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Ubiquitin release from eL40 is required for cytoplasmic maturation and function of 60S ribosomal subunits in *Saccharomyces cerevisiae*

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Ubiquitin is generated by proteolytic cleavage of precursor proteins in which it is fused either to itself, constituting a linear polyubiquitin protein of head-to-tail monomers, or as a single N-terminal moiety to one of two ribosomal proteins, eL40 (Ubi1/2 precursors) and eS31 (Ubi3 precursor). It has been proposed that the ubiquitin moiety fused to these ribosomal proteins could act as a chaperone by facilitating their efficient production, folding and ribosome assembly in *Saccharomyces cerevisiae*. We have previously shown that ubiquitin release from eS31 is required for yeast viability and that noncleaved Ubi3 can get incorporated into translation-competent 40S subunits. In this study, we have analysed the effects of mutations that partially or totally impair cleavage of the ubiquitin-eL40A fusion protein. While noncleaved Ubi1 is not able to support growth when it is the sole cellular source of eL40, it can assemble into nascent pre-60S particles. However, Ubi1-containing 60S ribosomal subunits are not competent for translation. This is likely due to a steric interference of the unprocessed ubiquitin with the binding and function of factors that interact with the ribosome's GTPase-associated centre. In agreement with this suggestion, Ubi1-containing ribosomes affect the efficient recycling of the anti-association factor Tif6 and have a reduced presence of translation elongation factors. We conclude that the removal of the ubiquitin moiety from ribosomal protein eL40 is an essential prerequisite for both the cytoplasmic maturation and the functionality of 60S ribosomal subunits.

Introduction

Ubiquitin is a highly conserved small protein of 76 amino acids that is restricted to eukaryotes, although ubiquitin-like proteins have been identified in prokaryotes [1]. Ubiquitin functions as a reversible post-translational modifier of proteins to regulate many different cellular processes, most notably proteasome-dependent protein degradation (for a review see Refs [2,3]). Normally, conjugation with ubiquitin occurs via the formation of an isopeptide bond between the α-carboxyl group of the C-terminal

Abbreviations
r-proteins, ribosomal proteins; r-subunits, ribosomal subunits; SD, synthetic dextrose (minimal yeast medium containing glucose); SGal, synthetic galactose (minimal yeast medium containing galactose); sg, slow-growth; YPD, yeast extract peptone dextrose (rich yeast medium containing glucose); YPGal, yeast extract peptone galactose (rich yeast medium containing galactose).

glycine of a ubiquitin molecule with an ε-amino group of a specific lysine residue within the target protein [2,3]. This process is known as ubiquitination and requires ubiquitin ligases; in turn, the removal of ubiquitin from their targets is known as deubiquitination and involves the action of specific proteases [4].

In all eukaryotes studied, ubiquitin is synthesised by proteolytic maturation from precursor proteins. In *Saccharomyces cerevisiae* (thereafter yeast), but also in mammals, ubiquitin is encoded by four different genes [5]. The yeast *UBI4* gene (human *UBB* and *UBC* genes) encodes a polyubiquitin precursor protein and is the main source of ubiquitin when cells enter the stationary phase or when they are exposed to an array of different adverse environmental conditions (i.e. heat shock, oxidative stress, nutrient depletion, DNA-damaging agents, DNA replication inhibitors or heavy metals, etc.) [6–10]. The paralogous *UBI1* and *UBI2* yeast genes (human *UBA52* gene) encode a single ubiquitin moiety fused to the 60S ribosomal protein (r-protein) eL40A and eL40B respectively. In turn, the yeast *UBI3* gene (human *RPS27A*/*UBA80* gene) codes for a single copy of ubiquitin fused to the 40S r-protein eS31 [5,7]; in growing yeast, most of the cellular ubiquitin originates from these three r-protein fusion genes [6].

Understanding the assembly and function of the different r-proteins is a major challenge to the ribosome biogenesis field (for reviews see Refs [11–13]). Both eS31 and eL40 are eukaryotic-specific r-proteins, although homologues of these proteins could be found in some archaea [14]. Experimental evidence indicates that while eS31 is a quasi-essential r-protein that assembles in the nucleus, most likely into early 90S pre-ribosomal particles [15,16], eL40 is an essential r-protein that associates in the cytoplasm with late pre-60S r-particles [17,18]. Under wild-type conditions, the Ubi1/2 and Ubi3 precursor proteins could so far never be detected, suggesting that their proteolytic maturation occurs very rapidly, likely co-translationally, and therefore before the assembly of the respective r-proteins into pre-ribosomal particles [5,15]. In agreement, the ubiquitin moiety of the Ubi1/2 and Ubi3 precursors can be deleted without causing a deleterious phenotype as long as the unfused r-proteins are expressed at elevated dosage ([5,19], and our unpublished results). However, single copy expression of either eS31 or eL40, not containing the N-terminal ubiquitin fusion, confers a pronounced slow-growth (sg) phenotype and a shortage of 40S or 60S r-subunits respectively ([5,19], and our unpublished results). Thus, the N-terminal ubiquitin moiety fused to eS31 and eL40 could act as a chaperone to facilitate the correct folding and hence the efficient synthesis of these r-proteins. We have previously studied the consequences of introducing mutations in the intersection between ubiquitin and eS31 within the Ubi3 precursor that partially or totally impair ubiquitin removal [19]. The obtained results indicate that the presence of ubiquitin hinders the assembly of eS31 into pre-40S r-particles, consequently, assembly of eS31 is favoured over that of noncleaved Ubi3 [19]. Moreover, cleavage-deficient Ubi3 variants [e.g. due to an additional proline between the ubiquitin moiety and eS31 (+P77)] are only stably expressed and get incorporated into 40S subunits upon depletion of wild-type Ubi3/eS31; intriguingly however, these mutants are unable to support growth even though such Ubi3-containing ribosomes can engage in translation [19]. In this study, we describe an equivalent characterisation of *ubi1* mutants with reduced or eliminated ubiquitin release. Our results indicate that noncleaved Ubi1 variants confer a lethal phenotype when expressed as the sole source of eL40 in a *ubi1*Δ *ubi2*Δ double knockout strain. These mutant proteins get incorporated into nascent pre-60S particles, although much less efficiently than processed eL40 derived from a wild-type Ubi1 precursor. However, these particles are mildly impaired in their cytoplasmic maturation (i.e. Tif6 recycling) and are unable to engage in translation elongation. Given the location of eL40 within mature 60S r-subunits in close proximity to the binding site of translational GTPases (Fig. 1A), we speculate that the assembly of uncleaved Ubi1 likely obstructs the binding and/or impedes the activity of this class of GTPases; thus, preventing the corresponding (pre-)60S r-subunits to efficiently mature and/or engage in translation.

Results

Phenotypic analysis of mutated ubiquitin-eL40 fusion variants

We are ultimately interested in understanding the role of the N-terminally fused ubiquitin moiety within Ubi1/2, the natural ubiquitin-eL40 fusion proteins, with respect to its relevance for eL40 synthesis and for the maturation and functionality of 60S r-subunits. To assess how the forced retention of the ubiquitin moiety might affect 60S r-subunit biogenesis and function, we constructed different *ubi1* mutants where the release of ubiquitin was expected to be partially or totally impaired. To this end, we introduced single G75A and G76A or double G75A G76A (G75,76A) mutations at the C-terminal end of the ubiquitin moiety within the Ubi1 precursor. We also inserted a supernumerary

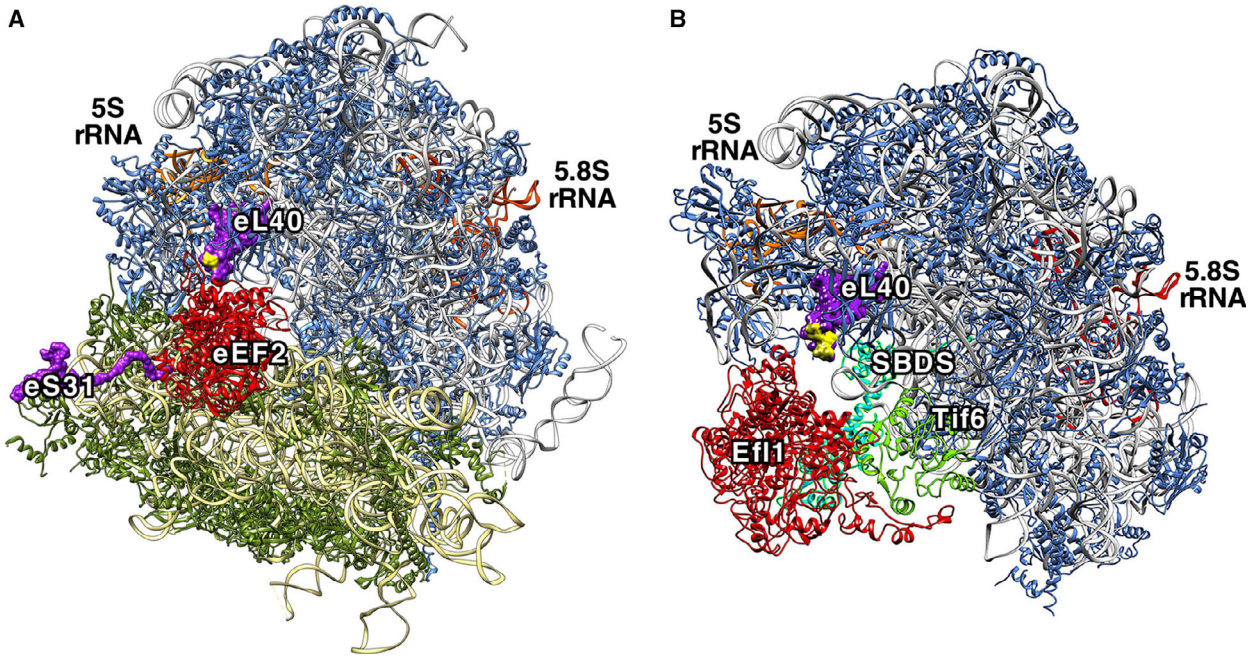

Fig. 1. Interaction of GTPases eEF2 and Efl1 with the ribosome. (A) Position of eEF2 (red) in the 80S ribosome from *Saccharomyces cerevisiae*. Ribosomal proteins eL40 and eS31 (purple) within the large and small r-subunit, respectively, have been highlighted. The remaining r-proteins are coloured in blue (large r-subunit) or green (small r-subunit); 25S rRNA is coloured in pale grey, 5.8S and 5S rRNAs in orange, and 18S rRNA in pale yellow. The representation was generated with the UCSF CHIMERA program, using the atomic model for the cryo-EM structure V of the yeast 80S ribosome bound to the Taura syndrome virus IRES (not shown) and eEF2·GTP bound to sordarin (PDB ID: 5JUU; [62]). eL40 could be modelled from the third residue E79, which is coloured in yellow, to the last K128 residue. (B) Position of Efl1 (red), SBDS (turquoise), and Tif6 (pale green) in a pre-60S particle from *Dictyostelium discoideum* (PDB ID: 5ANB; [27]). By using the UCSF CHIMERA program, we superimposed on the structure of this particle that of the yeast 60S r-subunit (PDB ID: 5APN; [63]); then, all common proteins from *D. discoideum* were removed from the model. eL40 (purple), the rest of large subunit r-proteins (blue), 25S rRNA (pale grey), 5.8S rRNA (orange), and 5S rRNA (orange) are highlighted. The first three N-terminal residues of eL40 (I77, I78 and E79) are coloured in yellow. Note that, due to the position of eL40 within 60S r-subunits, an assembled noncleaved Ubi1 protein is likely to sterically interfere with the binding of eEF2 and Efl1 to the ribosomal GTPase-associated centre.

proline at the junction between ubiquitin and eL40A (+P77). These mutations have been previously shown to interfere with the proteolytic maturation of the natural ubiquitin-eS31 fusion protein Ubi3 [19] and artificial ubiquitin fusion reporter constructs [20,21]. Figure 2A shows a scheme of the Ubi1 precursor protein, the nature of the introduced mutations, and the expected mature products. All these mutant variants were cloned into a yeast centromeric vector, under the control of the *UBI1* promoter and terminator. As also indicated in Fig. 2A, we also constructed variants where the eL40A moiety was additionally tagged with a single HA epitope at its C-terminal end for western detection. All the plasmids were transformed into a *UBI1/2* shuffle strain, which expresses, as a sole source of eL40, the eL40A protein from an instable centromeric *URA3* plasmid (see Materials and methods). Transformants were subjected to plasmid shuffling on plates containing 5-FOA, revealing that only the single *ubi1*[G75A] and *ubi1*[G76A] mutations supported growth. A detailed examination of their growth phenotypes on YPD plates showed that, compared to the wild-type counterpart, the *ubi1*[G75A] and *ubi1*[G76A] mutations conferred a mild or severe sg phenotype respectively (Fig. 2B). Growth was also analysed for the *UBI1*-HA, *ubi1*[G75A]-HA and *ubi1*[G76A]-HA strains. As also shown in Fig. 2B, the presence of the HA epitope slightly affected their growth, likely being the result of an adverse effect of the 11 extra amino acids on the interaction environment of the C terminus of eL40A within the ribosome (see Discussion). In agreement, doubling times of 2.4, 2.6 and 3.4 h for the *UBI1*, *ubi1*[G75A] and *ubi1*[G76A] strains and of 2.5, 3.1 and 3.6 h for the *UBI1*-HA, *ubi1*[G75A]-HA and *ubi1*[G76A]-HA strains were obtained in liquid selective SD medium at 30 °C respectively. The other two studied mutations, *ubi1*[G75,76A] and *ubi1*+P77, either HA-tagged or untagged, did not support growth,

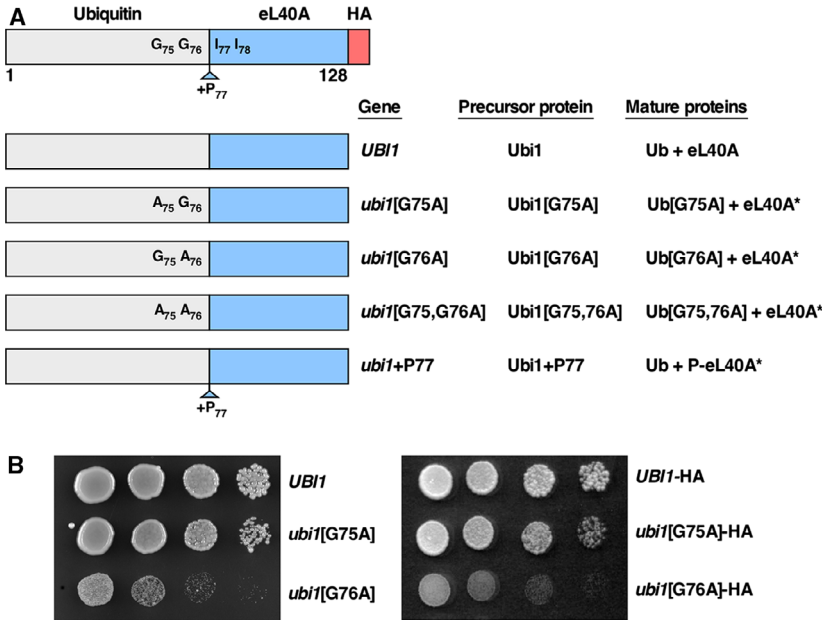

Fig. 2. Growth analysis of viable *ubi1 cleavage* mutants. (A) Schematic representation of the ubiquitin-eL40A fusion protein Ubi1. Ubi1 consists of an N-terminal ubiquitin moiety fused to the r-protein eL40A. A peptide bond between the C-terminal glycine of ubiquitin (G76) and the N-terminal isoleucine of eL40A (I77) connects the two proteins. Relevant residues and *ubi1* mutant variants thereof used in this study are also indicated. The respective alleles as well as the precursor protein and mature protein names are also indicated. The asterisk denotes that the exact nature of the mature proteins derived from these precursor proteins has not been studied; thus, the indicated mature proteins correspond to the most likely cleavage products. A C-terminal 1xHA tag was added for western detection of the Ubi1 and/or eL40 protein variants. (B) YCplac111-UBI1, YCplac111-UBI1-HA, and the indicated *ubi1* and *ubi1*-HA mutants were transformed into TAY001, the *UBI1/2* shuffle strain. Transformants were selected on SD-Leu plates and, after plasmid shuffling on plates containing 5-FOA, viable mutants were spotted in fivefold serial dilution steps onto YPD plates, which were incubated for 2 days at 30 °C.

indicating that these mutations conferred a lethal phenotype. For their phenotypic analysis, plasmids expressing these variants from the *UBI1* promoter were transformed into a conditional *GAL::UBI1* strain where the expression of plasmid-borne *UBI1,* which constitutes the sole source of eL40 in these cells, is driven from a *GAL1-10* promoter. In this setting, cells containing the *ubi1*[G75,76A] and *ubi1*+P77 variants can grow in media containing galactose as carbon source (SGal) but are unable to grow in media containing glucose as carbon source (SD; Fig. 3 and data not shown). Moreover, the expression of neither of the two mutant variants had an adverse effect on growth on SGal medium, as compared to the control transformed with an empty plasmid, indicating that these mutations are not conferring a dominant-negative phenotype.

Proteolytic processing of the Ubi1 variant proteins into ubiquitin and eL40A

Next, we determined whether the severity of the growth defect observed for the distinct *ubi1* mutants correlated with an increased impairment of the processing of the Ubi1 precursor. To this end, we monitored the extent of cleavage by comparing the amounts of uncleaved Ubi1-HA *versus* that of mature eL40A-HA. First, we analysed ubiquitin cleavage in the viable haploid *UBI1*-HA, *ubi1*[G75A]-HA, and *ubi1*[G76A]-HA strains, which were grown in liquid SD-Leu medium to mid exponential phase. Then, cell extracts were prepared and equivalent amounts of each of these were analysed by western blotting using anti-HA antibodies. As shown in Fig. 4A, no Ubi1-HA precursor and only mature eL40A-HA was detected in the wild-type *UBI1*-HA strain; however, some precursor protein could be detected in the *ubi1*[G75A]-HA mutant and, even more prominently, in the *ubi1*[G76A]-HA mutant. Despite this accumulation of uncleaved Ubi1 precursor, mature eL40A-HA was apparently present to the wild-type extent in both mutants. We conclude that introduction of either a single G75A or G76A exchange at the C terminus of ubiquitin reduces, albeit to different extents, the efficiency of Ubi1 proteolytic processing into ubiquitin and eL40.

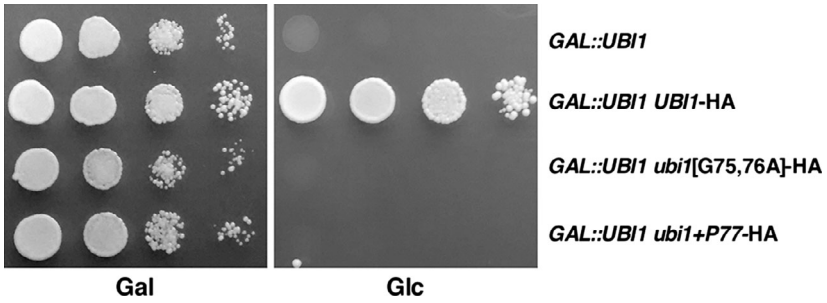

Fig. 3. Growth analysis of nonviable *ubi1 cleavage* mutants. A conditional *UBI1/2* shuffle strain harbouring the pGAL22-UBI1 plasmid was transformed with YCplac111-UBI1-HA and the indicated *ubi1*-HA mutants. Transformants were selected on SGal-Leu plates and then spotted in fivefold serial dilution steps onto SGal-Leu (Gal) and SD-Leu (Glc) plates, which were incubated for 3 days at 30 °C.

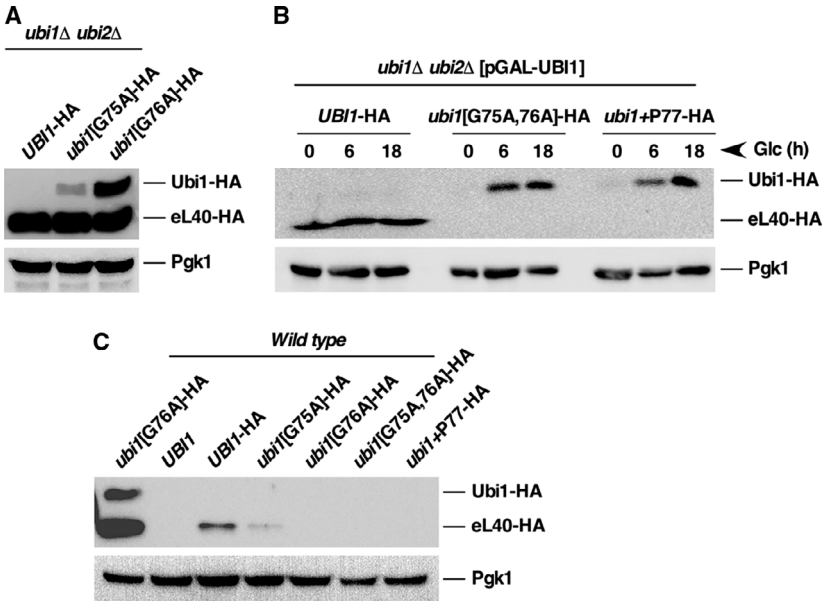

Fig. 4. Ubi1 cleavage *in vivo*. (A) Analysis of the Ubi1-HA protein in the viable *ubi1* cleavage mutants. Haploid *ubi1*Δ *ubi2*Δ cells harbouring wild-type YCplac111-UBI1-HA or the indicated *ubi1*-HA mutants were grown in liquid YPD medium at 30 °C. Total cell extracts were prepared and subjected to western blot analysis using anti-HA antibodies. Phosphoglycerate kinase was used as a loading control and detected with anti-Pgk1 antibodies. (B) Analysis of Ubi1 cleavage in the lethal *ubi1* mutants. Haploid *ubi1*Δ *ubi2*Δ cells harbouring pGAL22-UBI1 and expressing Ubi1-HA, Ubi1[G75,76A]-HA or Ubi1+P77-HA from YCplac111 were grown at 30 °C in liquid SGal-Leu media and then shifted for the indicated times to SD-Leu media. Total cell extracts were prepared and subjected to western blot analysis using anti-HA and anti-Pgk1 antibodies. (C) Analysis of *UBI1*-HA and the different *ubi1*-HA cleavage mutants in a haploid wild-type strain. YCplac111-based plasmids containing the indicated constructs were transformed into the W303-1A strain, transformants were then grown in liquid SD-Leu at 30 °C. Total cell extracts were prepared and subjected to western blot analysis as above. As a positive control for the detection of the Ubi1-HA precursor and processed eL40-HA, a cell extract from a strain expressing Ubi1[G76A]-HA as a sole source of Ubi1/2 was included (first lane from the left).

To assess the processing of the noncomplementing Ubi1[G75,76A]-HA and Ubi1+P77-HA precursor variants, we expressed the corresponding constructs in the above-described *GAL::UBI1* strain (Fig. 3). Then, extracts were prepared from cells grown in liquid selective SGal or shifted for 6 and 18 h to selective SD medium and subjected to western blot analysis using anti-HA antibodies. As shown in Fig. 4B, precursors were not detected in SGal medium but accumulated at both time points upon the shift to SD medium; however, no mature eL40A-HA could be detected. This result indicates that processing of the Ubi1 precursor into ubiquitin and eL40A is fully inhibited upon the introduction of a double G75,76A mutation at the C terminus of ubiquitin, as well as upon the insertion of a supernumerary proline at the intersection between

ubiquitin and eL40A. As no mutant precursors were detected in SGal medium, we suspected that their stability is reduced when co-expressed with wild-type Ubi1. Thus, to further evaluate this hypothesis, we transformed a wild-type strain with the plasmids harbouring wild-type *UBI1*-HA or any of the above *ubi1*-HA variants. Transformants were grown in the appropriate selective SD medium to maintain the plasmids to mid-log phase, and then, cell extracts were prepared and analysed by western blotting using anti-HA antibodies. As shown in Fig. 4C, the Ubi1-HA precursor was not detected in any of the transformants; in contrast, the mature eL40A-HA r-protein was clearly observed in cells expressing wild-type Ubi1-HA and could further only be faintly seen in cells expressing the Ubi1[G75A] variant. This observation strongly suggests that Ubi1 processing occurs rapidly, likely co-translationally, and normally before assembly of the eL40A r-protein into pre-60S r-particles. Notably, the noncleaved Ubi1 precursor variants can only be detected when no additional wild-type Ubi1/eL40A is present, indicating that they might be efficiently degraded when not assembled into 60S r-particles.

We conclude that there is a positive correlation between the extent of noncleaved Ubi1 accumulation and the decrease in the growth rate of the different *ubi1* cleavage mutants. Moreover, we assume that the growth defects of the four different *ubi1* mutants, when representing the sole source of eL40 r-protein, likely results from a negative effect directly associated with the incorporation of the noncleaved Ubi1 into ribosomes (see also below).

Noncleaved Ubi1 precursors can assemble into 60S r-particles that cannot engage in translation

To test the above prediction, we checked whether the *ubi1* cleavage mutants show defects in the production and function of 60S r-subunits. We first analysed the polysome profiles from cell extracts obtained from a wild-type *UBI1*-HA strain or the viable *ubi1*[G75A]-HA and *ubi1*[G76A]-HA mutants grown in YPD to logarithmic phase at 30 °C. This analysis revealed that the *ubi1*[G75A]-HA mutant exhibited a polysome profile that is indistinguishable from that of the wild-type strain (Fig. 5A). In contrast, the *ubi1*[G76A]-HA mutant displayed a profile where a slight increase in the amount of free 60S r-subunits as well as a mild accumulation of half-mer polysomes could be clearly observed (Fig. 5A). This type of profile is characteristic for mutants partially impaired in 60S to 40S r-subunit joining [17,22–26]. Second, we addressed whether the noncleaved Ubi1[G75A]-HA and Ubi1[G76A]-HA precursors get incorporated into ribosomal particles. To do so, cell extracts obtained from the mutants grown in liquid YPD to mid-log phase at 30 °C were subjected to sucrose gradient centrifugation and fractionation. Then, the fractions were analysed by SDS/PAGE and western blotting using anti-HA antibodies. As shown in Fig. 5B, cleaved eL40A-HA distributed in the 60S, 80S and polysome fractions in these mutants, similarly as another 60S r-protein, uL1. While no Ubi1[G75A]-HA precursor could be detected either in the total cell extract or in the fractions (data not shown), the Ubi1[G76A]-HA precursor, while only faintly detectable in the total extract, could be clearly revealed in the 60S r-subunit peak. Altogether, these data indicated that the Ubi1 precursor of the viable *ubi1*[G75A]-HA and *ubi1*[G76A]-HA mutants might be processed or degraded during the preparation of cell extracts for polysome analysis under native conditions (see Discussion). Nevertheless, it appears that some Ubi1[G76A]-HA precursor gets incorporated into 60S r-particles, but, to the extent the sensitivity of our blots allows this speculation, these particles seem not to be engaged in translation. Apparently, equal amounts of mature eL40A are associated with polysomes in *ubi1*[G76A]-HA and wild-type *UBI1*-HA cells (Fig. 5B). Thus, it is likely that the sg phenotype of the *ubi1*[G76A]-HA mutant is not directly related to possible defects in assembly of eL40 into 60S r-subunits, but may on the one hand be due to an interference of the Ubi1[G76A] precursor with efficient subunit joining (see above). Alternatively, the sg phenotype could also originate from the interference of the released UbG76A ubiquitin with the ubiquitin system, as we have previously suggested to occur in the case of the equivalent *ubi3*[G75A,76A] mutant, which also displays a sg phenotype [19].

Polysome profile analysis was also carried out with cell extracts obtained from conditional strains expressing the lethal Ubi1[G75,76A]-HA and Ubi1+P77-HA variants. For this purpose, *ubi1*Δ *ubi2*Δ cells carrying only pGAL22-*UBI1* or simultaneously co-expressing, under the control of the *UBI1* promoter, either wild-type Ubi1-HA, Ubi1[G75,76A]-HA or Ubi1+P77-HA precursors were grown in selective liquid SGal medium and then shifted for 18 h to selective liquid SD medium. Then, cell extracts were prepared and subjected to sucrose gradient centrifugation and fractionation. As shown in Fig. 6A and as expected [17], depletion of eL40 led to an aberrant polysome profile showing a general decrease in polysomes and the appearance of half-mer polysomes; these defects were fully complemented by the expression of wild-type Ubi1-HA. Expression of the mutant Ubi1[G75,76A]-HA and

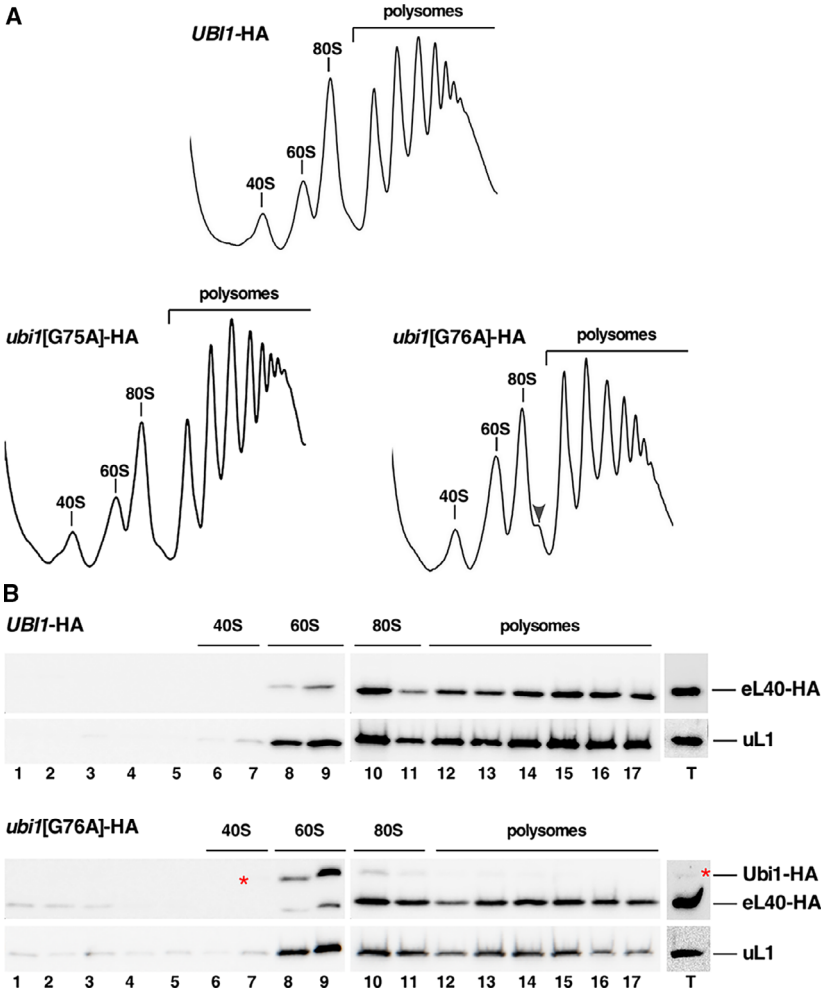

Fig. 5. Noncleaved Ubi1[G76A] is not incorporated into translating polyribosomes. (A) Haploid *ubi1*Δ *ubi2*Δ cells harbouring YCplac111-UBI1-HA (*UBI1*-HA), YCplac111-ubi1.G75A-HA (*ubi1*[G75A]) or YCplac111-ubi1.G76A-HA (*ubi1*[G76A]) were grown in liquid YPD medium at 30 °C to an OD_{600} of around 0.8. Cell extracts were prepared and ten A_{260} units of each extract were resolved in 7–50% sucrose gradients. The A_{254} was continuously measured. Sedimentation is from left to right. The peaks of free 40S and 60S r-subunits, 80S vacant couples/monosomes, and polysomes are indicated. Half-mers are labelled by arrows. (B) Fractions were collected from the above gradients, then proteins were extracted from each fraction, and equal volumes were subjected to western blot analysis using antibodies against the HA tag or the r-protein uL1. T, total cell extract. The asterisk marks the Ubi1 precursor protein.

Ubi1+P77-HA precursors resulted in polysome profiles very similar to those obtained upon the depletion of eL40 (Fig. 6A). Compared to eL40 depletion, however, there was a clear increase in the amounts of free 60S *versus* 40S r-subunits, indicating that the observed half-mers are due to a pronounced subunit-joining defect. Moreover, the dramatic decrease in the height of successive polysome peaks might be suggestive of an impairment in the elongation phase of translation. Another corollary that can be deduced from the polysome profile analysis of the lethal *ubi1*[G75,76A]-HA and *ubi1*+P77-HA mutants is that the quantitative production of 60S r-subunits is not selectively affected by these mutations, suggesting that, as previously reported for the depletion of eL40 [17], assembly and pre-rRNA processing are not significantly impaired. When the distribution of the mutant proteins was followed by fractionation analysis, the noncleaved precursors strongly peaked in the 60S and 80S fractions, but were, compared to the 60S r-subunit control protein uL1, much less abundant in or absent from the polysomal fractions (Fig. 6B). This result clearly indicates that the noncleaved Ubi1 precursor variants can assemble into pre-60S r-particles, provided that they are the sole source of cellular eL40. Importantly, assembly of uncleaved Ubi1 alters 60S r-subunits in a way that prevents them from efficiently engaging in subunit joining and possibly also translation elongation.

Assembly of noncleaved Ubi1 precursor interferes with the binding and function of translational GTPases

To further explore a possible impairment of translation elongation caused by the different uncleaved Ubi1 mutants, we determined the efficiency of *in vivo* binding of two translation elongation factors, eEF1A and eEF2, to ribosomes containing either the Ubi1 [G75,76A]-HA or Ubi1+P77-HA variant proteins and

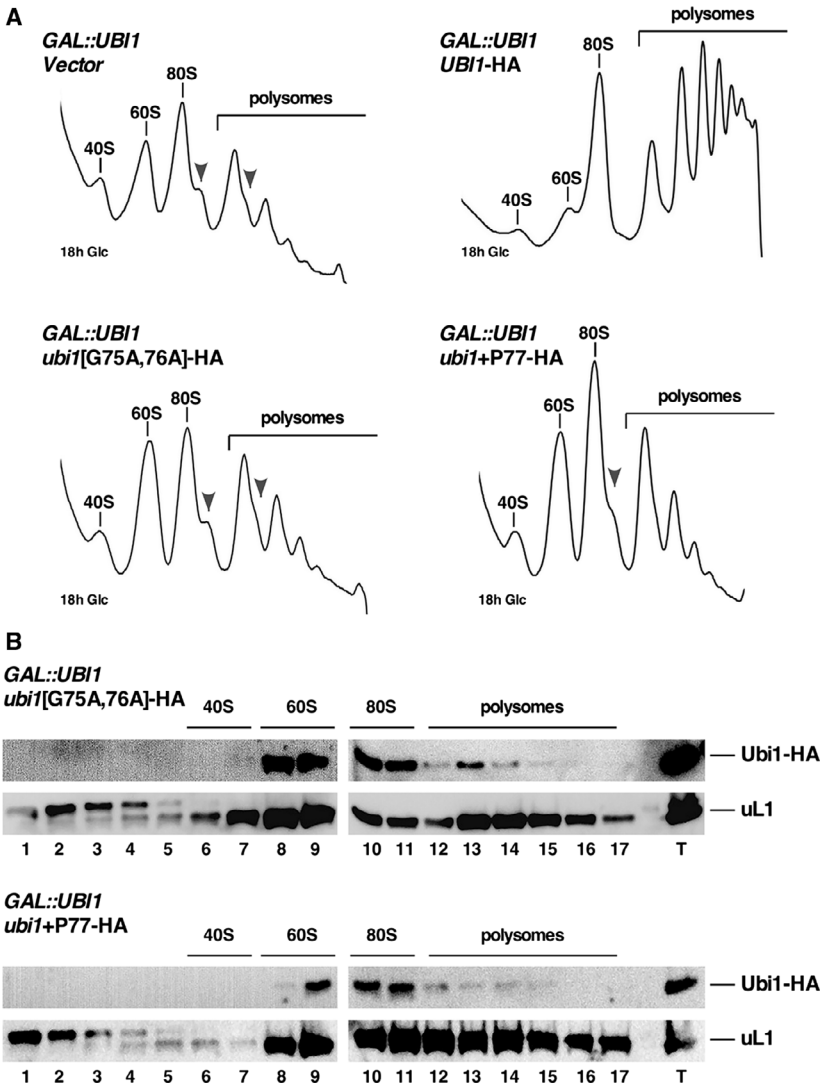

Fig. 6. Expression of noncleaved Ubi1 precursors leads to a 60S r-subunit joining defect and impairs translation elongation. (A) Haploid *ubi1*Δ *ubi2*Δ cells simultaneously harbouring pGAL22-UBI1 and either empty YCplac111 (*GAL::UBI1 Vector*), YCplac111-UBI1-HA (*GAL::UBI1 UBI1*-HA), YCplac111-ubi1.G75,76A-HA (*GAL::UBI1 ubi1* [G75,76A]-HA), or YCplac111-ubi1+P77-HA (*GAL::UBI1 ubi1*+P77-HA) were grown in liquid SGal-Trp-Leu media at 30 °C, then shifted for 18 h to liquid SD-Trp-Leu media, and harvested at an OD_{600} of around 0.8. Cell extracts were prepared and ten A_{260} units of each extract were resolved in 7–50% sucrose gradients. The A_{254} was continuously measured. Sedimentation is from left to right. The peaks of free 40S and 60S r-subunits, 80S free couples/monosomes, and polysomes are indicated. Half-mers are labelled by arrows. (B) Fractions were collected from the above gradients, then proteins were extracted from each fraction, and equal volumes were subjected to western blot analysis using antibodies against the HA tag or the r-protein uL1. T, total cell extract.

compared it to the efficiency of ribosomes containing wild-type eL40A-HA or depleted of eL40. To assess binding, *ubi1*Δ *ubi2*Δ cells either carrying only pGAL22-*UBI1* or harbouring pGAL22-*UBI1* and a second plasmid expressing the Ubi1-HA, Ubi1 [G75,76A]-HA or Ubi1+P77-HA precursors were grown in selective liquid SGal medium and then shifted for 6 h to selective liquid SD medium. Then, cell extracts were prepared and samples enriched in r-particles were obtained by ultracentrifugation (see Materials and methods). As shown in Fig. 7, western blot analysis and quantification confirmed that the noncleaved Ubi1 variants got incorporated into 60S r-particles, although apparently only into around 25% of these; thus, suggesting that the majority of the enriched r-particles could correspond to ones either still containing wild-type eL40, due to the short depletion time, or not having incorporated the uncleaved Ubi1 proteins. Still, we notably observed a mild reduction in the stable association of both translation elongation GTPases, eEF1A and eEF2, but not of the initiation factor eIF4A, used as control, to ribosomes containing Ubi1 [G75,76A]-HA or Ubi1+P77-HA, compared to the association of the same factors to ribosomes containing eL40A-HA or depleted of eL40.

Efl1/Ria1 is another GTPase that binds to the translational GTPase-associated centre [27]. It is well established that the release of the *trans*-acting factor Tif6 from cytoplasmic pre-60S r-particles is dependent on the activity of Efl1; consistently, upon loss-of-function of Efl1, the otherwise nuclear steady-state localisation of a Tif6-GFP reporter becomes cytoplasmic ([28], see also [29]). Therefore, we tested whether eL40 could be involved in Efl1-driven Tif6 release. In agreement with

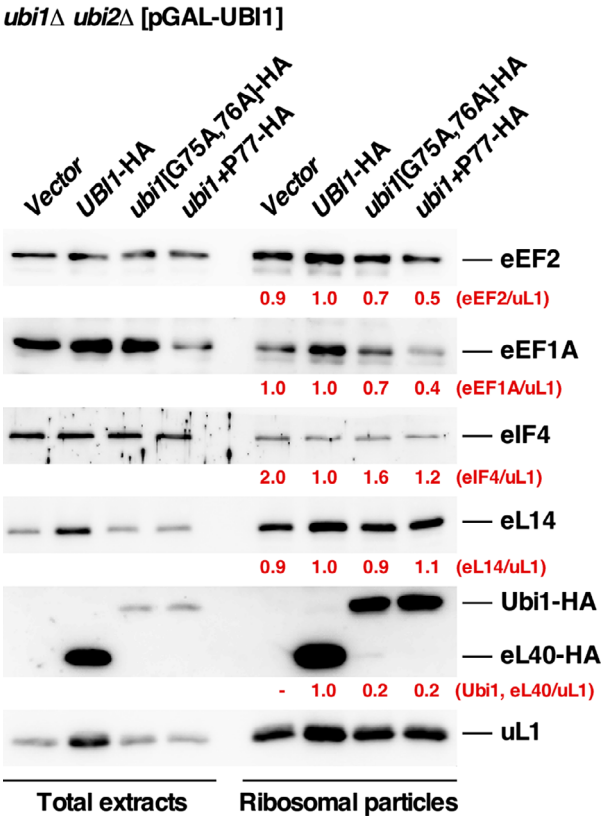

Fig. 7. Reduced binding of GTPases eEF1A and eEF2 to ribosomes containing the Ubi1 precursor protein. Haploid *ubi1*Δ *ubi2*Δ cells simultaneously harbouring pGAL22-UBI1 and either empty YCplac111 (*Vector*), YCplac111-UBI1-HA (*UBI1*-HA), YCplac111-ubi1.G75,76A-HA (*ubi1*[G75,76A]-HA), or YCplac111-ubi1+P77-HA (*ubi1*+P77-HA) were grown in liquid SGal-Trp-Leu media at 30 °C, then shifted for 6 h to liquid SD-Trp-Leu media, and harvested at an OD_{600} of around 0.8. Total extracts were prepared from each strain and used for purification of ribosomal particles. Samples containing equal amounts of protein were resolved by SDS/PAGE and analysed by western blotting. Antibodies against the HA tag or the uL1, eL14, eIF4A, eEF1A, and eEF2 proteins were used. The relative intensities of the specific bands from these western blots were quantified, and the ratios of the relative intensities between each of the detected proteins and the corresponding uL1 protein were calculated. The ratios obtained for the *UBI1*-HA sample, which were arbitrarily set to 1.0, were used for normalisation.

an impaired function of Efl1, expression of the uncleaved Ubi1[G75,76A]-HA or Ubi1+P77-HA variant proteins, but not of wild-type Ubi1-HA, in eL40-depleted cells, resulted in the cytoplasmic mislocalisation of plasmid-encoded Tif6-GFP (Fig. 8). As no such mislocalisation was observed upon eL40 depletion, the function of Efl1 seems not to be dependent on the presence of eL40, but appears to be specifically affected by the retained ubiquitin moiety at the N terminus of eL40. Moreover, this effect appears to be specific for Tif6-GFP as we did not observe any deficiency in cytoplasmic recycling of Mrt4-GFP (Fig. 8), which also displays a nucle(ol)ar steady-state localisation and whose Yvh1-promoted release enables cytoplasmic assembly of the r-protein uL10/P0 [30–33].

Altogether, these results suggest that the presence of uncleaved Ubi1 variant proteins in 60S r-particles may obstruct the recruitment of GTPases, which bind to the GTPase-associated centre of ribosomes, and therefore interfere with their proper activity; as a consequence, both final maturation of pre-60S r-particles and translation elongation processes are impaired.

Discussion

Ubiquitin is a small ubiquitous protein essential for protein degradation, although it also plays roles in a wide variety of other cellular processes [34]. In nearly all eukaryotes, ubiquitin is expressed as a polyubiquitin precursor, formed by few tandem repeats of ubiquitin, and as a fusion protein between a ubiquitin monomer and the r-proteins eL40 or eS31; only in few rare cases, ubiquitin appears to be fused to different proteins such as r-protein P1 or actin [35]. Free ubiquitin is generated by post-translational cleavage of the fusion proteins by specific, but still unidentified deubiquitinases. In exponentially growing yeast cells, ubiquitin is mostly derived from the r-protein fusions [7], suggesting a cross-talk between synthesis and degradation of proteins during active growth conditions. Moreover, the selection of eS31 and eL40 as the practically exclusive r-proteins fused to ubiquitin is almost invariant among eukaryotes, indicating that the ubiquitin moiety might play a direct role in the synthesis, ribosomal assembly and/or function of these two r-proteins. Strikingly, the particular location of these r-proteins in ribosomes, both approaching the binding site of translational GTPases (Fig. 1A), also raises the possibility of an intentional choice that may fulfil a regulatory role during translation.

In yeast, the proteolytic processing of the ubiquitin moiety from the linear Ubi1/2 and Ubi3 precursor proteins is a very rapid process ([5,19], this work). Several lines of evidence strongly suggest that the ubiquitin moiety of these precursors may act as a co-translational chaperone for proper synthesis of the corresponding r-proteins, thereby indirectly contributing to the efficient production of r-subunits [5,19]. Indeed, the expression levels of eS31 and eL40 are reduced when these are expressed form their genomic loci without the N-terminal ubiquitin moiety; accordingly, *ubi3*Δ*ub* and *ubi1*Δ*ub ubi2*Δ mutant cells display a severe sg phenotype due to a shortage of 40S and 60S r-

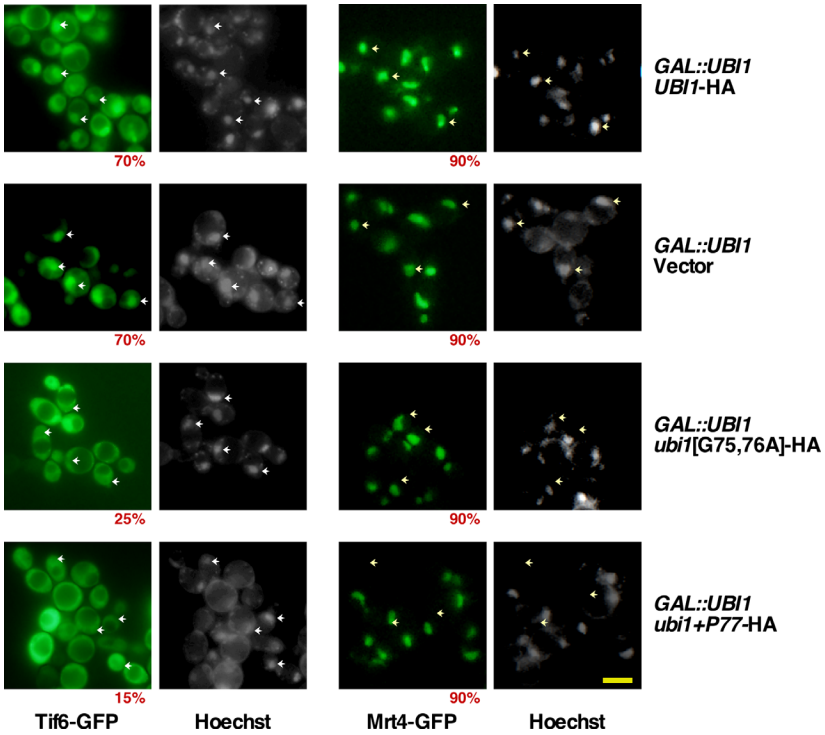

Fig. 8. Expression of the noncleaved Ubi1 precursor impairs cytoplasmic maturation of pre-60S particles. Haploid *ubi1*Δ *ubi2*Δ cells simultaneously harbouring pGAL22-UBI1 and either empty YCplac111 (*Vector*), YCplac111-UBI1-HA (*UBI1*-HA), YCplac111-ubi1.G75,76A-HA (*ubi1*[G75,76A]-HA), or YCplac111-ubi1+P77-HA (*ubi1*+P77-HA) were transformed with plasmids expressing C-terminally GFP-tagged Tif6 or Mrt4. Cells were grown in liquid SGal-Trp-Leu-Ura media at 30 °C and then shifted for 6 h to liquid SD-Trp-Leu-Ura media up to an OD_{600} of around 0.8. Hoechst was used to stain chromatin DNA. Then, cells were inspected by fluorescence microscopy. Arrows point to nuclear fluorescence. Approximately 200 cells were examined for each reporter; percentage of cells showing nuclear fluorescence for each GFP-tagged reporter are shown. Note that around 90% and 70% of cells of all four strains showed nuclear Tif6-GFP and Mrt4-GFP fluorescence, respectively, in selective SGal medium (not shown). Scale bar, 5 μm.

subunits respectively ([5,19], and our unpublished results). On the other hand, expression of these constructs from centromeric plasmids almost completely restores r-protein synthesis, ribosome production and cell growth ([5,19], and our unpublished results). Moreover, Deuerling and co-workers [36] have reported that the ribosome-associated Ssb-RAC and NAC chaperone complexes cooperatively assist in the *de novo* folding of r-proteins; in the absence of these chaperone systems practically all r-proteins tend to aggregate, with the apparent exception of eS31 and eL40. In agreement with these observations, addition of an N-terminal ubiquitin or a ubiquitin-like moiety is known to increase the expression and favour the solubility of different fusion proteins [37].

In contrast to this role as a chaperone, an active contribution of the ubiquitin moiety to ribosome biogenesis prior to cleavage is controversial and likely negligible. We have previously demonstrated that assembly of eS31 and eL40 into pre-ribosomal particles preferentially occurs in the nucleus and the cytoplasm respectively [15,17]. However, in no circumstance either the wild-type Ubi3 or Ubi1/2 fusion precursors could be detected by western blot, suggesting that the processing of the precursors into ubiquitin and the respective r-protein occurs very rapidly, possibly even co-translationally, and as a corollary before their assembly into the respective pre-ribosomal particles. Our experiments demonstrate that noncleaved variants of Ubi3 or Ubi1 can be incorporated into pre-ribosomal particles; however, this only occurs when these mutant precursors are not in direct competition with the processed wild-type r-proteins for their pre-ribosomal binding sites ([19] and this work). Thus, the presence of ubiquitin rather hampers than enables the assembly of eS31 and eL40 into pre-ribosomal particles.

While this observation applies to both noncleaved Ubi1 and Ubi3 variants, this study identifies two significant differences when comparing the phenotypes of the noncleaved *ubi1* mutants with those previously reported for the noncleaved *ubi3* mutants. First, the *ubi1*[G75,76A]-HA mutant is not viable, while the equivalent *ubi3*[G75,76A]-HA mutant only displays a sg phenotype [19]. Consistently, the Ubi3[G75,76A]-HA protein is partially processed into ubiquitin and eS31-HA [19], whereas the Ubi1[G75,76A]-HA variant protein is unable to produce the mature eL40A-HA r-protein. This dissimilarity is likely due to the different sequence around the junction between the ubiquitin moiety and the N-terminal end of both r-proteins, which consists of two adjacent glycines (G75–G76) followed by either an isoleucine (I77) in the case of Ubi1 or a third glycine (G77) in the case of Ubi3. Thus, it is possible that the G77 of the Ubi3[G75,76A]-HA variant protein is being used as an alternative cleavage site. In agreement with this possibility, the triple G75,76,77A mutation confers a lethal phenotype and

an almost complete impairment of the processing of the corresponding Ubi3[G75,76,77A]-HA variant protein into ubiquitin and eS31-HA [19]. Second, while the assembly of noncleaved Ubi3[G75,76A]-HA and Ubi3+P77-HA proteins yields mature 40S r-subunits that are competent to associate with 60S r-subunits and engage in active translation [19], the 60S r-subunits containing noncleaved Ubi1[G75,76A]-HA and Ubi1+P77-HA proteins are only inefficiently entering the pool of translating ribosomes. Moreover, while the cleavage-affecting *ubi3* mutations appear to minorly affect translation initiation, the corresponding *ubi1* mutations compromise the efficiency of translation elongation.

Our results reveal that the elongation defect could be due to the interference of the unprocessed ubiquitin present in Ubi1-containing 60S r-subunits with the binding and function of the elongation factors eEF1A and eEF2, which are recruited during translation to the GTPase-associated centre and are thus located in close proximity to eL40 (Fig. 1A). This finding therefore provides experimental evidence to a previous deduction, based on the analysis of the 60S r-subunit crystal structure from *Tetrahymena thermophila*, by Klinge *et al.* [38]. The GTPase Efl1 is structurally closely related to eEF2 [28,29], and it cooperates with Sdo1 to facilitate the release and recycling of Tif6, a ribosome biogenesis factor exhibiting ribosomal anti-association activity; thus, preventing premature and functionally inactive cytoplasmic pre-60S r-particles from engaging in translation [39–41]. Efl1 interacts with the 60S r-subunits in a similar manner as eEF2 [27,42] (see also, Fig. 1B); hence, it is expected that the unprocessed ubiquitin present in Ubi1-containing pre-60S r-particles also sterically hinders the binding of Efl1, as well as other translational GTPases, to ribosomes. Indeed, our results suggest a reduced activity of Efl1 within Ubi1-containing pre-60S particles since the release of Tif6 from cytoplasmic r-particles, which is promoted by Efl1, appears to be impaired in the noncleaved *ubi1* mutants. As Tif6 prevents r-subunit association [40,41,43], this fact could also contribute to the inability of Ubi1-containing 60S r-particles to efficiently enter the pool of translating ribosomes. This seems to be different in the case of preribosomal particles deprived of eL40, as our results suggest that neither the extent of eEF1A and eEF2 association with ribosomes nor Tif6 recycling are significantly affected upon eL40 depletion.

In summary, we have investigated in this study the *cis*-acting Ubi1-cleavage requirements and how the forced retention of the ubiquitin moiety on r-protein eL40 affects the biogenesis and function of 60S r-subunits. Our *in vivo* results strongly suggest that proteolytic removal of ubiquitin occurs prior to the assembly of Ubi1's r-protein component eL40 into pre-60S subunits. Additionally, they indicate that the retention of ubiquitin interferes with the efficient incorporation of eL40 into 60S r-particles and confers a lethal phenotype, which is, as suggested by our results, presumably due to the observed deficiency of Ubi1-containing 60S r-particles in subunit joining and global translation. Accordingly, the assembly of uncleaved Ubi1 partially impairs the recruitment and/or function of translational GTPases, likely due to a steric effect of the retained ubiquitin moiety, which projects into the location of the GTPase-associated centre. It will be of interest to learn whether similar defects will also be observed upon imposing a retention of the ubiquitin moiety on the eS31 and eL40 r-proteins in other, especially higher, eukaryotes. While a natural noncleaved form of Ubi3 has been described in the protozoan parasite *Giardia lamblia* as part of mature ribosomes [44], there is so far no evidence for naturally occurring noncleaved Ubi1 variants that assemble into functional ribosomes; these could potentially be very useful for clarifying how exactly the retained ubiquitin moiety interferes with the cytoplasmic maturation and/or translational function of 60S r-subunits. Finally, even though there is no experimental indication that the proteolytic removal of ubiquitin from eS31 and eL40 may serve to couple the supply of ubiquitin to the production and/or function of ribosomes, we believe it is reasonable to speculate, based on (a) the high evolutionary conservation of the Ubi1 and Ubi3 precursors, (b) the fact that these precursors generate r-protein constituents of both r-subunits, (c) the suspiciously proximal positions of eS31 and eL40 nearby the same functional centre within 80S ribosomes, and (d) the evidence that, at least in yeast, ubiquitin is mostly derived from the r-protein fusions in conditions of exponential growth, that this particular organisation may have evolved as a regulatory network for integrating and coordinating the synthesis and degradation of proteins in eukaryotes.

Materials and methods

Strains and microbiological methods

All yeast strains used in this study are derived from W303 (*MAT***a**/*MAT*α *ade2-1*/*ade2-1 his3-11,15*/*his3-11,15 leu2-3,112*/*leu2-3,112 trp1-1*/*trp1-1 ura3-1*/*ura3-1*) [45]. JDY925 (*MAT***a** *ubi1*::kanMX4 *ubi2*::kanMX4 [pAS24-RPL40A]) is a *rpl40* null strain that harbours the plasmid pAS24-RPL40A [17], which expresses Ubi1 under the transcriptional control of the regulatable *GAL1-10* promoter. TAY001, also named the *UBI1/2* shuffle strain, is a

segregant of the genetic cross between JDY925 and YDK11-5A (*MAT*α *ade3*::kanMX4) [25]. This strain is genotypically *MAT***a** *ubi1*::kanMX4 *ubi2*::kanMX4 *ade3*::kanMX4 and harbours the plasmid pHT4467Δ-UBI1; this strain forms red colonies and cannot grow on 5-FOA-containing plates since cells having lost this plasmid are not viable. To generate other strains, the *UBI1/2* shuffle strain was transformed with the indicated plasmids that carry wild-type or mutant alleles of *UBI1*, and transformants were restreaked on 5-FOA-containing plates to counterselect against the presence of pHT4467Δ-UBI1.

Yeast genetic techniques and growth media have been previously described [46]. Yeast transformation was done by the lithium acetate method [47]. Standard molecular biology techniques were as described [48]. For tetrad dissection, a Singer MSM 400 micromanipulator (Singer instruments, Roadwater, UK) was used. *Escherichia coli* DH5α was used for cloning and propagation of plasmids [48].

Plasmids

The plasmids used in this study are listed in Table 1. For most of the plasmids generated in this study, cloned DNA fragments were generated by PCR amplification. In particular, plasmids harbouring the various *ubi1* alleles were generated by a fusion PCR strategy. A single HA epitope was included at the C-terminal end of eL40A in wild-type and cleavage mutant versions of *UBI1*. pGAL22-UBI1, which allows the expression of Ubi1 under the control of the regulatable *GAL1-10* promoter, was generated by subcloning the ORF and terminator of *UBI1* into pGAL22 (CEN, *TRP1*). pTIF6-GFP [39], a gift from A. J. Warren, harbours wild-type *TIF6*, fused to the DNA sequence coding for GFP, in pRS316 (CEN, *URA3*). All constructs were verified by DNA sequencing. More information on the construction of the different plasmids will be available upon request. The description of the oligonucleotides used for cloning PCRs will also be provided upon request.

Protein extraction, western blot analyses and antibodies

Total yeast protein extracts were prepared by the method of Yaffe and Schatz [49], which immediately 'freezes' the *in vivo* protein content, thus, preventing rapid protein turnover reactions. Briefly, cultures were grown to an OD_{600} of around 0.8; about one OD_{600} of cells was harvested by centrifugation in Eppendorf tubes, washed with ice-cold distilled water and resuspended in 150 μL of a fresh 1.85 M NaOH/7.4% 2-mercaptoethanol solution. This mixture was held on ice for 10 min, and proteins were then precipitated by the addition of 150 μL of 50% (w/v) trichloroacetic acid. After a further 10 min on ice, tubes were centrifuged at full speed in an Eppendorf centrifuge for 2 min at 4 °C. Pellets were then washed with 1.5 mL of ice-cold acetone, and proteins were solubilised with Laemmli sample buffer for 4 min at 95 °C. The insoluble material was removed by centrifugation and proteins in the supernatants were resolved by SDS/PAGE on 14% Tris-Glycine-SDS polyacrylamide gels. Western blotting was performed by a standard procedure using the following primary antibodies: mouse monoclonal anti-HA 16B12 (Covance Inc., Pricenton, NJ, USA) and anti-Pgk1 (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA); rabbit polyclonal anti-uL1 (gift from F. Lacroute) [50], anti-eL14 (gift from G. Dieci) [51], anti-eIF4A (gift from P. Linder), anti-eEF1A (gift from T. G. Kinzy) [52], and anti-eEF2 (gift from T. G. Kinzy) [53]. Goat anti-mouse or anti-rabbit horseradish

Table 1. Plasmids used in this study

Name (collection name)	Relevant information	Reference
pHT4467Δ	*CEN6 (instable), URA3, ADE3*	[59]
pHT4467Δ-UBI1	*CEN6 (instable), URA3, ADE3*. Wild-type Ubi1; promoter and terminator *UBI1*	This study
pAS24-RPL40A	CEN; LEU2. Wild-type Ubi1; promoter *GAL1-10*; terminator *UBI1*	[17]
pGAL22	CEN; *TRP1;* promoter *GAL1-10*;	[60]
pGAL22-UBI1 (pDK4165)	CEN; *TRP1*. Wild-type Ubi1; promoter *GAL1-10*; terminator *UBI1*	This study
pTIF6-GFP	CEN; *URA3*. C-terminally GFP-tagged Tif6; promoter *TIF6* and terminator *ADH1*	[39]
YCplac33-MRT4-eGFP	CEN; *URA3*. C-terminally GFP-tagged Mrt4; promoter and terminator *MRT4*	[32]
YCplac111	CEN, *LEU2*	[61]
YCplac111-UBI1 (pDK4067)	CEN, *LEU2*. Wild-type Ubi1; promoter and terminator *UBI1*	This study
YCplac111-ubi1.G75A (pDK4034)	CEN, *LEU2*. Mutant Ubi1[G75A]; promoter and terminator *UBI1*	This study
YCplac111-ubi1.G76A (pDK4035)	CEN, *LEU2*. Mutant Ubi1[G76A]; promoter and terminator *UBI1*	This study
YCplac111-ubi1.G75,76A (pDK4036)	CEN, *LEU2*. Mutant Ubi1[G75,76A]; promoter and terminator *UBI1*	This study
YCplac111-ubi1+P77 (pDK4037)	CEN, *LEU2*. Mutant Ubi1+P77; promoter and terminator *UBI1*	This study
YCplac111-UBI1-HA (pDK4131)	CEN, *LEU2*. C-terminally 1xHA-tagged eL40A; promoter and terminator *UBI1*	This study
YCplac111-ubi1.G75A-HA (pDK4188)	CEN, *LEU2*. Mutant Ubi1[G75A]-HA; promoter and terminator *UBI1*	This study
YCplac111-ubi1.G76A-HA (pDK4189)	CEN, *LEU2*. Mutant Ubi1[G76A]-HA; promoter and terminator *UBI1*	This study
YCplac111-ubi1.G75,76A-HA (pDK4190)	CEN, *LEU2*. Mutant Ubi1[G75,76A]-HA; promoter and terminator *UBI1*	This study
YCplac111-ubi1+P77-HA (pDK4191)	CEN, *LEU2*. Mutant Ubi1+P77-HA; promoter and terminator *UBI1*	This study

peroxidase-conjugated antibodies (Bio-Rad laboratories, Hercules, CA, USA) were used as secondary antibodies. Immune complexes were visualised using a chemiluminescence detection kit (Super-Signal West Pico; Pierce; Thermo Fisher Scientific, WA, Massachusetts, USA) and a Chemi-Doc™ MP imaging system (Bio-Rad). Quantification of band intensities was done with the GELQUANT.NET software provided by biochemlabsolutions.com.

Ribosome preparations

Ribosomes were enriched from cell extracts as described previously [17,54]. Cells (200 mL cultures at mid-log phase) of the appropriate strains were washed and concentrated in 500 μL of ice-cold buffer 1 (10 mM Tris-HCl, pH 7.4; 20 mM KCl; 12.5 mM $MgCl_2$; 5 mM 2-mercaptoethanol) containing a protease inhibitor mixture (Complete EDTA-free; Roche Applied Science, Penzberg, Germany). Cells were disrupted by vigorous shaking with glass beads in a Precellys 24 homogenizer (Bertin Technologies; CNIM group, Paris, France) at 4 °C. Clarified fractions were obtained for each strain by centrifuging the extracts at maximum speed for 20 min at 4 °C in an Eppendorf microcentrifuge. These fractions were centrifuged in a TLA110 rotor in a Beckman-Coulter Optima™ Max centrifuge (Beckman-Coulter, Brea, CA, USA) at 351 956 *g* for 60 min at 4 °C. The pellets were resuspended in buffer 1 and stored at −80 °C before use. Ribosome concentration was determined by A_{260} and the Bradford assay (Bradford reagent; Bio-Rad). Proteins were separated by SDS/PAGE and assayed by western blot using the above-described antibodies.

Sucrose gradient centrifugation and fractionation

Cell extracts for polysome analyses were prepared according to Foiani *et al.* [55], as previously described [56], using an ISCO UA-6 system equipped to continuously monitor A_{254}. When needed, fractions of 0.5 mL were collected from the gradients, then proteins were extracted from each fraction by TCA precipitation as *exactly* described [57], and equal volumes were subjected to western blot analyses as described above.

Inspection of ribosome structures

Images of the r-particles bound to eEF2 or Efl1 were generated using the UCSF CHIMERA package [58], using the atomic model for selected cryo-EM structures deposited in the Protein Data Bank.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

DK and JC conceived and designed the experiments; SM-V, AF-P and JF-F performed the experiments; SM-V, AF-P, JF-F, DK and JC analysed the data; AF-P, DK and JC wrote the paper.

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