Yeast Uri1p promotes translation initiation and may provide a link to cotranslational quality control

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Translation initiation in eukaryotes is accomplished by a large set of translation initiation factors, some of which are regulated by signals monitoring intracellular and environmental conditions. Here, we show that Uri1p is required for efficient translation initiation in budding yeast. Indeed, *uri1Δ* cells are slow growing, sensitive to translation inhibitors and they exhibit an increased 80S peak in polysome profiles. Moreover, *GCN4* translation is derepressed in *uri1Δ* cells, strongly supporting an initiation defect. Genetic and biochemical experiments indicate that Uri1p interacts with the translation initiation factor eIF1A and promotes ternary complex (TC) recruitment to the 40S subunit. Interestingly, we found that Uri1p is also part of a chaperone-network, including the prefoldin Pfd6p and several other proteins involved in cotranslational quality control such as the ribosome-associated Hsp70 chaperone Ssb1p, the Hsp40 Sis1p and the translation elongation factor eEF1A. Together with genetic data, these interactions indicate that Uri1p may coordinate translation initiation and cotranslational quality control.

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Introduction

Regulated protein synthesis is a fundamental process that is carried out by ribosomes and a large number of translation factors. Chaperone complexes assist in the cotranslational folding of nascent polypeptides into their functional conformations (reviewed in Frydman, 2001). Protein translation can

be divided into three successive events. First, translation initiation leads to the correct positioning of the 80S ribosome at the AUG start codon, which in eukaryotes is accomplished by a plethora of well-described initiation factors. The process begins with the recruitment of a ternary complex (TC) consisting of the initiator methionyl-tRNA (Met-tRNA_i^{Met}), the initiation factor eIF2 and GTP to the 40S ribosomal subunit. The initiation factor eIF1A promotes and stabilizes TC binding to the 40S subunit, resulting in the formation of the 43S preinitiation complex (PIC), which besides the 40S subunit and the TC contains many other associated factors. The PIC is then recruited to the mRNA through the eIF4F-initiation factors that are associated with the 5' cap structure of the mRNA. The resulting complex scans the mRNA for the AUG codon, and recognition of the start codon triggers GTP hydrolysis by eIF2 followed by release of the initiation factors and joining of the 60S subunit. These rearrangements result in an elongation-competent ribosome, which is able to begin synthesis of the polypeptide (reviewed in Dever, 2002; Gebauer and Hentze, 2004).

The second step of protein synthesis, translation elongation, ensures the processive addition of amino acids to the growing polypeptide. Elongation is followed by translation termination, which leads to the release of the protein at the stop codon. Nascent and newly synthesized proteins are targets of co- and post-translational quality control mechanisms. In eukaryotes, several sets of chaperones assist the folding of nascent chains, including the nascent chain-associated complex, the Hsp70 system, the TriC chaperonin system and prefoldins (PFDs) (reviewed in Frydman, 2001). Strikingly, it has been estimated that up to 50% of newly synthesized proteins are cotranslationally degraded by the proteasome (Schubert *et al.*, 2000; Turner and Varshavsky, 2000), implying that efficient quality control mechanisms must exist that couple protein translation, protein folding and proteasomal degradation. Mistranslated and misfolded proteins may be detrimental for the cell and need to be removed at an early stage. However, to date, the mechanisms and coordination between translation and quality control remain poorly understood.

Protein synthesis is regulated in response to extracellular signals and intracellular growth and stress conditions, primarily at the level of translation initiation. Moreover, several chaperone systems are upregulated in response to stress conditions to cope with the increase of misfolded or denatured proteins. The two best-described mechanisms that control translation initiation include first, sequestration of the cap-binding eIF4-complex by eIF4E-binding proteins (4E-BPs), which is reversed under nutrient-rich conditions by phosphorylation of 4E-BP by the TORC1 kinase. The second initiation control mechanism involves Gcn2p-dependent phosphorylation of eIF2 α , which converts it into an inhibitor of its own guanine-nucleotide exchange factor (GEF) eIF2B

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Figure 1 Uri1p regulates *GCN4* translation independently of eIF2 α (Sui2p) phosphorylation. (A) Diagram representing regulation of *GCN4* translation and general translation by ternary complex (TC) levels. (B, C) The indicated strains transformed with *GCN4-lacZ* (uORFs 1–4) (pBL199) or *xxxxGCN4-lacZ* (uORFs with mutated initiation codons) (pBL201) reporters were grown under rich conditions, and β -galactosidase activity in extracts was quantified in Miller units. Error bars represent standard deviations of three (B) or four (C) individual transformants. The following strains were analysed: wild-type (YAD219), *uri1* Δ (YAD211), *gcn2* Δ (YAD352), *gcn2* Δ *uri1* Δ (YAD310), *sui2* Δ (p*SUI2*(S51A)) (YAD23) and *uri1* Δ *sui2* Δ (p*SUI2*(S51A)) (YAD324). (D) Protein extracts of non-starved wild-type (YAD135), *uri1* Δ (YAD327), *gcn2* Δ (YAD353), *gcn2* Δ *uri1* Δ (YAD355), *sui2* Δ (p*SUI2*(S51A)) (YAD359) and *uri1* Δ *sui2* Δ (p*SUI2*(S51A)) (YAD357) cells carrying TAP-tagged Gcn4p expressed from its genomic locus were blotted and probed with antibodies specific for Uri1p, proteinA (recognizing Gcn4p-TAP) and actin. (E) Exponentially growing wild-type (YAD135) and *uri1* Δ (YAD327) cells harbouring endogenously TAP-tagged Gcn4p were pelleted and resuspended in starvation medium lacking amino acids. TCA extracts were prepared at the indicated time points and the levels of Gcn4p-TAP and S51-phosphorylated eIF α was analysed by immunoblotting. A non-specific band serves as a loading control (bottom panel). (F) Serial dilutions of an equal number of *his1-29 gcn2-101 gcn3-101* (YAD37), *his1-29 gcn2-101 gcn3-101 gcd10-503* (YAD36) and *his1-29 gcn2-101 gcn3-101 uri1::Nat* (YAD379) cells were spotted on plates with complete SD medium or SD medium lacking histidin containing 10 mM 3-aminotriazole (3-AT). Plates were grown for 1–3 days at 30°C.

(Figure 1A) (reviewed in Dever, 2002; Gebauer and Hentze, 2004). Although the latter mechanism inhibits translation of most proteins, reduced TC levels are required to activate translation of a few stress-regulated proteins such as the transcription factors ATF4 in mammals and Gcn4p in yeast. Gcn4p controls the expression of > 500 target genes, among them many involved in amino acid biosynthesis. Its protein levels are upregulated in response to amino acid deprivation and other types of starvation or stress conditions by an elaborate translation regulation mechanism involving four upstream open reading frames (uORFs) in the 5'UTR of *GCN4*. Under rich conditions, when TC concentration is high, scanning 40S subunits are efficiently reloaded with TC after uORF1 and restart translation at uORFs 2, 3 or 4. Because of the GC-rich sequences surrounding the uORF4

stop codon, 80S ribosomes disassemble and do not reform at the *GCN4* start site, resulting in low levels of translated Gcn4p. Starvation conditions reduce TC levels, thereby causing less efficient TC re-loading after uORF1. Under these conditions, uORFs 2–4 are mostly by-passed and the PIC is not able to form before scanning has advanced to the *GCN4* start codon, resulting in increased *GCN4* translation under starvation conditions (reviewed in Hinnebusch, 2005).

This regulated mechanism of *GCN4* translation was successfully used as a genetic tool to identify factors involved in translation initiation. Indeed, *GCN4* expression under rich conditions indicates defects in translation initiation and several so-called Gcd[–] mutations (general control derepressed) were identified where the amount of TC bound to the 40S subunit was reduced and therefore *GCN4* translation

was increased. Typical Gcd^- mutants lead to *GCN4* derepression even in the absence of functional Gcn2p, suggesting that they affect proteins that regulate *GCN4* translation downstream or independently of Gcn2p (reviewed in Hinnebusch, 2005).

It has been shown earlier that *GCN4* is constitutively expressed in yeast cells lacking *URI1* (Unconventional pre-foldin Rpb5 Interactor, also known as *BUD27*) (Gstaiger *et al*, 2003). On the basis of the observation that yeast *uri1Δ* cells exhibit starvation-like phenotypes and that hURI is regulated by the TOR pathway in mammalian cells, it has been suggested that Uri1p has a conserved function in nutritional signalling. The Uri1 protein contains evolutionary conserved Rpb5p-binding and PFD domains, which have been shown to mediate binding of Uri1p to Rpb5p and Pfd6p, respectively. PFD domains are typical for PFDs, which in the PFD/GimC complex are known to function as molecular chaperones that assist in the folding of newly synthesized actin and tubulin (Geissler *et al*, 1998; Siegers *et al*, 1999). However, Uri1p is not a subunit of the canonical GimC complex and so far no functional connection to other PFDs has been reported. Human URI forms a PFD/GimC-like complex together with the PFD-proteins STAP1, PFD2 and PFD4-related (PFD4r) as well as the non-PFDs RPB5, TIP48 and TIP49 (Gstaiger *et al*, 2003). Moreover, hURI was found to associate with PP1 γ at mitochondria, where it is involved in a feedback loop to inhibit S6 kinase 1 on growth factor stimulation (Djouder *et al*, 2007). At present it is not clear whether these different human URI complexes are functionally connected.

In this study, we analysed the function of yeast Uri1p, starting from the observation that *uri1Δ* cells confer a Gcd^- phenotype. We found that Uri1p is involved in translation initiation, where it promotes TC recruitment to the 40S subunit. Moreover, Uri1p interacts with several nascent chain-associated proteins, and deletion of *URI1* renders chaperone mutants resistant to the misfolding inducing drug azetidine-2-carboxylic acid (AZC). Thus, we propose that Uri1p may be involved in coordinating translation initiation with cotranslational quality control.

Results

Uri1p is required to repress GCN4 translation independently of eIF2 α -(Sui2p) phosphorylation

Earlier studies have shown that Gcn4p is upregulated in *uri1Δ* cells by a mechanism that involves the uORFs in the 5'UTR of *GCN4* (Gstaiger *et al*, 2003). To investigate the molecular basis of this regulation, we compared the expression of wild type (*GCN4-lacZ*) and *GCN4-lacZ* reporter constructs harbouring mutations in the initiation codons of all four upstream uORFs (*xxxxGCN4-lacZ*) (Mueller and Hinnebusch, 1986). *GCN4-lacZ* expression was repressed in wild type but not *uri1Δ* cells (Figure 1B), whereas the *xxxxGCN4-lacZ* reporter was expressed at similar levels in both strains. These data imply that *GCN4* derepression in *uri1Δ* cells is not dependent on the promoter region, which is shared by both constructs, but requires functional uORFs.

A well-known mechanism to upregulate *GCN4* translation involves Gcn2p-dependent phosphorylation of eIF2 α on Ser51 (Figure 1A). However, deletion of *URI1* leads to a strong *GCN4-lacZ* induction in *gcn2Δ* cells, indicating that Uri1p function is not dependent on Gcn2p (Figure 1C; Gstaiger *et al*,

2003). Moreover, *GCN4-lacZ* expression was also induced in *uri1Δ* cells expressing a non-phosphorylatable S51A mutant of eIF2 α (encoded by *SUI2*) (Figure 1C), excluding the possibility that a kinase other than Gcn2p may phosphorylate Ser51 under these conditions. Immunoblotting experiments confirmed that TAP-tagged Gcn4p is expressed in *uri1Δ*, *uri1Δ gcn2Δ* and *uri1Δ sui2(S51A)* cells grown in nutrient-rich conditions (Figure 1D), whereas it was barely detectable in wild type, *gcn2Δ* and *sui2(S51A)* cells. We could not detect increased levels of eIF2 α phosphorylation in a *uri1Δ* strain under non-starvation conditions, whereas phosphorylation was efficiently induced on amino acid starvation in both wild-type and *uri1Δ* cells (Figure 1E).

Gcn2p-independent derepression of *GCN4* translation under rich conditions is typical for Gcd^- mutants (Harashima and Hinnebusch, 1986). Originally, Gcd^- mutants were isolated as suppressors of the inability of *gcn2-101 gcn3-101* double mutants to activate *GCN4* and its target genes in the histidine biosynthetic pathway. In a *his1-29* background, *gcn2-101 gcn3-101* double mutants are hypersensitive to 3-amino triazole (3-AT), an inhibitor of histidine biosynthesis, and Gcd^- mutants can suppress this sensitivity (Harashima and Hinnebusch, 1986). Indeed, deletion of the *URI1* gene in a *gcn2-101 gcn3-101 his1-29* strain restored its ability to grow on 3-AT-containing media, similarly to *gcd10-503* controls (Figure 1F). Taken together, these results show that, similar to known Gcd -proteins, Uri1p is required to repress *GCN4* translation under nutrient-rich conditions.

Cells lacking Uri1p are slow growing, hypersensitive to translation inhibitors and exhibit a translation initiation defect

Defects in protein translation generally cause reduced fitness such as slow growth and temperature sensitivity (Cuesta *et al*, 1998). Consistent with a role for Uri1p in protein translation, *uri1Δ* cells divided with a doubling time of 2.34 h compared with 1.52 h for isogenic wild-type controls (Figure 2A). Moreover, *uri1Δ* cells display reduced growth at 37°C and were hypersensitive to the translation inhibitors cycloheximide, paramomycin and hygromycin B (Figure 2B, data not shown). In contrast, *uri1Δ* cells were not hypersensitive to the DNA damaging agent methyl methan sulfonate, indicating that the sensitivity to translation inhibitors is specific and not caused by a general uptake defect (Supplementary Figure 1). All these sensitivities could be rescued by transforming *uri1Δ* cells with a plasmid carrying the *URI1* gene. As Gcn4p levels are induced in *uri1Δ* cells leading to upregulation of Gcn4p targets such as Gln3p and Gat1p (Gstaiger *et al*, 2003), we analysed the phenotype of *gcn4Δ uri1Δ* double mutants. *GCN4* deletion was unable to rescue the sensitivities and slow growth defects of *uri1Δ* cells (Figure 2A and B), indicating that these defects are not caused by upregulated Gcn4p targets.

To test which conserved domains of Uri1p are involved in the uncovered translation function of Uri1p, we transformed *uri1Δ* cells with plasmids expressing full-length Uri1p or the indicated Uri1p domains, and tested their ability to complement the temperature sensitivity, *GCN4* derepression and sensitivity to translation inhibitors (Figure 2C). Surprisingly, neither the PFD nor the Rpb5p-binding domain (RPB5) were functionally important *in vivo*, whereas the acidic region of Uri1p was necessary and sufficient to rescue all tested translation defects of *uri1Δ* cells.

Figure 2 Deletion of *URI1* affects general translation. (A) Over night cultures of wild-type (YAD219), *uri1Δ* (YAD211) and *uri1Δ gcn4Δ* (YAD308) strains were diluted to OD₆₀₀ 0.2 and grown in YPD for 9 h. OD₆₀₀ was measured at the indicated time points, and plotted against time (in hours) in semi-logarithmic graphical form. Bold lines represent exponential trend lines. Error bars represent the standard deviation of three independent experiments. Growth rates are wild-type, 1.5 h; *uri1Δ*, 2.34 h; *uri1Δ gcn4Δ*, 2.15 h. (B) Wild-type (YAD219), *uri1Δ* (YAD211) and *gcn4Δ uri1Δ* (YAD308) strains were transformed with an empty control vector or a plasmid expressing Uri1p from the *ADH*-promoter (pAD89). An equal number of cells were spotted in serial dilutions on YPD plates containing the indicated drugs in the following concentrations: cycloheximide 0.1 µg/ml, hygromycin B 0.05 mM. YPD plates without drugs were incubated for 2 days at 30 and 37°C, respectively, strains on drug plates were grown at 30°C for 3 days. (C) *uri1Δ* cells (YAD213) were transformed with plasmids expressing the indicated Uri1p fragments from the *ADH*-promoter, and tested for their ability to grow at 37°C (ts) or on plates containing the indicated drugs. The transformants were also tested for their ability to repress *HIS4*-LacZ expression (GCN4). In the lacZ-assay, increasing numbers of + indicate higher GCN4 expression, whereas for the sensitivity assays, more + indicate increased sensitivity. cx, cycloheximide; hygB, hygromycin B; GCN4, *HIS4*-lacZ assay. (D) Representative polysome traces of wild-type (YAD219), *uri1Δ* (YAD211) and *gcn4Δ uri1Δ* (YAD308) cells carrying an empty vector (pAD49), and *uri1Δ* cells transformed with a plasmid expressing *URI1* from the *ADH*-promoter (pAD89). Extracts were prepared as described in Materials and methods. Samples were centrifuged in low salt (100 mM NaCl) buffer on 15 to 50% sucrose gradients, and polysome profiles were obtained by measuring absorbation at 254 nm. Polysome/80S ratios are indicated.

To directly assess the role of Uri1p in protein synthesis, we analysed the proportion of actively translating ribosomes in polysome profiles of wild-type and *uri1Δ* cells. Yeast cell extracts were centrifuged through sucrose gradients to separate free ribosomal subunits from 80S ribosomes and polysomes. Interestingly, the 80S peak was considerably higher in extracts prepared from *uri1Δ* cells compared with wild-type controls, and the corresponding polysomes/80S ratio was significantly reduced (Figure 2D). A wild-type polysome profile was restored after introducing a plasmid carrying *URI1*, showing that this translation defect is indeed caused by the absence of Uri1p. Consistent with the results described

above, deletion of the *GCN4* gene in *uri1Δ* cells did not suppress the translation effect analysed by polysome gradients. Taken together, we conclude that Uri1p is required for efficient protein translation, by a mechanism independent of Gcn4p.

Uri1p regulates translation initiation by promoting efficient recruitment of the TC to 40S subunits

The observed translation defects in *uri1Δ* cells support a role of Uri1p in translation initiation, possibly by promoting recruitment of the TC to the 40S ribosome. We thus tested whether the Gcd⁻ phenotype of *uri1Δ* cells could be suppressed by a

multicopy plasmid (hcTC) overexpressing all components of the TC, consisting of three eIF2 subunits and Met-tRNA^{Met} (Asano *et al*, 1999). GCN4 expression was measured indirectly by following the activity of its target gene *HIS4*, either using a specific reporter construct (*HIS4-lacZ*), where Gcn4p drives the expression of β -galactosidase (Figure 3A), or the sensitivity to the histidine-biosynthesis inhibitor 3-AT (Figure 3B). Interestingly, TC overexpression reduced *HIS4-lacZ* levels in *uri1* Δ strains almost three-fold (Figure 3A), and likewise, TC

overexpression rescued the 3-AT-resistance of *uri1* Δ cells (Figure 3B). Together, these results provide genetic evidence that Uri1p may promote TC recruitment to 40S ribosomes (as suggested for eIF1A in Olsen *et al*, 2003).

As another indicator of TC levels, we examined diazaborine-induced halfmers in polysome profiles. The drug diazaborine inhibits 27S pre-rRNA processing, leading to a decrease in 60S levels, which in turn produces an excess of 40S subunits and PICs. As a result, cells exposed to diaza-

Figure 3 Uri1p has a function in TC recruitment. (A) Gcn4p activity was determined by measuring β -galactosidase activity of *HIS4-lacZ* expressed from its own promoter in wild-type (YBL320) or *uri1* Δ (YBL323) cells transformed with an empty control plasmid (pAD111), or the plasmids pADH-URI (pAD89) or hcTC (pAD116). (B) Serial dilutions of *his1-29 gcn2-101 gcn3-101* (YAD37) and *his1-29 gcn2-101 gcn3-101 uri1::Nat* (YAD379) cells, transformed with an empty vector (pAD111) or hcTC (pAD116) were spotted on plates containing selective medium with or without 5 mM 3-amino triazole (3-AT) to reveal the Gcd⁻ phenotype. Plates were photographed after 4 days at 30°C. (C) *gcd10-503* (YAD 36) and isogenic wild-type cells (YAD37) were treated with 10 μ g/ml diazaborine for 2 h before incubation with cycloheximide and cell lysis. Cell extracts were loaded on sucrose gradients and polysome profiles were obtained as described for Figure 2D. Halfmer shoulders are indicated by arrows. (D) Wild-type (YAD211) and *uri1* Δ (YAD219) cells were treated with the indicated amounts of diazaborine for two hours and processed as described for Figure 3C. (E) TCA extracts of wild-type (YAD219) and *uri1* Δ (YAD211) strains were separated by SDS-PAGE and analysed by ponceau staining (upper panel) or immunoblotting using antibodies specific for Uri1p, Rps3p and Rpl3p. (F) Wild-type (YAD211) and *uri1* Δ (YAD219) cells were grown without diazaborine (left panel) or with 10 μ g/ml diazaborine for 2 h. Cells were lysed without previous cycloheximide treatment in buffer devoid of Mg²⁺ and extracts were loaded on 7.5–30% sucrose gradients. Polysome profiles were obtained as described earlier, 60S/40S ratios are indicated.

borine accumulate additional PICs on actively translating mRNAs, which can be visualized as halfmers (small peaks on the heavy side of the monosomal and polysomal major peaks) in polysome profiles (Pertschy *et al*, 2004). As PIC formation on mRNAs involves binding of TC to the 40S subunit, we expect that halfmer formation induced by diazaborine should be reduced in mutants harbouring TC-recruitment defects. We tested this assumption using mutants in two well-described initiation factors. Gcd10p is required for tRNA^{Met} modification, and therefore *gcd10-503* cells exhibit reduced levels of TC, leading to derepression of *GCN4* (Anderson *et al*, 1998). Likewise, Gcd6p, a subunit of the initiation factor eIF2B, which acts as a GEF for eIF2 (Bushman *et al*, 1993), is required for high TC levels. Wild-type cells treated with 10 µg/ml diazaborine induce articulate halfmers, which indeed were reduced in *gcd10-503* and to a smaller extent in *gcd6-1* cells (Figure 3C; Supplementary Figure 2). Importantly, the same effect could be detected with *uri1Δ* cells on treatment with 5 or 10 µg/ml diazaborine (Figure 3D). To exclude the possibility that reduced 40S levels may explain this reduced halfmer formation, we first confirmed that the levels of the ribosomal proteins Rpl3p and Rps3p are comparable in *uri1Δ* compared with wild-type cells (Figure 3E). Moreover, we quantified the 60S/40S ratio in the absence and presence of diazaborine. For this purpose, we performed polysome profiles in the absence of cycloheximide to allow ribosome runoff and in the absence of Mg²⁺ to dissociate 40S from 60S subunits. As shown in Figure 3F, the 60S/40S ratio of *uri1Δ* strain is comparable to isogenic wild-type controls, suggesting that the reduction of halfmers on diazaborine treatment is likely caused by reduced PIC levels. Therefore, this result corroborates the interpretation that Uri1p promotes TC binding to 40S subunits *in vivo*.

To directly test this model, we biochemically measured binding of the TC-component eIF2α to 40S subunits. For this purpose, we treated cells with formaldehyde to cross-link PICs (Valasek *et al*, 2007), isolated 40S subunits by sucrose gradient centrifugation, and quantified 40S-associated eIF2α levels by western blots using the LI-COR imaging system. Indeed, *uri1Δ* cells exhibited a subtle but reproducible reduction of eIF2 levels associated with 40S ribosomes compared with wild-type controls (Figure 4A and B). This recruitment defect was further supported by the observation that Rps2p levels in the 40S fractions were significantly higher in *uri1Δ* cells. A similar effect has been described earlier for eIF1A mutants, which are defective for TC recruitment (Fekete *et al*, 2005, 2007). Together, these observations suggest that the number of uncharged 40S subunits is higher in *uri1Δ* cells compared with wild-type controls, supporting the notion that Uri1p promotes TC recruitment to 40S subunits.

Uri1p associates with ribosomes and interacts with eIF1A (Tif11p)

To examine the subcellular localization of Uri1p, we expressed a fully functional, amino-terminal GFP-fusion protein (GFP-Uri1p) from the *GPD*-promoter, and analysed its distribution by fluorescence microscopy. As shown in Figure 4C, GFP-Uri1p localized throughout the cytoplasm and was excluded from the nucleus and the vacuole, consistent with a role in protein translation.

We next tested whether Uri1p may associate with cytoplasmic ribosomes. To this end, we separated yeast cytosol

into a postribosomal supernatant and a ribosomal pellet in the presence of low or high salt concentrations (Figure 4D). As endogenous Uri1p levels were undetectable in this assay, we overexpressed HA-tagged Uri1p from the constitutive *ADH*-promoter. Interestingly, a significant fraction of HA-Uri1p was specifically recovered in the ribosomal pellet at low salt concentrations, but released into the supernatant under high salt conditions. As controls, we analysed the ribosome-associated chaperone Zuo1p, which like Uri1p is released under high salt conditions, and the core ribosomal protein Rpl16Ap, which remains stably bound to ribosomal particles under all conditions (Gautschi *et al*, 2001). These results indicate that at least part of Uri1p associates with ribosomes. The role of Uri1p in TC recruitment could indicate that the ribosome-associated fraction of Uri1p may bind to 40S subunits. However, although Uri1p was present in 40S fractions isolated by sucrose gradients (Supplementary Figure 3), it was not clear whether the presence of Uri1p in these fractions was due to specific association or due to trailing from the upper free protein fractions.

Interestingly, a cytoplasmic yeast two-hybrid screen previously identified a strong interaction between Uri1p and the translation initiation factor eIF1A (encoded by *TIF11*) (Möckli *et al*, 2007). eIF1A interacts with the acidic region and PFD domains of Uri1p (Supplementary Figure 4), suggesting that the interaction may be functionally important for translation initiation *in vivo*. Although *TIF11* is an essential gene, deletion of its C terminus confers phenotypes connected to translation initiation and TC recruitment (Fekete *et al*, 2005), which strongly resemble that of *uri1Δ* cells. Polysome profiles of *uri1Δ tif11Δ* double mutants revealed an additive phenotype compared with each individual mutant (Figure 5A), suggesting that both proteins may cooperate to ensure efficient translation initiation and TC recruitment *in vivo*. Consistent with this observation, *uri1Δ tif11Δ* double mutants were strongly impaired for growth, and hypersensitive to increased temperature stress and exposure to the translation inhibitors hygromycin B and cycloheximide (Figure 5B). Interestingly, we found that overexpression of full-length *TIF11* from a *GPD*-promoter was able to partially suppress the hygromycin B-sensitivity of *uri1Δ* cells, as shown by serial dilution spottings on rich medium (Figure 5C). Likewise, overexpression of eIF1A was able to suppress expression of the *GCN4-lacZ* reporter (Figure 5D), suggesting that increased levels of eIF1A can partially bypass the need for Uri1p in translation initiation. Taken together, the functional and physical interaction with eIF1A, which is known to facilitate TC recruitment, support the notion that Uri1p promotes TC binding to 40S subunits.

Uri1p physically interacts with multiple components involved in protein synthesis

In addition to eIF1A, the cytoplasmic yeast two-hybrid screen identified 14 other Uri1p interactors, among which at least eight can be linked to protein synthesis (Möckli *et al*, 2007). These candidates include the small ribosomal subunit protein Rps4A, the elongation factor eEF1A, and one of two subunits of eEF1B (Supplementary Table S1). Moreover, several chaperone proteins interacted with Uri1p by two-hybrid assay, including the Hsp70- and Hsp40 family members Ssb1p and Sis1p, both of which have been directly implicated in translation (Nelson *et al*, 1992; Zhong and Arndt, 1993; Rakwalska

Figure 4 Uri1p promotes TC recruitment to 40S ribosomal subunits. **(A, B)** TC levels on 40S subunits were measured by detecting eIF2α in 40S fractions of sucrose gradients. Wild-type (YAD219) and *uri1Δ* (YAD211) cells were transformed with a plasmid expressing Rps2p-GFP (pAD10). After cross-linking cells with HCHO, extracts were resolved by sedimentation through a 7.5–30% sucrose gradient, and the indicated proteins were detected by immunoblotting and quantified using the LI-COR imaging system. The fractions containing free 40S subunits are boxed. The ‘merge’-panel overlays Rps2p-GFP and the eIF2α signals. **(A)** Three independent experiments were quantified and shown as average values with standard deviation **(B)**. **(C)** The subcellular localization of Uri1p-GFP was determined by GFP-fluorescence microscopy in *uri1Δ* cells (YAD211) transformed with the plasmid pBL22. DNA was visualized by Hoechst-staining, and vacuoles with FM464. **(D)** Uri1p is loosely associated with ribosomes. Experimental strategy used for ribosomal association assay (left panel). Cell extracts of wild-type cells carrying a plasmid expressing *URI1* under control of the *ADH*-promoter were spun through sucrose cushions containing low (120 mM) or high (800 mM) salt concentrations. Equivalent portions of the supernatant (S), resuspended pellet (P) and total extract (TE) were immunoblotted with the indicated antibodies. A TCA extract prepared from wild-type cells was included as a control (C).

and Rospert, 2004). Finally, Uri1p interacted with the peptide-prolyl cis-trans isomerases Fpr1p and Crp1p, which in analogy to the bacterial trigger factor might be involved in folding of newly synthesized proteins. As expected from earlier results (Gstaiger *et al*, 2003), Uri1p also bound the PFD Pfd6p and the RNA polymerase subunit Rpb5p in this yeast two-hybrid system (Möckli *et al*, 2007). To biochemically confirm these interactions, we immunoprecipitated the

HA-tagged candidate proteins and monitored their binding to myc-tagged Uri1p expressed from its endogenous locus (Möckli *et al*, 2007). In addition, we co-immunoprecipitated GFP-tagged Uri1p expressed in strains carrying TAP-tagged Ssb1p, Sis1p, eEF1Bγ (Tef4p) and for control Pdf6p, expressed from their endogenous promoters. Indeed, we observed a specific interaction of Uri1p with eEF1A (Tef1p), eEF1Bγ (Tef4p) and Ssb1p (Figure 6A; Möckli *et al*, 2007).

Figure 5 Uri1p interacts with eIF1A (Tif11p). (A) Representative polysome traces of *tif11Δ* (pTIF11ΔC) (YAD407) and *uri1Δ tif11Δ* (pTIF11ΔC) (YAD399) prepared as described in the legend of Figure 2D. The polysome to 60S ratio was quantified and listed below. (B) Wild-type (YAD211) and *uri1Δ* (YAD219) cells transformed with an empty vector (pAD48) and *tif11Δ* (pTIF11) (YAD403), *tif11Δ* (pTIF11ΔC) (YAD407), *uri1Δ tif11Δ* (pTIF11) (YAD414) and *uri1Δ tif11Δ* (pTIF11ΔC) (YAD399) cells were grown in selective medium, and serial dilutions were spotted on YPD plates containing the indicated drugs in the following concentrations: hygromycin B 0.08 mM, cycloheximide 0.07 μg/ml. Plates were grown for 3 days at 30 and 37°C, respectively. (C, D) Wild-type (YAD219) and *uri1Δ* (YAD211) strains were transformed with an empty vector (pAD48), or plasmids expressing Uri1p from the *ADH*-promoter (pAD89) or *TIF11* from the *GPD*-promoter (pAD118). Spotting conditions are described in Figure 5B. (D) The strains were also tested for the expression of the *GCN4-lacZ* reporter construct (pBL199). Error bars represent standard deviations of eight individual transformants.

To establish the relationship among the Uri1p-BPs, we examined the interactions among full-length Uri1p, Ssb1p, Sis1p, Pfd6p, Rpb5p and eEF1A by cytoplasmic two-hybrid assays. As negative controls, we used the empty vectors (pASO and NubG), and the three PFDs Pfd4p, Pfd2p and Pfd1p, which did not interact with Uri1p. Interestingly, Ssb1p, eEF1A, Pfd6p and Uri1p formed the core of a small Uri1p interaction network, as each of them showed strong interactions with at least three of the other proteins (Figure 6B; Supplementary Figure 5). Consistent with the co-immunoprecipitation experiments, where the interaction of Uri1p with Sis1p was not detectable, binding of Sis1p to Ssb1p and Pfd6p was stronger compared with its interaction with Uri1p, suggesting that one of these proteins may bridge the interaction of Sis1p and Uri1p. Together, these results indicate that Uri1p functions in a network at the interface of translation and protein quality control together with Ssb1p, eEF1A and Pfd6p.

Functional interaction of Uri1p with the CLIPS Pdf6p and Ssb1/2p

Ssb1/2p and the GimC PFD complex have recently been classified as chaperones linked to protein synthesis (CLIPS)

(Albanese *et al*, 2006). Cells lacking CLIPS function are viable but hypersensitive to translation inhibitors, similar to *uri1Δ* cells. To examine a possible functional relationship between Uri1p and CLIPS chaperones, we compared the phenotypes of single and *uri1Δ* double mutants. As expected (Albanese *et al*, 2006), we found that both the *pfd6Δ* and *ssb1Δ ssb2Δ* strains were sensitive to the translation inhibitor cycloheximide and the misfolding-inducing agent AZC (Figure 6C), supporting the notion that they function as CLIPS. The combined deletion strain *uri1Δ ssb1Δ ssb2Δ* was clearly sicker than the *uri1Δ* or the *ssb1Δ ssb2Δ* strains, and this triple deletion strain was extremely sensitive to the translation inhibitor cycloheximide. Similarly, *uri1Δ pfd6Δ* cells were hypersensitive to cycloheximide, suggesting that the functions of Uri1p and these chaperones in protein translation are additive. In accordance with this result, the chaperone mutants did not affect *GCN4-lacZ* expression (Figure 6D), indicating that unlike Uri1p they are not involved in TC recruitment. Moreover, in contrast to *uri1Δ*, strains carrying deletions in any of the PFD genes are sensitive to the microtubule-depolymerizing drug benomyl (Figure 7B), consistent with the known role of the GimC PFD complex

Figure 6 Uri1p interacts with proteins involved in protein quality control. **(A)** Co-immunoprecipitation assays with endogenously tagged Uri1p-interacting proteins. TAP-tagged Pfd6p (YAD349), Ssb1p (YAD155), Sis1p (YAD156), eEF1B γ (YAD119) and a non-tagged wild-type control strain (YAD219) were transformed with a plasmid expressing *URI1-GFP* (pBL22) expressed from the *GPD*-promoter. TAP-tagged proteins were immunoprecipitated, and associated Uri1p-GFP was detected by immunoblotting (upper panel). Arrowheads indicate the immunoprecipitated TAP-tagged proteins; an unspecific band migrating at the same size as Ssb1p-TAP are indicated with an asterisk. **(B)** Schematic representation of the Uri1p interaction network as revealed by cytoplasmic two-hybrid analysis (for source data see Supplementary Figure 5). Bold arrows mark stronger interactions; arrowheads at both ends indicate that the interaction was detected in both directions. Rounded arrows highlight components that interact with themselves. **(C)** Wild-type (YAD219), *uri1* Δ (YAD211), *ssb1* Δ *ssb2* Δ (YAD194), *pfd6* Δ (YAD282), *uri1* Δ *pfd6* Δ (YAD262) and *ssb1* Δ *ssb2* Δ *uri1* Δ (YAD234) cells were spotted in serial dilutions on YPD containing as indicated cycloheximide 0.07 μ g/ml, hygromycin B 0.08 mM or AZC 1 mg/ml. Plates were incubated at 30 or 37°C for 2 or 3 days. **(D)** Expression of the *GCN4-lacZ* reporter construct (pBL199) was measured in wild-type (YAD219), *uri1* Δ (YAD211), *ssb1* Δ *ssb2* Δ (YAD194) and *pfd6* Δ (YAD282) strains. Error bars represent standard deviations of five individual transformants. **(E)** Wild-type (YAD219), *uri1* Δ (YAD211), *pfd6* Δ (YAD282), *uri1* Δ *pfd6* Δ (YAD262) cells carrying an empty vector as well as *uri1* Δ *pfd6* Δ cells transformed with plasmids carrying full-length *URI1* (pNM100) or the indicated domains (pNM107, pNM103) were spotted in serial dilutions on SD-Leu plates containing as indicated cycloheximide (0.07 μ g/ml) or AZC (0.2 mg/ml).

in tubulin folding (Geissler *et al*, 1998). Although *ssb1* Δ *ssb2* Δ and *pfd6* Δ cells were sensitive to AZC, *uri1* Δ *ssb1* Δ *ssb2* Δ cells were slightly resistant to AZC compared to the

ssb1 Δ *ssb2* Δ strain. Strikingly deletion of *URI1* restored full resistance of *pfd6* Δ cells to this drug. In contrast to the PFD domain, this effect was reverted by plasmids expressing

Figure 7 Suppression of AZC sensitivity of *pfd6Δ* cells by deletion of *URI1* may be caused by a slowdown in translation. (A) Wild-type (YAD219), *uri1Δ* (YAD211), *pfd6Δ* (YAD282), *uri1Δ pfd6Δ* (YAD262), *pfd6Δ gcn4Δ* (YAD485) and *pfd6Δ gcn4Δ uri1Δ* (YAD460) strains were spotted in serial dilutions on YPD plates containing the indicated drugs AZC (1 mg/ml), cycloheximide (0.07 μg/ml), benomyl (25 μg/ml) and incubated for 3 days at 30 or 37°C, respectively. (B) Serial dilutions of wild-type (YAD219), *uri1Δ* (YAD211), *pfd6Δ* (YAD282), *uri1Δ pfd6Δ* (YAD262), *pfd4Δ* (YAD426), *uri1Δ pfd4Δ* (YAD428), *pfd5Δ* (YAD291) and *uri1Δ pfd5Δ* (YAD431) strains were spotted on YPD plates as described for panel (A). (C) *tif11Δ* (pTIF11) (YAD462), *tif11Δ*(pTIF11ΔC) (YAD464), *pfd6Δ tif11Δ*(pTIF11) (YAD468), *pfd6Δ tif11Δ*(pTIF11ΔC) (YAD470) strains were spotted under the same conditions as described in (A) and (B). (D) A model illustrating the functions of Uri1p in protein translation. (a) Uri1p promotes TC loading during translation initiation and interacts with the translation initiation factor eIF1A. (b) In addition, Uri1p interacts with several components involved in cotranslational quality control. We speculate that misfolded nascent chains may recruit Uri1p to the cotranslational quality control machinery, which in turn might lead to reduction of the translation initiation function of Uri1p. Black, Uri1p; dark grey, Uri1p interactors; white, other factors involved in translation initiation.

full-length or the acidic domain of Uri1p (Figure 6E). Importantly, this AZC resistance is not altered by deletion of *GCN4* (Figure 7A), excluding the possibility that Gcn4p-induced increase of prolin biosynthesis (Li and Brandriss, 1992) might reduce incorporation of the prolin analog AZC. To test whether suppression of AZC sensitivity by deletion of *URI1* was specific for Uri1p interactors, we compared AZC sensitivity of strains deleted for other PFDs that do not physically interact with Uri1p (Supplementary Figure 6), either singly or in combination with deletion of *URI1*. Similar to *pfd6Δ*, deletion of *URI1* also suppressed the AZC sensitivities of *pfd4Δ* and *pfd5Δ* cells (Figure 7B), suggesting that this phenotype may be explained by a general mechanism such as the reduction of translation initiation. Indeed, the *tif11ΔC* mutation, which causes a strong translation initiation defect, also suppressed AZC sensitivity of *pfd6Δ* cells (Figure 7C).

Discussion

In this study, we show that yeast Uri1p is required for efficient translation initiation, most likely by promoting TC recruitment to 40S subunits. Moreover, Uri1p interacts with eEF1A and the CLIPS Ssb1,2p and Pfd6p, which are involved in folding and degrading newly synthesized proteins. Our results thus suggest that Uri1p may mediate between translation initiation and quality control.

Uri1p regulates translation initiation by promoting recruitment of TC to 40S subunits

Translation initiation requires that the TC, which consists of the methionine-charged initiator tRNA and GTP-bound initia-

tion factor eIF2, is recruited to the 40S subunit. Several lines of evidence strongly suggest that Uri1p promotes this step. First, *GCN4* translation is upregulated in *uri1Δ* cells under rich conditions, and this phenotype is suppressed by a plasmid overexpressing all components of the TC (Figure 3A and B). Second, the formation of aberrant halfmers induced by diazaborine treatment is reduced in *uri1Δ* cells (Figure 3D). Halfmers in polysome profiles reflect mRNAs with one or several ribosomes and an additional 40S subunit and can be caused by a reduction of 60S subunits or an excess of 40S subunits. As the ratio of the 60S and 40S peaks in polysome profiles, and Rpl3p and Rps3p levels by western blots are unchanged in wild-type and *uri1Δ* cells (Figure 3E and F), the first two possibilities are unlikely. Moreover, because the free 40S peak on diazaborine treatment is increased in *uri1Δ* compared with wild-type cells (Figure 3D), a negative effect on 40S ribosome biogenesis is unlikely. However, the reduction of halfmers on diazaborine treatment can be explained by inefficient TC recruitment to 40S subunits, which leads to reduced PIC levels, thus supporting a role of Uri1p in TC loading. Third, sucrose-gradient analysis of cross-linked protein extracts revealed reduced levels of the TC component eIF2α in 40S fractions in *uri1Δ* cells (Figure 4A and B). This relatively modest biochemical effect indicates that Uri1p is not a central translation initiation factor, but rather increases the efficiency of this process. Finally, Uri1p genetically and physically interacts with the translation initiation factor eIF1A (Figure 5), which was earlier found to function in TC recruitment, PIC assembly, ribosomal scanning and AUG selection (Olsen *et al*, 2003; Fekete *et al*, 2005, 2007). The carboxy-terminus of eIF1A is

required for efficient TC recruitment (Olsen *et al*, 2003; Fekete *et al*, 2005), and this defect is worsened in cells lacking Uri1p function. Conversely, overexpression of eIF1A can partially suppress the hygromycin B sensitivity of *uri1Δ* cells (Figure 5C), suggesting that Uri1p and eIF1A have overlapping functions in the recruitment of TC to the 40S subunit (PIC assembly). Taken together, our results thus suggest that Uri1p may support translation initiation by directly promoting TC recruitment and/or activating initiation factors such as eIF1A. Although under normal conditions lack of Uri1p results in a global reduction of translation initiation, Uri1p is required to cope with stress conditions such as high temperature or the presence of translation inhibitors (Figure 2B).

Uri1p interacts with CLIPS

Our results together with earlier published data (Gstaiger *et al*, 2003; Möckli *et al*, 2007) also revealed a physical and functional interaction of Uri1p with several factors involved in protein synthesis downstream of translation initiation, including the translation elongation factors eEF1A (Tef1p, Tef2p) and eEF1B γ (Tef4p), the Hsp70 chaperone Ssb1, 2p and the Gim-complex component Pfd6p. Both Pfd6p and Ssb1, 2p belong to a set of chaperones called CLIPS, which in contrast to heat-shock chaperones, but similar to various translation components, are downregulated at the transcriptional level in response to heat shock (Albanese *et al*, 2006). Interestingly, *URI1* is also downregulated on heat-shock treatment as judged by microarray data (Gasch *et al*, 2000). However, in contrast to cells deleted for bona fide members of the CLIPS family, *uri1Δ* cells are not sensitive to the misfolding-inducing agent AZC (Figures 6 and 7). Moreover, unlike deletion strains of all yeast PFDs, *uri1Δ* cells are not sensitive to the presence of the microtubule-destabilizing drug benomyl (Figure 7A–C). Finally, the PFD domain is not required for the function of Uri1p *in vivo* (Figure 2C). Together, these data suggest that Uri1p may not directly participate in protein folding, but rather functions as a regulator at the interface between translation and protein quality control.

Uri1p may link translation initiation with cotranslational quality control

What could be the role of a non-essential factor promoting translation initiation that interacts with several components of the quality control machinery? It is possible that Uri1p is involved in a feedback mechanism that actively prevents translation initiation under conditions when misfolded proteins accumulate. For example, Uri1p may be recruited to nascent chains by its interactors eEF1A (Tef1p), Pfd6p, Ssb1,2p and Sis1p under conditions when increased levels of misfolded proteins are being synthesized, thereby reducing translation initiation (Figure 7D). Indeed, reducing translation initiation by deletion of *URI1* or eIF1A mutations restores growth of chaperone mutants such as *pdf6Δ* cells on media containing AZC. A comparable feedback mechanism known as the ‘unfolded protein response’ (UPR) exists in the endoplasmic reticulum of higher eukaryotes. Accumulation of unfolded proteins in the ER dissociates the Hsp70 chaperone BIP from the transmembrane kinase PERK, which in turn phosphorylates and inhibits eIF2 α in the cytosol and thereby inhibits translation initiation by the same mechanism as scGcn2p (Harding *et al*, 2000; reviewed in Schroder and Kaufman, 2005).

In addition to promoting correct folding of newly synthesized proteins, quality control mechanisms also trigger degradation of misfolded proteins. In the endoplasmic reticulum, the UPR is closely coupled to ERAD (ER-associated degradation) (reviewed in Buck *et al*, 2007). Likewise, nascent peptides in the cytoplasm can be ubiquitinated cotranslationally (references in Chuang and Madura, 2005), but it remains unclear how the ubiquitination machinery recognizes these proteins. A role in cotranslational protein degradation has been suggested earlier for the Uri1p-interacting proteins eEF1A (Tef1p, Tef2p) as well as Ssb1,2p and Sis1p (Zhong and Arndt, 1993; Gonen *et al*, 1994; Ohba, 1997; Rakwalska and Rospert, 2004; Chuang *et al*, 2005). It is interesting to note in this context that human Uri1p was identified as a protein interacting through STAP1 with Skp2p, which functions in a cullin1-based E3 ubiquitin ligase (Gstaiger *et al*, 2003). It is thus possible that Uri1p may help to recruit the ubiquitination machinery to mistranslated proteins by interacting with Ssb1,2p, eEF1A and Pfd6p. This might lead to reduced levels of Uri1p functioning in translation initiation and contribute to slowing down translation initiation to allow cells to cope with difficult conditions.

Materials and methods

Strains (Supplementary Table S2) and plasmids (Supplementary Table S3) are described in Supplementary data.

Antibodies

The following antibodies were used: Actin (Chemicon), myc (Gramsch Laboratories), PAP (peroxidase anti-peroxidase) (Sigma), GFP (Roche), HA-11 (Convance), eIF2 α -P (Genetex), Zuo1p (S Rospert), Rpl16p (S Rospert), Sui2p (T Dever), Rps3 (M Seedorf), Rpl3 (J Warner). Uri1p-specific antibodies were generated by immunizing rabbits with a recombinant Uri1p-fragment encompassing amino acids 165–331. Antibodies were affinity purified and characterized as shown in Figures 1D and 3E.

LacZ-assays

β -galactosidase activity in strains bearing a *GCN4-lacZ* (uORFs 1–4) (pBL199) or *xxxxGCN4-lacZ* (mutated uORFs) (pBL201) reporter plasmid or strains carrying endogenous *HIS4-lacZ* (YBL320) was measured using standard assays detecting ONPG (o-nitrophenyl- β -D-galactoside) cleavage by β -galactosidase, as described earlier (Valtz and Peter, 1997). β -galactose levels were quantified as Miller units $(OD_{600} \times 1000)/(OD_{420} \times \text{min})$, where OD_{600} indicates the density of the culture and absorption at OD_{420} denotes the amount of cleaved ONPG in a given time [min].

Polysome analysis and diazaborine experiments

Yeast cultures (100 or 200 ml) were grown to exponential phase, and treated with 100 μ g/ml cycloheximide for 10 min on ice before centrifugation. Pellets were washed twice and resuspended in 500–800 μ l lysis buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 30 mM MgCl₂, 100 μ g/ml cycloheximide, 0.2 mg/ml heparin, 0.2 μ l/ml DEPC). Cells were lysed by bead-beating and lysates (10 OD_{260} absorption units) were loaded on 12.2 ml 15–50% linear sucrose gradients prepared in 50 mM Tris-acetate (pH 7.5), 50 mM NH₄Cl, 12 mM MgCl₂. The gradients were centrifuged at 39 000 rpm in a Beckman SW41 rotor at 4°C for 2 h 45 min, followed by analysis with an ISCO UV-6 gradient collector. The polysome to 80S ratio (P/80) was determined using ImageJ software (<http://rsb.info.nih.gov/ij/>).

For diazaborine experiments, 50 ml cultures were grown to OD_{600} 0.5, at which time 5 or 10 μ g/ml diazaborine (kindly provided by Gregor Hoegenauer) was added for 2 h at 30°C. Cycloheximide was added to a final concentration of 100 μ g/ml, and polysome profiles were obtained as described above.

To determine the 60S/40S ratio by polysome profiling, 100 ml wild-type (YAD219) and *uri1Δ* (YAD211) cells were grown to exponential phase and incubated with 10 μ g/ml diazaborine without cycloheximide addition before lysis. Cells were lysed using the

same procedure as described above, but $MgCl_2$ was omitted in the lysis buffer. Extracts were loaded on a 7.5–30% sucrose gradient, centrifuged and analysed as described.

PIC cross-linking using formaldehyde (HCHO)

In total, 100 ml wild-type (YAD219) and *uri1Δ* (YAD211) cells transformed with a plasmid expressing Rps2p-GFP (pAD10) were grown to exponential phase and incubated on ice with 1% formaldehyde for 1 h as described (Valasek *et al*, 2007). Cross-linking was stopped by the addition of glycine to 0.1 M final concentration. Cells were centrifuged, and one volume cell was resuspended in one volume lysis buffer (20 mM Tris (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, protease inhibitor cocktail (Roche), 5 mM NaF, 1 mM DTT, 1 mM PMSF). Subsequently, cells were lysed with one volume glass beads using a beat beater. About 20 OD₂₆₀ units of lysate were applied on 7.5–30% sucrose-gradients and centrifuged for 5 h at 41 000 rpm. A measure of 500 µl fractions were collected and precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 20%. Pellets were washed in ice-cold acetone and resuspended in loading buffer. Western blot signals were quantified using the LI-COR imaging system and ImageJ software. Input-ratios ($Rps2_{input}(wt)/Rps2_{input}(uri1Δ)$) and $eIF2α_{input}(wt)/eIF2α_{input}(uri1Δ)$ were used to normalize the 40S signals and adjust differences in loading. Then, ratios of 40S fractions were calculated ($Rps2_{40S}(uri1Δ)/Rps2_{40S}(wt)$) and ($eIF2α_{40S}(uri1Δ)/eIF2α_{40S}(wt)$). Average values and standard deviation of three independent experiments were calculated.

Microscopy

Localization of pGPD-GFP-URI1 was determined in *uri1Δ* cells, which were grown to exponential phase on minimal medium lacking uracil at 30°C. Vacuoles were stained with FM460 (final conc. 16 µM), incubated for 30 min at 30°C, followed by washing with SD-Ura medium and further incubation for 30 min at 30°C. During the second incubation, the DNA-stain Hoechst (Hoechst 33342) was added (2 µg/ml final conc.). Samples were analysed on a Zeiss Axiovert 200 M fluorescence microscope.

Ribosome-binding assay

Ribosome binding of Uri1p was examined by ultracentrifugation through a sucrose cushion containing low (120 mM) or high (800 mM) concentrations of potassium acetate. Wild-type (YAD219) and *uri1Δ* (YAD211) cells were grown in 100 ml selective medium, pelleted and resuspended in 2 ml lysis buffer (10 mM Tris pH 7.5, 50 mM HEPES pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 10%

glycerol, 0.1% TritonX-100, 1 mM DTT, protease inhibitor cocktail (Roche)). Cells were broken using a one shot cell disrupter (constant systems LTD), and the lysate was cleared by centrifugation at 4°C for 15 min at full speed in an eppendorf-centrifuge (5417R), followed by 30 min spinning at 80 000g using a TLA 120.2 rotor in a Beckman coulter Optima LE-80K ultracentrifuge. A measure of 60 µl extract were removed for the 'total'-sample, and 60 µl were loaded on a 90 µl low-salt (120 mM potassium acetate) or high-salt (800 mM potassium acetate) sucrose cushion (25% sucrose, 2 mM DTT, 5 mM magnesium acetate, protease inhibitor cocktail (Roche)). After centrifugation for 2–4 h at 200 000g in a TLA100 rotor, cytoplasmic supernatants were collected and ribosomal pellets resuspended in 1 × buffer (2 mM DTT, 5 mM magnesium acetate, 120 mM potassium acetate). 'Total'-samples, supernatant fractions and resuspended pellets were precipitated by TCA (final concentration 5%) and resuspended in loading buffer. Corresponding amounts were analysed by SDS-PAGE and immunoblotting.

Immunoprecipitation experiments

For immunoprecipitation of endogenously TAP-tagged proteins, 100 ml yeast cultures were grown and one cell volume was resuspended in one volume RIPA buffer (5 mM HEPES pH 7.4, 1% Triton, 1% NaDeoxycholate, 0.1% SDS, 100 mM NaCl, protease inhibitor cocktail (Roche), 1.8 mM NaVa, 72 mM β-glycerophosphate) and lysed by bead beating. Equivalent amounts of protein (around 2–3 mg in 300 µl) was added to 30 µl IgG Sepharose beads (GE Healthcare) and incubated at 4°C for 90 min. Beads were washed five times with RIPA buffer before elution in 40 µl loading buffer.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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