Yeast Upf1 CH domain interacts with Rps26 of the 40S ribosomal subunit

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ABSTRACT

The central nonsense-mediated mRNA decay (NMD) regulator, Upf1, selectively targets nonsense-containing mRNAs for rapid degradation. In yeast, Upf1 preferentially associates with mRNAs that are NMD substrates, but the mechanism of its selective retention on these mRNAs has yet to be elucidated. Previously, we demonstrated that Upf1 associates with 40S ribosomal subunits. Here, we define more precisely the nature of this association using conventional and affinity-based purification of ribosomal subunits, and a two-hybrid screen to identify Upf1-interacting ribosomal proteins. Upf1 coimmunoprecipitates specifically with epitope-tagged 40S ribosomal subunits, and Upf1 association with high-salt washed or puromycin-released 40S subunits was found to occur without simultaneous eRF1, eRF3, Upf2, or Upf3 association. Two-hybrid analyses and *in vitro* binding assays identified a specific interaction between Upf1 and Rps26. Using mutations in domains of *UPF1* known to be crucial for its function, we found that Upf1:40S association is modulated by ATP, and Upf1:Rps26 interaction is dependent on the N-terminal Upf1 CH domain. The specific association of Upf1 with the 40S subunit is consistent with the notion that this RNA helicase not only triggers rapid decay of nonsense-containing mRNAs, but may also have an important role in dissociation of the premature termination complex.

Keywords: NMD; RNA helicase; ribosomal proteins

INTRODUCTION

Nonsense-mediated mRNA decay (NMD), a cytoplasmic mRNA surveillance pathway, targets transcripts harboring a premature translation termination codon (PTC) or a termination codon in a context characteristic of premature termination (Jacobson and Izaurralde 2007; Kervestin and Jacobson 2012; Kervestin et al. 2012). In part, NMD ensures that the potentially toxic polypeptide products of its substrate mRNAs do not accumulate in the cell (Jacobson and Izaurralde 2007; Kervestin and Jacobson 2012; Kervestin et al. 2012). Factors that regulate NMD include Upf1, Upf2, and Upf3, the principal regulators in all eukaryotes, as well as Smg-1 and Smg-5–9, proteins that play additional regulatory roles in metazoans (Kervestin and Jacobson 2012; Schoenberg and Maquat 2012). The three Upf proteins form a complex with Upf2 acting as a bridge between Upf1 and Upf3 (He et al. 1997; Serin et al. 2001; Chamieh et al. 2008). Single or multiple deletions of the *UPF* genes have similar mRNA decay phenotypes, suggesting that these proteins function in a common pathway (He et al. 1997; Maderazo et al. 2000; Wang et al. 2001; He et al. 2003), albeit

one that may be branched under some circumstances (Chan et al. 2007; Huang et al. 2011).

Upf1, the key effector of NMD, has a cysteine- and histidine-rich zinc-finger domain (CH domain) at its N terminus and a helicase domain comprised of 12 conserved motifs common to the members of helicase superfamily I (SF1) (Fairman-Williams et al. 2010). Upf1 shows RNA-dependent ATPase and 5'-to-3' RNA helicase activities, both of which, although critical for NMD (Czapinski et al. 1998; Wang et al. 2001), have unresolved roles in the process. In yeast, ATPase-deficient Upf1 accumulates with NMD substrates in cytoplasmic processing bodies (Sheth and Parker 2006); and in human cells, ATPase- or helicase-deficient Upf1 leads to the accumulation of partially degraded 3' decay intermediates (Franks et al. 2010). These reports and others (Ghosh et al. 2010) suggest a role for Upf1 in disassembling post-termination mRNPs in a mechanism that requires its ATPase and helicase activities. Notably, the maximal activation of these activities is stimulated by a complex of Upf2 and Upf3 (Chamieh et al. 2008; Chakrabarti et al. 2011).

Numerous studies have shown that NMD is inhibited by drugs, mutations, or mRNA structures that inhibit translation (Jacobson and Izaurralde 2007), or by suppressor tRNAs (Losson and Lacroute 1979; Gozalbo and Hohmann 1990; Belgrader et al. 1993), indicating that a premature stop codon must be recognized by translating ribosomes for NMD to

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occur. This conclusion is reinforced by experiments demonstrating a direct correlation between the extent of ribosome occupancy at a PTC and the degree to which NMD is activated (Gaba et al. 2005). Additional links between translation and NMD include the observations that decapping and degradation of nonsense-containing transcripts occur while the transcript is associated with polyribosomes (Mangus and Jacobson 1999; Hu et al. 2010) and that the Upf factors colocalize with polyribosomes, 80S ribosomes, and ribosomal subunits (Peltz et al. 1993; Atkin et al. 1995, 1997; Mangus and Jacobson 1999; Ghosh et al. 2010). Consistent with a dependence of NMD on translation termination, Upf1 interacts with the release factors eRF1 and eRF3 in humans and yeast, possibly to function in termination events using its helicase and RNA binding activities (Czaplinski et al. 1998; Wang et al. 2001; Ghosh et al. 2010). In vertebrates, Upf1 and its regulator Smg-1 interact with the release factors forming the SURF (Smg-1-Upf1-Release factors) complex, and this SURF-associated Upf1 appears to be able to interact with Upf2-Upf3 bound to the exon junction complex (Yamashita et al. 2009; Kashima et al. 2010).

Although nonsense codon recognition by the ribosome and Upf1 interaction with the release factors are steps that are incorporated into most models of NMD (Kervestin and Jacobson 2012), there is some uncertainty about the timing and specificity of Upf1 interaction with terminating mRNPs. Some models and some experimental results indicate that mechanistic distinctions between premature and normal termination lead to preferential association of Upf1 with mRNPs undergoing premature termination (Amrani et al. 2004, 2006; Bühler et al. 2006; Johansson et al. 2007; Kervestin and Jacobson 2012; Kervestin et al. 2012). Other models and other data suggest that Upf1 associates with all terminating ribosomes, and specificity for premature termination events is achieved by subsequent Upf1 interactions with factors that could only remain mRNA-associated if termination had occurred upstream of its normal site (Peltz et al. 1993; Le Hir et al. 2000; Sun and Maquat 2000). Additional studies imply that specificity for hUpf1 is imparted by mRNA 3'-UTR length (Hogg and Goff 2010). Here, we address the Upf1 localization problem by providing biochemical and genetic evidence for the association of Upf1 with ribosomal subunits in the budding yeast *Saccharomyces cerevisiae*. First, we utilized independent approaches to confirm our earlier observations of Upf1 association with the 40S ribosomal subunit (Ghosh et al. 2010). Having confirmed such interactions, we then defined the domains of Upf1 on which they depend as well as a specific ribosomal protein with which Upf1 interacts.

RESULTS

Upf1 binds to affinity-purified 40S ribosomal subunits

Our previous experiments demonstrated that yeast Upf1 associates with purified 40S ribosomal subunits (Ghosh et al.

2010). We have now explored this interaction further by testing whether Upf1 is retained by an alternative method of ribosome isolation, namely affinity purification. We constructed yeast strains harboring epitope-tagged Rps13 or Rpl25 (Table 1), which would be incorporated into 40S or 60S ribosomal subunits, respectively. These proteins were selected for modification based on their accessibility on the solvent side of the ribosome (Spahn et al. 2001; Inada et al. 2002; Ben-Shem et al. 2011). We inserted a hemagglutinin (HA) tag at the C terminus of Rps13 and a c-Myc tag at the C terminus of Rpl25. The tags did not appear to disrupt the functions of the respective proteins since the tagged strains exhibited growth characteristics comparable to those of untagged strains in liquid yeast extract peptone dextrose (YEPD) culture at 25°C (Fig. 1A) or on multiple solid media at 25°C or 30°C (Fig. 1B). Immunoprecipitations with antibodies targeting the HA or c-Myc epitopes showed efficient capture of the corresponding ribosomal protein from cell extracts (Fig. 2A,B, cf. lane 5 in top panels). Importantly, we found Upf1 to be present in these immunoprecipitates but absent in immunoprecipitates from untagged strains (Fig. 2A,B, bottom panels, cf. lane 5 to lane 2). These results demonstrate that Upf1 is also associated with a ribosomal complex using extraction conditions that differ from those used in our original experiments (Ghosh et al. 2010). Cell-free extracts treated with puromycin to promote ribosome dissociation (Lawford 1969; Azzam and Algranati 1973; Algire et al. 2002) allowed for a test of the subunit-specific association of Upf1 (Fig. 2A,B). Under these conditions, we observed significant enrichment of Upf1 in the HA-specific immunoprecipitates (targeting Rps13-HA) (Fig. 2A, bottom panel, lane 8)

TABLE 1. Yeast strains used in this study

Strains	Genotype	Reference
MBS	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 [rho+]</i> L-o, M-o	Amrani et al. 2004
NAY101	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 [rho+]</i> L-o, M-o, <i>upf1 :: HIS3</i>	Amrani et al. 2004
NAY215	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 [rho+]</i> L-o, M-o, <i>RPL25-c-MYC</i>	This study
NAY217	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 [rho+]</i> L-o, M-o, <i>upf1 :: HIS3, RPL25-c-MYC</i>	This study
NAY223	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 [rho+]</i> L-o, M-o, <i>RPS13-HA</i>	This study
NAY225	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 [rho+]</i> L-o, M-o, <i>upf1 :: HIS3, RPS13-HA</i>	This study
GGY1::171	<i>gal4Δ gal80Δ URA3::GAL1-lacZ his3 leu2</i>	He et al. 1996

FIGURE 1. Yeast strains harboring HA- or c-Myc-tagged *RPS13* or *RPL25* genes grow at wild-type (WT) rates. (A) Growth curves for yeast strains in liquid YEPD medium at 25°C. (◆) WT; (▲) *RPS13*-HA; (■) *RPL25*-c-Myc. (B) Liquid cultures of yeast strains WT, *RPS13*-HA, and *RPL25*-c-Myc were serially diluted and incubated on YEPD plates and plates containing G418 at the temperatures shown.

but not in the c-Myc-specific immunoprecipitates (targeting Rpl25-c-Myc) (Fig. 2B, bottom panel, lane 8). These results demonstrate that, in an independent assay, Upf1 still associates with 40S ribosomal subunits and not with 60S subunits. To determine whether this association was mediated indirectly by Upf1 interaction with mRNA, cell lysates were treated with micrococcal nuclease prior to ribosome immunoprecipitation. These experiments showed significant retention of Upf1 in the immunoprecipitates (Fig. 2C, lane 6), implying that Upf1 is associated directly with ribosomes. The absence of poly(A)-binding protein (Pab1), a factor known to be associated with the mRNA 3' poly(A) tail (Sachs and Davis 1989), in the immunoprecipitates further supported the idea that Upf1 is associated with ribosomes.

Upf1 association with 40S ribosomal subunits requires its ATPase activity, but not its ATP-binding or RNA-binding activities, or the functions of the other NMD factors

To further understand the basis of Upf1 association with 40S ribosomal subunits, we employed domain-specific mutations to determine whether some of the biochemical activities known to be associated with those Upf1 domains might be required for this interaction. High-salt washed 40S and 60S ribosomal subunits were purified with or without prior puromycin release from cells harboring wild-type *UPF1*, or different *upf1* alleles (Fig. 3A), and tested for Upf1:40S association by western blotting. The concurrent retention of Rps6 in the

purified ribosomal subunits served as a control for 40S subunit integrity and 60S subunit purification. As shown in Figure 3B, neither the C62Y or C84S mutations in the N-terminal CH domain nor the K436E or RR973AA mutations in the C-terminal helicase domain (that respectively inactivate ATP binding or RNA binding) had any significant effect on the association of Upf1 with high-salt washed 40S subunits prepared with or without puromycin-mediated release. In contrast, Upf1 derived from the *upf1* DE572AA ATP hydrolysis mutant failed to associate to any significant extent with 40S subunits even though Rps6 was amply recovered and Upf1 from the DE572AA mutant was expressed at levels comparable to wild-type Upf1 (Fig. 3C). Wild-type Upf1, analyzed in the same manner as the five mutant versions of the protein, retained its preference for 40S subunit association (Fig. 3B).

FIGURE 2. Treatment with puromycin diminishes coimmunoprecipitation of Upf1 with Rpl25-c-Myc, but not with Rps13-HA. Lysates from WT, *RPS13*-HA-WT, and *RPS13*-HA-*upf1*Δ cells (A), and WT, *RPL25*-c-MYC-WT, and *RPL25*-c-MYC-*upf1*Δ cells (B) prepared with (+) or without (−) puromycin treatment were immunoprecipitated with anti-HA antibody or anti-c-Myc antibody, respectively. (C) Micrococcal nuclease-treated lysates from WT, *RPL25*-c-MYC-WT, and *RPL25*-c-MYC-*upf1*Δ cells were immunoprecipitated with anti-c-Myc antibodies. Input (I), flowthrough (FT), and eluate (E) were analyzed by western blotting for the presence of Rps13-HA, Rpl25-c-Myc, Upf1, or Pab1 with specific antibodies.

FIGURE 3. Upf1:40S ribosomal subunit association is modulated by ATP and is independent of Upf2, Upf3, or translation termination release factors eRF1/Sup45 and eRF3/Sup35. (A) Schematic representation of amino acid substitutions in the functional domains of Upf1. (B,D,E) Ribosomal subunits were prepared under high-salt conditions with or without prior puromycin treatment from yeast strains of the indicated genotypes. Increasing amounts (5 µg and 10 µg) of purified subunits were analyzed by SDS-PAGE and western blots were probed with specific antibodies; (B) strains harboring different *upf1* alleles; (D) strains harboring *upf2Δ*, *upf3Δ*, or *upf2Δupf3Δ* mutations, and (E) wild-type cells. In E, lanes marked *upf1Δ* or wild-type are lysates from the respective cells. (C) Western blot for Upf1 from equal amounts of cell lysates of the indicated genotypes.

Additional insight into the determinants of Upf1 association with 40S subunits was obtained by evaluating whether the other two Upf proteins were required for this association. High-salt washed 40S and 60S ribosomal subunits were purified with or without prior puromycin treatment from cells lacking Upf2, Upf3, or both proteins, and then assayed by western blotting for retention of Upf1 (Fig. 3D). These experiments showed that Upf1 copurified predominantly with 40S ribosomal subunits in *upf2Δ*, *upf3Δ*, and *upf2Δupf3Δ* strains, similar to the results observed in wild-type strains and consistent with earlier studies showing that polysome-association of Upf1 is independent of the other NMD factors (Atkin et al. 1997). In several of the strains analyzed, a modest amount of Upf1 association with 60S subunits was observed; however, as monitored by Rps6 recovery, this appeared to re-

flect 40S subunit contamination in the 60S subunit preparations (Fig. 3D). Collectively, these data indicate that the association of Upf1 with 40S ribosomal subunits is neither bridged by nor dependent on Upf2 or Upf3.

Several reports of experiments done in yeast and mammalian cells have indicated that Upf1 association with a prematurely terminating ribosome may be attributable to direct interactions with the release factors, eRF1 and eRF3 (Czapinski et al. 1998; Kashima et al. 2006; Ivanov et al. 2008; Singh et al. 2008). Since we observed Upf1 association with 40S subunits purified after puromycin release (Fig. 3C,D), it seemed unlikely that maintenance of Upf1:40S interaction depended on the eRFs. Nevertheless, we tested directly whether either of the release factors was present in the purified 40S ribosomal subunits. Western blotting analyses

showed that the same subunit preparations that had substantial bound Upf1 were devoid of detectable levels of Sup45/eRF1 or Sup35/eRF3 (Fig. 3E), indicating that Upf1 can maintain an association with 40S ribosomal subunits independent of the two release factors. However, whether Upf1's initial association with the 40S subunits depended on these factors remains to be ascertained.

Upf1 interacts with 40S ribosomal proteins

Upf1 association with 40S ribosomal subunits suggests two possible types of interactions that may mediate this association: protein–protein and/or protein–rRNA. Evidence for Upf1:ribosomal protein association has been obtained from coimmunoprecipitation experiments in higher eukaryotes (Yamashita et al. 2009). This precedent, and our observation that Upf1's RNA-binding activity was dispensable for 40S subunit association (Fig. 3B), led us to test for Upf1 interaction with specific small subunit ribosomal proteins using a directed yeast two-hybrid screen. Using full-length Upf1 as bait (fused to the *GAL4* DNA binding domain) and thirty-two 40S ribosomal proteins as prey (fused to the *GAL4* activation domain) (He et al. 1996; Valášek et al. 2003), bait and prey plasmids were cotransformed into a *S. cerevisiae* GGY1::171 strain that contains an integrated *GAL1-lacZ* reporter construct. In these strains, the expression of the reporter is directly related to the extent of interaction of the bait and prey proteins. With a *GAL4-UPF2* fusion serving as a positive control for two-hybrid interaction, five ribosomal proteins were found to manifest significant *lacZ* expression, indicating an interaction with Upf1 (Fig. 4, left panel). In both qualita-

tive plate-based and quantitative liquid β -galactosidase assays, the strongest interactions were observed with Rps26. Significantly, the Upf1:Rps26 interaction was as strong qualitatively and quantitatively as the well-characterized Upf1:Upf2 interaction (Fig. 4, left panel; He and Jacobson 1995; He et al. 1996). Weaker Upf1 interactions were observed with Rps6, Rps9, Rps10, and Rps13 (Fig. 4, left panel).

Although our experiments with purified subunits show that Upf1 association with the 40S subunit is independent of Upf2 and Upf3, we tested if the two-hybrid interactions with the individual ribosomal proteins were mediated by either of these NMD factors or by some aspect of Upf1 multimerization. We assayed the interactions between Upf1 and the candidate ribosomal proteins in two-hybrid yeast strains harboring deletions of *UPF1*, *UPF2*, or *UPF3* and found no change in the interactions between Upf1 and the candidate ribosomal proteins in any of these strains (Fig. 4, right panel, and data not shown). These results indicate that Upf1:40S ribosomal protein interactions are not bridged by other NMD factors and are likely to be direct.

Point mutations in the CH domain of Upf1 alter its interaction with Rps26

To gain further insight into the Upf1 domain that interacts with Rps26, its strongest 40S ribosomal protein interactor, we analyzed Upf1:Rps26 interaction using five full-length *upf1* loss-of-function alleles (Fig. 3A). Figure 5B shows that two-hybrid interaction between Upf1 and Rps26 was lost completely in strains harboring the C62Y or C84S mutations. The K436E mutation showed a modest decrease in *lacZ* expression, whereas the DE572AA and RR793AA mutants did not exhibit any change in two-hybrid interaction. The loss of interaction with Rps26 in the C62Y or C84S alleles suggests that Upf1's N terminus mediates interaction with Rps26. The domain encompassing the K436E mutation in hUpf1 has been demonstrated to form a rigid association with the CH domain (Clerici et al. 2009), which could explain the partial loss of interaction in the K436E mutation. Interestingly, neither the C62Y nor the C84S mutations affect the association of Upf1 with polysomes or 40S ribosomal subunits (Fig. 3B; Atkin et al. 1997; S Ghosh and A Jacobson, unpubl.), suggesting that Upf1:40S association may be a multivalent interaction involving multiple interacting epitopes.

Upf1 N terminus is required for its interaction with Rps26

Mutations in the Upf1 N terminus completely disrupted its interaction with Rps26 (Fig. 5B). However, the ability of two different Upf1 proteins with C-terminal amino acid substitutions (DE572AA or RR793AA) to retain interaction with Rps26 does not rule out the possibility that the Upf1 C terminus also interacts with Rps26. To define more precisely the domain(s) of Upf1 that mediate interaction with Rps26, we

FIGURE 4. Upf1 interacts with 40S ribosomal proteins. Directed yeast two-hybrid assays were performed using *UPF1-GAL4* (DNA-binding domain) as bait and a library of *RPS-GAL4* (activation domain) fusion proteins as prey. Individual transformants were assayed for β -galactosidase activity qualitatively on plates and quantitatively in liquid assays. Interactions were tested in GGY1::171 wild-type (left) and GGY1::171 *upf2*Δ (right) strains. Values for β -galactosidase assays represent mean $\pm$ standard deviation for three independent experiments performed on cultures from independent transformants.

FIGURE 5. The CH domain of Upf1 mediates interaction with Rps26. (A) Schematic representation of Upf1 fragments tested for interaction with Rps26. (B,C) Two-hybrid assays were performed in the GGY1::171 *upf1Δ* strain to prevent any effects of possible oligomerization with endogenous Upf1 protein. Interaction of Rps26 with either full-length Upf1 mutant alleles (B) or indicated truncations of Upf1 (C) are shown. Values for β -galactosidase assays represent mean $\pm$ standard deviation for three independent experiments performed on cultures from independent transformants.

further tested the interactions of a series of previously characterized N- and C-terminal Upf1 truncations (Fig. 5A; He et al. 1997). We first tested Upf1(1–289), which harbors only the first 289 amino acid residues of Upf1, and Upf1(290–971), which lacks the first 289 amino acids of Upf1. Upf1(1–289), which includes the CH domain but lacks the helicase domains of Upf1, still showed interaction with Rps26 (Fig. 5C). Upf1(290–971), on the other hand, failed to show any interaction with Rps26 (Fig. 5C), confirming that the C-terminal domains of Upf1 are not important for binding to Rps26. To further narrow down the minimal interaction domain on Upf1, we tested N- and C-terminal truncations of the Upf1(1–289) fragment. Since residues 62 and 84 were found to be important for Rps26 interaction (Fig. 5B), fragments tested spanned residues 1–181, 1–207, 62–181, 62–207, and 62–289 (Fig. 5A). The shortest Upf1 fragment that still retained the ability to interact with Rps26 was comprised of residues 62–207 (Fig. 5C). Interestingly, the interaction of Upf1(1–289) with Rps26 was weaker than that observed with either full-length Upf1, or the shortest Upf1(62–207) fragment.

Rps26 interacts with Upf1 in vitro

Two-hybrid interactions can be bridged (Bartel et al. 1993; Bartel and Fields 1995; He et al. 1997), so we sought to deter-

mine whether Upf1:Rps26 interaction could be confirmed biochemically, using an in vitro binding assay. Purified yeast FLAG-Upf1 (Fig. 6A) and lysates from *Escherichia coli* expressing recombinant His-tagged Rps26 (Fig. 6B) were incubated and subjected to immunoprecipitation using anti-FLAG beads. An *E. coli* strain not expressing any His-tagged protein was used as a control for background binding. Analysis of the immunoprecipitates showed coimmunoprecipitation of His-Rps26 with FLAG-Upf1 and not with FLAG beads alone (Fig. 6C, cf. lanes 8 and 12). This in vitro data, in addition to the two-hybrid analyses, demonstrates that Upf1 association with the 40S subunit is mediated at least in part by a direct interaction with 40S ribosomal protein Rps26 and does not involve any endogenous yeast protein bridging the interaction.

DISCUSSION

Upf1 plays a central role in triggering NMD as well as in promoting several processes ancillary to this quality control pathway, including translational repression, dissociation of the translating mRNP, degradation of the nascent polypeptide, and inhibition of pre-mRNA splicing (Kervestin and Jacobson 2012). Accordingly, the platform from which Upf1 launches these diverse activities is of considerable interest. Notwithstanding disagreement about the involvement of

FIGURE 6. Upf1 interacts with Rps26 in vitro. In vitro binding assays were performed with *E. coli* BL21(DE3) cells expressing His-Rps26. (A) Coomassie-stained SDS-PAGE gel showing purity of yeast FLAG-Upf1. (B) Time course of His-Rps26 expression after IPTG induction in *E. coli* BL21 (DE3) cells. (C) Western blot analyses of anti-FLAG immunoprecipitation assays utilized anti-His and anti-Upf1 antibodies on input (I), flowthrough (FT), wash (W), and eluate (E) fractions.

all termination events or just those which are premature, several models suggest that an early event in NMD is the interaction of Upf1 with the release factors localized at the A site of a terminating ribosome (Kervestin and Jacobson 2012). The implication of such models is that Upf1, and perhaps the other Upfs, should be associated with ribosomes during at least one phase of their functional lifetimes. This notion was supported by early experiments demonstrating polysomal localization of the Upfs (Peltz et al. 1993; Atkin et al. 1995, 1997), as well as by more recent studies which have localized Upf1 to the SURF complex (Yamashita et al. 2009) or to purified 40S ribosomal subunits (Ghosh et al. 2010). To understand the mechanistic basis for Upf1's association with the termination complex, we have pursued details of its mode of interaction with yeast ribosomes.

Using affinity purification of tagged ribosomal subunits, we demonstrate here that Upf1 is specifically immunoprecipitated with tagged 40S ribosomal subunits and not 60S subunits (Fig. 2), consistent with our previous results (Ghosh et al. 2010). The significant enrichment of Upf1 in ribosomal pulldowns of micrococcal nuclease-treated lysates, as well as the failure to detect poly(A)-binding protein (Pab1) in these pulldowns, indicates that ribosome association of Upf1 is likely mediated by a direct interaction with the 40S subunit and not through residual ribosome-associated mRNA. Importantly, this conclusion is reinforced by the observation that the association of Upf1 with the 40S ribosome is sufficiently stable to resist either high salt-induced or puromycin-triggered ribosomal dissociation (Fig. 3). Stable association of Upf1 with the 40S subunit appears to be modulated by ATP, and independent of the simultaneous presence of Upf2 or Upf3, or translation termination factors eRF1 and eRF3 (Fig. 3).

The demonstration of specific Upf1 association with the 40S subunit led us to screen for Upf1:40S ribosomal protein interactions, an experimental approach that identified Rps26

as a strong interacting partner of Upf1 (Figs. 4, 6). The interaction between Upf1 and Rps26 was observed to be specific, direct, and independent of the other Upf factors (Figs. 4, 6). Consistent with the latter result, the association of Upf1 with purified subunits was also found to be independent of Upf2 and/or Upf3 (Fig. 3D). Two-hybrid analyses that exploited different *upf1* alleles showed that the RR793AA RNA-binding mutant had no effect on Upf1:Rps26 interaction, whereas single point mutations in the CH domain abrogated the ability of Upf1 to interact with Rps26 completely (Fig. 5B). The requirement for a functional Upf1 N-terminal CH domain in Upf1:Rps26 interaction was further evident from deletion analyses which showed

that only those Upf1 fragments containing the CH domain could interact with Rps26 (Fig. 5C). Interestingly, although the C62Y and C84S mutation in the CH domain of Upf1 abolished the Upf1:Rps26 interaction, these mutations did not significantly affect Upf1's association with the 40S ribosomal subunit. This observation suggests that Upf1 ribosomal association is likely mediated through multiple interaction epitopes. In support of this idea, we found Upf1 also interacts with Rps6, Rps9, Rps10, and Rps13 (Fig. 4).

The K436E mutation in the UPF1 ATP-binding domain also manifested impaired Upf1:Rps26 interaction, although not to the extent seen with the CH domain mutants (Fig. 5B). X-ray crystallographic analysis of hUpf1 has demonstrated a strong association between the CH domain (encompassing residues C62 and C84) and domain 1A (encompassing residues K436 and DE572), suggesting that Upf1 may adopt a folded conformation that allows these domains to not only interact with each other, but with Rps26 as well. Interestingly, we noted that the interaction between the DE752AA ATP hydrolysis mutant of Upf1 with Rps26 was not affected in the two-hybrid assay, whereas association of this mutant protein with the purified 40S subunits was lost (Fig. 3B). The lack of complete correspondence between the mutations that affect two-hybrid interaction and copurification with 40S subunits must take into account the differences between the two assays. Although copurification demands stable association with the subunit subsequent to treatment with high salt or puromycin, the two-hybrid assay is capable of registering a transient binding event that may be a precursor, for example, to the post-puromycin state. The former may have a readily detectable two-hybrid interaction (e.g., Rps26), whereas the latter may not. The DE572AA mutation may trap Upf1 in a conformation or state during its function that can bind to mRNA but not to the 40S ribosomal subunit. The possibility that Upf1 association with a translating mRNP involves complex mechanisms has been

FIGURE 7. Upf1-interacting ribosomal proteins and eIF3 binding site on the solvent side of the *S. cerevisiae* 40S subunit. The image showing ribosomal proteins only was created using PyMol (<http://www.pymol.org>) and is based on a Protein Data Bank entry (accession number 3U5C) (Ben-Shem et al. 2011). Ribosomal proteins interacting with Upf1 are highlighted using different shades of blue (dark blue denoting strong interaction and light blue representing weak interaction in the two-hybrid assay). The eIF3 binding site is highlighted in tan (Kouba et al. 2012). The asterisk denotes the mRNA entry channel and the mRNA is represented using a dotted line.

highlighted in recent studies. Cryo-EM structures of the exon junction complex (EJC)-UPF complex have positioned Upf1 on the 3' side of the EJC (Melero et al. 2012), contradictory to the current notion of Upf1 localization at the 5' side that is closer to the premature termination codon. It has also been reported that hUPF1 binds directly to mRNA, with binding varying as a function of mRNA 3'-UTR length (Hogg and Goff 2010; Kurosaki and Maquat 2013).

Rps26 is positioned on the solvent side of the 40S platform and is a crucial component of the 40S ribosome-binding site for mRNA (Ben-Shem et al. 2011; Sharifulin et al. 2012). Available structural data show that the binding site of eIF3 on the 40S subunit overlaps with Rps26 as well as the other Upf1-interacting ribosomal proteins (Fig. 7). This observation is of interest because temperature-sensitive lesions in the Prt1 subunit of eIF3 (eIF3b) antagonize NMD in yeast (Welch and Jacobson 1999), and phosphorylated Upf1 interacts with eIF3 and inhibits translation initiation in mam-

malian NMD (Isken and Maquat 2008). eIF3 has been implicated in efficient ribosome recycling after translation termination (Pisarev et al. 2007), and *upf1Δ* extracts have been shown to be defective in efficient ribosome recycling from a nonsense-containing mRNA (Ghosh et al. 2010). Collectively, these observations suggest a possible role for Upf1:eIF3 interaction in promoting ribosome dissociation and/or recycling from premature termination events.

MATERIALS AND METHODS

Construction of yeast strains with epitope-tagged ribosomal proteins

The chromosomal *RPL25* and *RPS13* genes were tagged with HA and c-Myc tags by homologous recombination at their 3' ends using a single step PCR-mediated technique (Longtine et al. 1998). The oligonucleotide primers NA193 and NA194 were used for tagging *RPL25* and oligonucleotide primers NA191 and NA192 were used for tagging *RPS13* (Table 2).

Plasmid construction

His-tagged ribosomal fusion proteins were constructed by PCR amplification using oligonucleotides listed in Table 2 and yeast genomic DNA as template. The PCR products were digested with *NdeI*/*Bam*HI and subcloned into the pET15b vector (Novagen).

Affinity purification of ribosomes and western blot analysis

Cell-free extracts were prepared as described previously (He et al. 2008). Extracts (15 A₂₆₀) from cells expressing c-Myc tagged *RPL25* or HA-tagged *RPS13* were mixed with an equal volume of Buffer A (100 mM Tris-HCl, pH 7.5, 24 mM Mg(OAc)₂, 1 mM dithiothreitol (DTT), 1 mM PMSF, 50 units/mL RNasin [Roche]) and with 30 μL of anti-HA or anti-c-MYC agarose slurry (Pierce). These mixtures were applied to spin columns, which were then incubated for 2 h at 4°C with gentle rocking. The columns were subjected to pulse centrifugation, and the respective flowthrough fractions were collected. The columns containing epitope-tagged ribosomal proteins were then washed three times with 0.8 mL Buffer W (100 mM Tris-HCl, pH 7.5, 24 mM Mg(OAc)₂, 1 mM DTT, 100 mM PMSF, 50 units/mL RNasin [Roche], protease inhibitor cocktail [Roche], 0.1% NP40) and were eluted with 2× nonreducing sample buffer (Pierce) for 5 min at 100°C on a heat block. The eluted

TABLE 2. Oligonucleotides used in this study

NA191	CTACGATGCTTTGGACATTGCTAACAGAATCGGTTACATTCGGATCCCCGGGTTAATTAA
NA192	GAAAAATTTAAAAATAATATTAATTTATTAATTAAACCAATTAGAGAATTCGAGCTCGTTTAAAC
NA193	CCAACTGGAAGTACGAATCCGCCACTGCCTCCGCTTTGGTCAACCGGATCCCCGGGTTAATTAA
NA194	CAAAATATACATATAAAATAATGAAATAAATGATTTAAACACTGAATTCGAGCTCGTTTAAAC
Rps26 Forward	CCCGGCATATGCCAAGAAGAAGCTTC
Rps26 Reverse	CCCGGGGATCCTTATAAAGCCTTCTTG

proteins were collected by pulse centrifugation, and 2 μ L of β -mercaptoethanol was added for SDS-PAGE analysis. For micrococcal nuclease treatment, 50 units of micrococcal nuclease (S7 nuclease, Roche) was preincubated with extract for 10 min at 25°C and digestion was then terminated by adding 100 mM of ethylene glycol tetraacetic acid (EGTA). Puromycin (1 mM, Sigma) pretreatment of extracts was performed for 15 min at 4°C and 10 min at 37°C. Aliquots of the initial extract as well as the supernatant and eluate of immunoprecipitations were subjected to SDS-PAGE analysis followed by western blotting using anti-Upf1 (1:5000) (Belk et al. 1999), anti-c-Myc (1:5000, Sigma), or anti-HA (1:5000, Sigma) antibody.

Purification of ribosomal subunits

Cytoplasmic extracts were prepared as described previously with the omission of cycloheximide and inclusion of high salt (0.5 M KCl) (Mangus and Jacobson 1999). Extracts (20 A_{260}) were centrifuged at 120,000g for 10 h at 4°C on 34 mL 15%–40% sucrose gradients containing 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 30 mM NH_4Cl , and 1 mM DTT. The gradients were scanned at A_{254} , and the resulting absorbance profiles were used to determine the positions of the ribosomal fractions. Fractions corresponding to the 40S and 60S peaks were concentrated separately in Amicon Ultra-15 100K NMWL filters (EMD Millipore). The concentrated samples were diluted with Buffer E (10 mM Tris-HCl, pH 7.4, 10 mM KCl, 1 mM $MgCl_2$) and reconcentrated as before. The subunits were aliquotted and stored at $-80^\circ C$. Concentrations of 40S and 60S subunits were determined by spectrophotometry, using 1 A_{260} = 50 nM for 40S and 1 A_{260} = 25 nM for 60S subunits (Matasova et al. 1991). The integrity and the quality of purified 40S and 60S subunits were determined by SDS-PAGE analysis followed by western blotting using anti-Rps6 (1:5000, Cell Signaling) and analysis of rRNA on denaturing agarose gels.

Yeast two-hybrid screening

Full-length yeast *UPF1* and mutant derivatives of the gene, all fused to the DNA binding domain of transcriptional activator *GAL4* (He and Jacobson 1995; He et al. 1996), were used as bait against a two-hybrid library of ribosomal proteins fused to the *GAL4* activation domain (Valášek et al. 2003). The *UPF1-GAL4* (DNA-binding domain) and *RPS-GAL4* (activation domain) constructs were cotransformed into the two-hybrid tester strain GGY1::171 (Table 1). Transformants were incubated for 3–5 d at 30°C and qualitative and quantitative β -galactosidase activity was assayed as described previously (He et al. 1996).

In vitro binding assay

E. coli BL21 (DE3) cells (New England BioLabs, Inc.) were transformed with pET-His-Rps26, and protein expression was induced by addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Cells expressing the His-Rps26 fusion protein were lysed in Talon Xtractor Buffer (Clontech Laboratories, Inc.). The lysate was centrifuged to remove cell debris, and the supernatant was incubated with purified yeast FLAG-Upf1 for 1 h at 4°C. Binding was followed by incubation with FLAG beads

(Sigma) for 4 h at 4°C. After extensive washing in Talon Wash Buffer (Clontech Laboratories, Inc.), FLAG-Upf1 was eluted with 3XFLAG peptide and samples were analyzed on SDS-PAGE followed by western blotting using anti-His (1:7000; Maine Biotechnology) or anti-Upf1 (1:5000) antibody (Belk et al. 1999).

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