

## eIF5A interacts functionally with eEF2

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**Abstract** eIF5A is highly conserved from archaea to mammals, essential for cell viability and the only protein known to contain the essential amino acid residue hypusine, generated by a unique posttranslational modification. eIF5A was originally identified as a translation initiation factor due to its ability to stimulate the formation of the first peptide bond. However, recent studies have shown that depletion of eIF5A causes a significant decrease in polysome run-off and an increase in the ribosome transit time, suggesting that eIF5A is actually involved in the elongation step of protein synthesis. We have previously shown that the depletion mutant *tif51A-3* (eIF5A<sup>C39Y/G118D</sup>) shows a sicker phenotype when combined with the dominant negative mutant *eft2*<sup>H699K</sup> of the elongation factor eEF2. In this study, we used the eIF5A<sup>K56A</sup> mutant to further investigate the relationship between eIF5A and eEF2. The eIF5A<sup>K56A</sup> mutant is temperature sensitive and has a defect in protein synthesis, but instead of causing depletion of the eIF5A protein, this mutant has a defect in hypusine modification. Like the mutant *tif51A-3*, the eIF5A<sup>K56A</sup> mutant is synthetic sick with the mutant *eft2*<sup>H699K</sup> of eEF2. High-copy eEF2 not only improves cell growth of the eIF5A<sup>K56A</sup> mutant, but also corrects its increased cell size defect.

Moreover, eEF2 suppression of the eIF5A<sup>K56A</sup> mutant is correlated with the improvement of total protein synthesis and with the increased resistance to the protein synthesis inhibitor hygromycin B. Finally, the polysome profile defect of the eIF5A<sup>K56A</sup> mutant is largely corrected by high-copy eEF2. Therefore, these results demonstrate that eIF5A is closely related to eEF2 function during translation elongation.

**Keywords** eIF5A · Hypusine · eEF2 · Translation elongation

### Abbreviations

|                          |   |
|--------------------------|---|
| eIF5A                    | Eukaryotic translation initiation factor 5A |
| eEF2                     | Eukaryotic translation elongation factor 2  |
| <i>TIF51A</i>            | Gene encoding eIF5A in yeast                |
| <i>EFT2</i>              | Gene encoding eEF2 in yeast                 |
| <i>PKC1</i>              | Gene encoding protein kinase C in yeast     |
| HIV                      | Human immunodeficiency virus                |
| [ <sup>3</sup> H]leucine | Tritium-radioactive leucine                 |
| c.p.m.                   | Counts per minute                           |

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### Introduction

The putative eukaryotic translation initiation factor 5A (eIF5A) is a highly conserved and essential protein present in all organisms from archaea to mammals, but not in eubacteria (Schnier et al. 1991; Chen and Liu 1997). eIF5A is the only protein known to contain the unusual amino acid residue hypusine (Chen and Liu 1997; Wolff et al. 2007). Hypusine is formed by the addition of a 4-aminobutyl moiety from spermidine to a specific lysine residue of

eIF5A, followed by its hydroxylation. Hypusine is essential for eIF5A function and several studies have aimed to characterize the mechanisms of the hypusine modification enzymes and the development of specific inhibitors (Wolff et al. 2007; Kerscher et al. 2010; Cano et al. 2010). This specific lysine converted to hypusine is located at the tip of a  $\sim 10$  amino acid loop in the N-terminus domain of the protein and is the most conserved region of eIF5A through evolution (Park et al. 2010). eIF5A is organized in two independently folded domains (Stiuss et al. 1999): an N-terminal SH3-like barrel, found in other proteins related to translation; and a C-terminal OB-fold, present in nucleic acid-binding proteins (<http://www.rcsb.org/pdb/>).

Despite being highly conserved and essential, the critical role of eIF5A in the cell remains unclear. Originally purified from ribosomes isolated from reticulocyte lysates, eIF5A was shown to stimulate methionyl-puromycin synthesis, indicating a role in the formation of the first peptide bond (Benne and Hershey 1978). However, eIF5A depletion in yeast caused a small decrease in cell protein synthesis, arguing against a role as a general translation initiation factor. Curiously, eIF5A depletion induced an increased number of G1-arrested yeast cells (Kang and Hershey 1994). This result, together with the fact that inhibitors of hypusine formation caused cell-cycle arrest in mammalian cells (Hanauske-Abel et al. 1994), led to the hypothesis that eIF5A may be important for translation of mRNAs encoding specific proteins required for cell-cycle progression (Park et al. 1997). In support of this idea, eIF5A function is necessary for polarized cell growth, a process essential for the G1/S transition in yeast (Zanelli and Valentini 2005). Furthermore, synthetic lethality was revealed between mutants of eIF5A and of Ypt1, which is a protein essential for vesicular trafficking and also for proper polarized growth in yeast (Frigieri et al. 2008). However, no specific subset of mRNAs whose translation is controlled by eIF5A has been identified so far.

eIF5A has also been implicated in nuclear export of HIV-1 Rev and mRNA decay, but these findings are controversial and may reflect secondary effects of eIF5A function (Huang et al. 2007). More specifically, the effect of eIF5A on mRNA decay seems to be secondary, as the arrest of cell growth in eIF5A temperature-sensitive mutants does not directly correlate with mRNA accumulation (Valentini et al. 2002).

More recently, it has been demonstrated that eIF5A interacts physically with the 80S ribosome as well as with the translation elongation factors eEF1A and eEF2 (Jao and Chen 2006; Zanelli et al. 2006). eIF5A was shown to co-fractionate with monosomes in a translation-dependent manner. Moreover, eIF5A mutant strains show accumulation of polysomes instead of polysome run-off and an increase in the average time necessary for ribosomes to

transit along mRNAs (Greggio et al. 2009; Saini et al. 2009). Therefore, these results not only support a role for eIF5A in translation, but also suggest that this factor plays a role in translation elongation rather than initiation. Furthermore, a function for eIF5A in translation elongation would explain the secondary effect of mRNA stabilization and a block in the formation of P bodies, observed in eIF5A mutants as a result of mRNA trapping in polysomes (Valentini et al. 2002; Greggio et al. 2009).

Herein, we use the eIF5A<sup>K56A</sup> mutant to further investigate the relationship between eIF5A and eEF2.

## Materials and methods

### Yeast strains, plasmids and standard procedures

Procedures for cell growth and genetic manipulations were carried out according to standard protocols (Guthrie and Fink 1991). *Saccharomyces cerevisiae* strains used in this study are: wild type eIF5A (VZL838—MAT $\alpha$  *leu2 ura3 trp1 his3 tif51A::HIS3 tif51B::KanMX4 [TIF51A/TRP1/CEN]*), eIF5A<sup>K56A</sup> mutant (VZL987—MAT $\alpha$  *leu2 ura3 trp1 his3 tif51A::HIS3 tif51B::KanMX4 [tif51A-K56A/TRP1/CEN]*) and eIF5A<sup>P83S</sup> (*tif51A-1*) mutant (VZL904—MAT $\alpha$  *leu2 ura3 trp1 his3 tif51A::HIS3 tif51B::KanMX4 [tif51A-1/TRP1/CEN]*) (Valentini et al. 2002). The plasmids used are: high-copy vectors pRS426 and pRS425, high-copy eEF2 (pSV262—*EFT2/URA3/2 $\mu$* ), high-copy Pkc1 (pSV181—*PKC1/URA3/2 $\mu$* ) and dominant negative *eft2*<sup>H699K</sup> allele of eEF2 (pVZ1104—*eft2*<sup>H699K</sup>/*LEU2/2 $\mu$* ) (Ortiz and Kinzy 2005).

### Flow cytometry analysis

Strains were grown to mid-log phase (OD<sub>600nm</sub> = 0.5) at 25°C in 5 mL of Synthetic Complete medium lacking uracil (SC-ura) and then were shifted to the restrictive temperature (38°C) for 4 h. The graph under each micrograph was obtained by flow cytometry analysis of granularity (side light scatter or SSC—y axis) in function of cell volume (forward light scatter or FSC—x axis) using a BD FACSCanto apparatus and the BD FACSDiva software. The percentages under the dot plot graphs represent the relative number of cells (dots) inside the area delimited in each graph. The delimited area was determined as the area representing the cell dispersion of the wild type strain (eIF5A).

### Protein synthesis measurement

Cells were grown to mid-log phase (OD<sub>600nm</sub> = 0.5) at 25°C in 5 mL of synthetic complete medium lacking uracil (SC-ura). [<sup>3</sup>H]leucine (PerkinElmer, MA, USA) was added

to the medium to a final concentration of 2  $\mu\text{Ci/mL}$  and the cultures were shifted to the restrictive temperature (38°C) for 4 h, harvested at 4°C and frozen at −80°C. All frozen cell pellets were resuspended in 15% cold trichloroacetic acid solution and incubated on ice for 15 min. The samples were heated at 72°C for 30 min and then incubated on ice for 15 min. Trichloroacetic acid precipitates were collected by centrifugation (15,000  $g$ , 4°C, 10 min) and washed four times with 10% trichloroacetic acid to remove free [ $^3\text{H}$ ]leucine. The final washed pellets were resuspended in 100  $\mu\text{L}$  of 0.2 M NaOH, and 50  $\mu\text{L}$  aliquots were used to determine the radioactivity incorporated into protein synthesis in a Beckman Scintillation Counter (Beckman Coulter, Brea, CA, USA). Total protein concentration was determined by the BCA protein assay (Thermo Scientific, IL, USA), using 5  $\mu\text{L}$  aliquots of each sample. The amount of total protein synthesis was calculated for each sample as c.p.m.  $\mu\text{g}^{-1}$  total protein and then expressed as percentage of total protein synthesis with the wild type (eIF5A) strain arbitrarily set to 100% (Table 1).

### Polysome profiling

Cells from 100 mL cultures ( $\text{OD}_{600\text{nm}} = 0.3\text{--}0.6$ ) were shifted to 38°C for 4 h and used for each sucrose gradient. At the time of harvesting, the translational machinery was blocked by the addition of 0.1 mg/mL cycloheximide for 5 min at 38°C. Briefly, 10  $\text{A}_{260\text{nm}}$  units of cell lysates were layered onto 10–50% sucrose gradients and centrifuged for 3 h at 39,000 $g$  at 4°C in a Beckman SW41-Ti rotor. The gradients were then fractionated by upward displacement with 60% (w/v) sucrose using a gradient fractionator connected to a Control Unit UV-1 monitor (Amersham Pharmacia Biotech) for continuous measurement of the absorbance at 254 nm. The polysomal profile fractions were quantified with the aid of NIH Image J software.

## Results and discussion

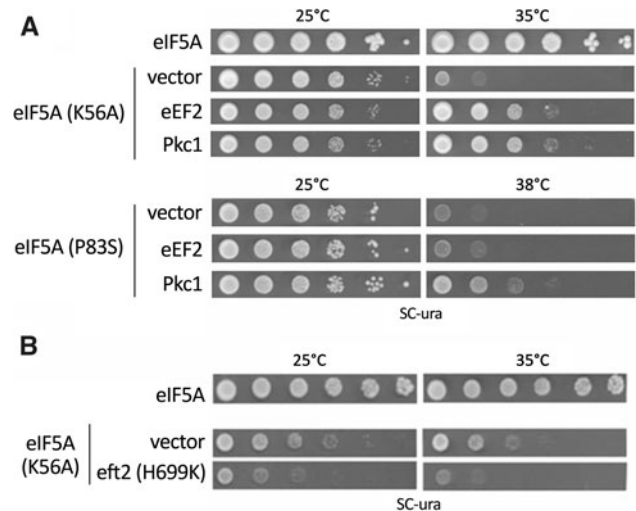
### High-copy eEF2 suppresses growth and cell size defects of yeast eIF5A<sup>K56A</sup> mutant

In order to better understand the function of eIF5A during protein synthesis, more specifically during translation

**Table 1** Measurement of total protein synthesis

| Samples               | Relative values (%) $\pm$ SD |
|-----------------------|------------------------------|
| eIF5A                 | 100.0 $\pm$ 1.0              |
| eIF5A (K56A) + vector | 59.6 $\pm$ 2.6               |
| eIF5A (K56A) + eEF2   | 85.5 $\pm$ 2.5               |

SD standard deviation



**Fig. 1** Genetic interactions between eIF5A and eEF2. **a** Tenfold serial dilutions of wild type (eIF5A) cells harboring the vector alone, the mutant strains eIF5A<sup>K56A</sup> or eIF5A<sup>P83S</sup> harboring the vector alone, or high-copy eEF2 or high-copy Pkc1 plasmid were plated onto SC-ura to determine the growth at permissive (25°C) or non-permissive temperatures (35°C to eIF5A<sup>K56A</sup> and 38°C to eIF5A<sup>P83S</sup>). **b** The wild type strain harboring the vector alone or the eIF5A<sup>K56A</sup> mutant strain harboring the vector alone or together with eEF2<sup>H699K</sup> dominant negative mutant plasmid were assayed as described in (a). The plates were photographed after 3–4 days of growth

elongation, we searched for yeast eIF5A temperature-sensitive mutants which would show genetic interactions with the elongation factor 2 (eEF2). Interestingly, we found that the temperature sensitivity of the eIF5A<sup>K56A</sup> mutant was suppressed by high-copy *EFT2*, the gene encoding the major form of eEF2 in yeast. As shown in Fig. 1a, both eIF5A<sup>P83S</sup> (*tif51A-1*) and eIF5A<sup>K56A</sup> mutants are suppressed by Pkc1. Overexpression of *PKC1* suppresses eIF5A mutant temperature sensitivity through overactivation of the cell integrity pathway and resultant bypass of the secondary effect of eIF5A in cell-cycle progression (Zanelli and Valentini 2005). Therefore, Pkc1 is a more general suppressor of eIF5A, since it overcomes the secondary effects caused by eIF5A loss of function in the cell (Zanelli and Valentini 2007). On the other hand, only the eIF5A<sup>K56A</sup> mutant is suppressed by eEF2, demonstrating an allele-specific genetic interaction. This suggests that a specific defect in eIF5A function impaired in the eIF5A<sup>K56A</sup> mutant is compensated by high-copy eEF2, as discussed in the next paragraph.

The eIF5A<sup>K56A</sup> mutant was previously shown to be defective in protein synthesis, similarly to other yeast eIF5A mutants (Dias et al. 2008; Gregio et al. 2009; Saini et al. 2009). Lysine 56 (K56) is the last residue of the amino acids stretch forming a hypusine-containing loop in the eIF5A N-terminal domain (Dias et al. 2008). It was shown that the eIF5A<sup>K56A</sup> mutant protein is less effectively

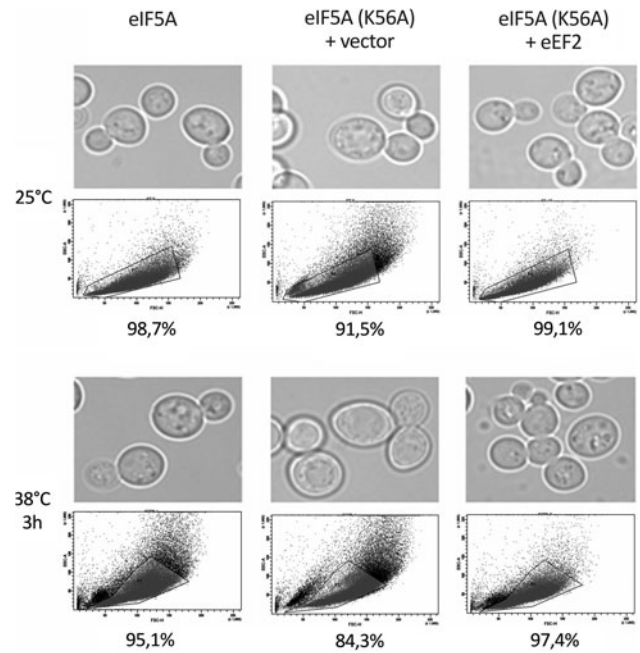
modified to the hypusine form than the wild type protein (roughly 50% of the wild type), while the eIF5A<sup>K56D</sup> mutant protein is not modified at all (Dias et al. 2008). Besides, the eIF5A<sup>K56A</sup> mutant does not show reduced levels of eIF5A protein due to degradation at the restrictive temperature, as displayed by most of the temperature sensitive eIF5A mutants described so far, including the eIF5A<sup>P83S</sup> (*tif51A-1*) mutant (Dias et al. 2008; Saini et al. 2009). Since eIF5A<sup>K56A</sup> is the only eIF5A mutant defective in hypusine modification and the only one suppressed by high-copy eEF2, we believe that high-copy eEF2 is able to compensate for the decreased availability of functional hypusine-containing eIF5A.

We also tested whether impairment in the function of eEF2 would interfere with eIF5A<sup>K56A</sup> mutant. For that purpose, we used the eEF2 dominant negative mutant *eft2*<sup>H699K</sup> (Ortiz and Kinzy 2005). As shown in Fig. 1b, growth of eIF5A<sup>K56A</sup> mutant strain is significantly reduced upon coexpression of the eEF2 dominant negative mutant protein *eft2*<sup>H699K</sup>. This synthetic sick phenotype is similar to that previously observed for the *tif51A-3* mutant of eIF5A (Greggio et al. 2009). Besides that, the genetic interaction between eIF5A and eEF2 mutants is correlated with the fact that another eIF5A mutant was shown to be sensitive to the eEF2 inhibitor drug sordarin (Saini et al. 2009). Therefore, the data presented here further support a close functional link between eIF5A and eEF2.

It has already been demonstrated that various yeast eIF5A mutants display a morphological phenotype of increased cell size (Kang and Hershey 1994; Zanelli and Valentini 2005), which is correlated with a defect in cell cycle events such as actin cytoskeleton polarization (Zanelli and Valentini 2005). As expected, the eIF5A<sup>K56A</sup> mutant also shows increased cell size at both temperatures (Fig. 2, microscopy images). To better quantify this effect, we used flow cytometry analysis to determine the alterations in cell volume (Fig. 2, dot plot graphs). However, when high-copy eEF2 is present, the morphology is remarkably similar to the wild type (eIF5A) strain at both permissive and restrictive temperatures. These results further implicate a role for eIF5A in cell cycle progression (a secondary effect) and demonstrate that this function of eIF5A is also linked to eEF2 function.

Translation elongation defect in the eIF5A<sup>K56A</sup> mutant is also suppressed by high-copy eEF2

Since eEF2 has a well established function in translation elongation and eIF5A has recently been shown to have a role in protein synthesis as well, we tested whether high-copy eEF2 can also improve the levels of protein synthesis in the eIF5A<sup>K56A</sup> mutant strain. We have previously shown that the eIF5A<sup>K56A</sup> mutant has a defect in protein synthesis,

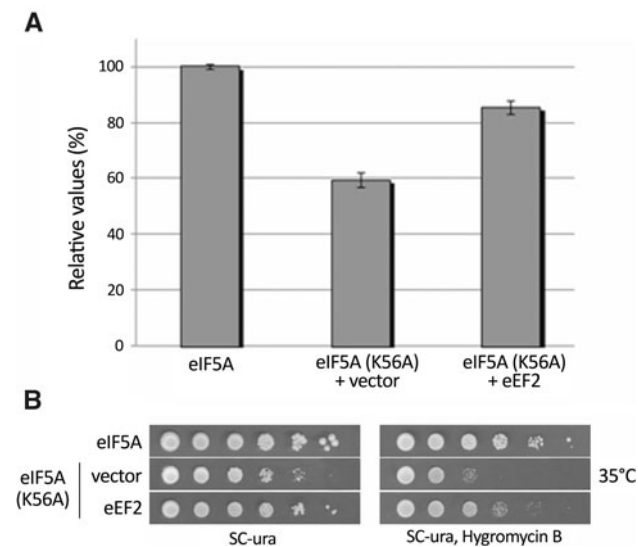


**Fig. 2** Suppression of cell size defect of the eIF5A<sup>K56A</sup> mutant by high-copy eEF2. Wild type (eIF5A) harboring the vector alone and the eIF5A<sup>K56A</sup> mutant strain harboring the vector alone or a high-copy eEF2 plasmid were grown at 25°C in SC-ura to mid-log phase and half of the cultures was then shifted to 38°C for 3 h. The microscopic images were then captured using a Nikon TE300 inverted microscope and a CCD camera. The graph under each micrograph was obtained by flow cytometry analysis of granulosity in function of cell volume using a BD FACSCanto and the BD FACSDiva software. The percentages under the dot plot graphs represent the relative number of cells (dots) inside the area delimited in each graph. The delimited area was determined as the area representing the cell dispersion of the wild type strain (eIF5A)

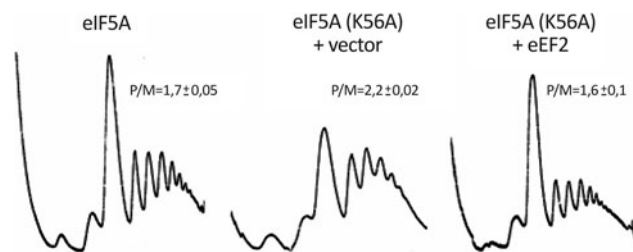
which is correlated with its temperature sensitivity for growth (Dias et al. 2008). As shown in Fig. 3a and Table 1, the defect in protein synthesis displayed by the eIF5A<sup>K56A</sup> mutant is considerably ameliorated by high-copy eEF2. This result strongly suggests that the phenotypic suppression promoted by high-copy eEF2 in the eIF5A<sup>K56A</sup> mutant is the result of a partial restoration of protein synthesis.

Mutants of factors involved in protein synthesis are expected to be more sensitive to translation inhibitors. In fact, several mutants of eIF5A are more sensitive to different antibiotics targeting translation (Zanelli et al. 2006) (and data not shown). We found that the eIF5A<sup>K56A</sup> mutant is hypersensitive to the aminoglycoside hygromycin B, which inhibits eEF2-dependent translocation of the ribosome (Gonzalez et al. 1978). Therefore, we tested the ability of high-copy eEF2 to attenuate hygromycin B sensitivity of the eIF5A<sup>K56A</sup> mutant and the result was positive as the growth was improved in the presence of high-copy eEF2 (Fig. 3b). These findings are in agreement with the idea that high-copy eEF2 acts through improvement in the process of protein synthesis to suppress the eIF5A<sup>K56A</sup> mutant.





**Fig. 3** Improvement of eIF5A<sup>K56A</sup> mutant protein synthesis by high-copy eEF2. **a** Total protein synthesis was measured as described and the amount of [<sup>3</sup>H]leucine incorporation into proteins is shown relatively to the wild type (100%). **b** Tenfold serial dilutions of wild type and eIF5A<sup>K56A</sup> harboring the vector alone or high-copy eEF2 were plated onto SC-ura or SC-ura containing 200 µg/mL of hygromycin B and incubated at 35°C for 3–4 days



**Fig. 4** Recovery of polysome profile defects of the eIF5A<sup>K56A</sup> mutant by high-copy eEF2. Extracts prepared from the wild type harboring the vector alone and eIF5A<sup>K56A</sup> mutant strain harboring the vector alone or the high-copy eEF2 plasmid were fractionated by ultracentrifugation in a sucrose density gradient. Optical scans (OD<sub>254nm</sub>) of the gradients are shown. The ratios of P/M were calculated by comparing the areas of the 80S and polysome peaks

Since eIF5A is known to be involved in translation elongation (Gregio et al. 2009; Saini et al. 2009), we decided to analyze the polysome profile of the eIF5A<sup>K56A</sup> mutant strain. As shown in Fig. 4, the polysome profile of the eIF5A<sup>K56A</sup> mutant shows increased polysome peaks and a decreased 80S peak at the restrictive temperature when compared with the wild type strain. Besides the evident difference between the shapes of wild type and eIF5A<sup>K56A</sup> mutant polysome profile graphs, quantification

of the area of the peaks clearly demonstrates the accumulation of polysomes in the eIF5A<sup>K56A</sup> mutant as the ratio polysome/monosome area (P/M) was increased. An increase in the ratio of polysome to monosome in the mutant strain is due to continuous translation initiation and reduced rates of polysome run-off (Peltz et al. 1992). This result is in accordance with the previously published data (Gregio et al. 2009; Saini et al. 2009). On the other hand, the polysome profile of the eIF5A<sup>K56A</sup> mutant harboring high-copy eEF2 is strikingly similar to the wild type strain. These results once more support a function for eIF5A in translation elongation and suggest that this function is closely related to eEF2.

Although the precise mechanism of action of eIF5A has not been elucidated so far, its involvement in the elongation step of translation has been well documented recently (Gregio et al. 2009; Saini et al. 2009; Patel et al. 2009; Li et al. 2010; Landau et al. 2010). The observation that increase in eEF2 function cooperates with the role of eIF5A during the elongation step of protein synthesis, reported herein for the first time, strongly supports the hypothesis that eIF5A interacts functionally with this elongation factor. Whether these proteins interact physically and where on the ribosome eIF5A binds are still open questions and the answers will conclusively help to elucidate the mechanistic aspects underlying the essential role of eIF5A in the cell.

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