amounts of cDNA for each target gene were quantified using Light Cycler 480 (Roche). The primers and probes used for qPCR analysis are described in Table S4. *RDN18-1* was used as a normalization control for the quantity of 40S ribosomes.

Table S1. Yeast strains, Related to Figures 1-7

S. cerevisiae strain: BY4741 (Yeast Knock Out parental strain)	Horizon Discovery	YSC1048
S. cerevisiae strain: *OTU2-3×Flag-kanMX4*	This study	YUT61
S. cerevisiae strain: *OTU2-3×Flag-kanMX4*, *RLI1-GFP-HIS3MX6*	This study	YUT37
S. cerevisiae strain: *uS15 (RPS13)-3×Flag-kanMX4*	This study	YUT141
S. cerevisiae strain: *otu2*Δ::*nat*, *uS15* (*RPS13*)*-3×Flag-kanMX4*	This study	YUT140
S. cerevisiae strain: *otu2*Δ::*nat*	This study	YUT8
S. cerevisiae strain: *ubp3*Δ::*hph*	This study	YUT10
S. cerevisiae strain: *otu2*Δ::*nat*, *ubp3*Δ::*hph*	This study	YUT13
S. cerevisiae strain: *eS7A* (*rps7a*)Δ::*kanMX4*	Horizon Discovery	YSC1053
S. cerevisiae strain: *eS7A*(*rps7a*)Δ::*kanMX4*, *otu2*Δ::*nat*	This study	YUT74
S. cerevisiae strain: *eS7A*(*rps7a*)Δ::*kanMX4*, *otu2*Δ::*nat*, *ubp3*Δ::*hph*	This study	YUT82
S. cerevisiae strain: *otu2*Δ::*nat*, *ubp3*Δ::*hph*, *ski2*Δ::*kan*, *eS7A* (*RPS7A*)*-3HA-HIS3MX6*	This study	YUT216
S. cerevisiae strain: *RLI1-GFP-HIS3MX6*	Thermo Fisher Scientific	95702
S. cerevisiae strain: *HCR1-GFP-HIS3MX6*	Thermo Fisher Scientific	95702
S. cerevisiae strain: *eIF3A* (*RPG1*)*-GFP-HIS3MX6*	Thermo Fisher Scientific	95702
S. cerevisiae strain: *eIF5* (*TIF5*)*-GFP-HIS3MX6*	Thermo Fisher Scientific	95702
S. cerevisiae strain: *eIF4A* (*TIF1*)*-GFP-HIS3MX6*	Thermo Fisher Scientific	95702
S. cerevisiae strain: *eIF4E* (*CDC33*)*-GFP-HIS3MX6*	Thermo Fisher Scientific	95702
S. cerevisiae strain: *eIF2***α** (*SUI2*)*-GFP-HIS3MX6*	This study	YT1659
S. cerevisiae strain: *eIF2***γ** (*GCD11*)*-GFP-HIS3MX6*	This study	YT1660

Table S2. Plasmids, Related to Figures 2-6

LEU2 ARS CEN	(Sikorski and Hieter, 1989)	pRS315
URA3 ARS CEN	(Sikorski and Hieter, 1989)	pRS316
*GAL1*p-Flag *URA3* 2μ	Gift from Dr. Saeki	pYF5
YEplac181-*GAL1*p-Myc *LEU2*	This study	pT607
pYES2-Flag	This study	pT611
pYF5-*OTU2*	This study	pAK1
pYF5-*OTU2-C178S*	This study	pAK10
pT611-*OTU2*	This study	pUT45
pT611-*OTU2-C178S*	This study	pUT46
pT611-6xHis-Ub	This study	pT623
pRS316-*OTU2*	This study	pUT136
pRS315-*OTU2*	This study	pUT55
pRS315-*UBP3*	This study	pUT56
pRS316-*OTU2-3×Flag-Tcyc1*	This study	pUT94
pRS316-*OTU2-3×Flag-Tcyc1-C178S*	This study	pUT95
pRS315-e*S7A* (*RPS7A*)-*HA*	Ikeuchi et al., 2019	p315-7A
pRS315-e*S7A-K56R-HA*	This study	pUT122
pRS315-e*S7A-K83R-HA*	This study	pUT156
pRS316-e*S7A*	This study	pUT64
pRS316-e*S7A-K83R*	This study	pUT65
pET28a (+)-His-*OTU2*	This study	pUT154
pET28a (+)-His-*OTU2-C178S*	This study	pUT153
pGEX-4T-1-GST-*UBP3*	This study	pUT150
pGEX-4T-1-GST-*UBP3-C469A*	This study	pUT165
pBluescriptIISK(+)-V5-Rluc-stop-MCS-Nluc-HA	Gift from Drs. Matsuo and Inada	pUT167
pYF-*LacZ*	This study	pT721
pYF-*GFP (S65T)*	This study	pT723

Table S3. Antibodies, Related to Figures 1, and 3-7

Mouse monoclonal anti-Ubiquitin (clone FK2)	Nippon Bio-Test Laboratories	Cat#0918-2, RRID: AB_10541840
Monoclonal Anti-FLAG M2 antibody	Sigma-Aldrich	Cat# F31665, RRID:AB_259529
Anti-FLAG M2 affinity gel antibody	Sigma-Aldrich	Cat# A2220, RRID:AB_10063035
Rabbit Anti-HA Tag Polyclonal Antibody	MBL	Cat# 561, RRID:AB_591839
Rabbit polyclonal anti-GFP	Our laboratory stock	N/A
PGK1 Mooclonal Antibody (22C5D8)	Thermo Fisher Scientific	Cat# 459250, RRID:AB_2532235
Peroxidase-AffiniPure Rabbit Anti-Mouse IgG + IgM (H+L) (min X Hu Sr Prot) antibody	Jackson ImmunoResearch Labs	Cat# 315-035-048, RRID:AB_2340069
Peroxidase-IgG Fraction Monoclonal Mouse Anti-Rabbit IgG, Light Chain Specific (min X Bov,Gt,Arm Hms,Hrs,Hu,Ms,Rat,Shp Ig) antibody	Jackson ImmunoResearch Labs	Cat#211-032-171, RRID:AB-2339149
Monoclonal Anti-V5 antibody	Thermo Fisher Scientific	Cat# R960-25, RRID: AB_2556564

Table S4. Probes and Primers for qPCR, Related to Figure 7

Universal ProbeLibrary Probes #40 for *RDN18-1*	Roche	04687990001
Universal ProbeLibrary Probes #25 for *ACT1*	Roche	04686993001
Universal ProbeLibrary Probes #100 for *PGK1*	Roche	04692187001
Universal ProbeLibrary Probes #131 for *eS27A*	Roche	04694155001
Universal ProbeLibrary Probes #9 for *eS27B*	Roche	04685075001
Forward primer for *RDN18-1* qPCR: 5′-AAACGGCTACCACATCCAAG-3′	This study	N/A
Reverse primer for *RDN18-1* qPCR: 5′-TCCCTGAATTAGGATTGGGTAAT-3′	This study	N/A
Forward primer for *ACT1* qPCR: 5′-CCATCTTCCATGAAGGTCAAG-3′	This study	N/A
Reverse primer for *ACT1* qPCR: 5′-CCACCAATCCAGACGGAGTA-3′	This study	N/A
Forward primer for *PGK1* qPCR: 5′-ATCAACGATGCCTTCGGTA-3′	This study	N/A
Reverse primer for *PGK1* qPCR: 5′-CAAGTCGAAACCGACCATAGA-3′	This study	N/A
Forward primer for *eS27A* qPCR: 5′-TCCAAGATCGTACTTCCTAGACG-3′	This study	N/A
Reverse primer for *eS27A* qPCR: 5′-GTTTGGGCGTGTGAGAAAAC-3′	This study	N/A
Forward primer for *eS27B* qPCR: 5′-GTCAAATGCCCAGGTTGTTT-3′	This study	N/A
Reverse primer for *eS27B* qPCR: 5′-TGACAGCAGTTTGAGCATGA-3′	This study	N/A

Supplemental References

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