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Mutational analysis reveals potential phosphorylation sites in eukaryotic elongation factor 1A that are important for its activity

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Previous studies have suggested that phosphorylation of translation elongation factor 1A (eEF1A) can alter its function, and large-scale phospho-proteomic analyses in *Saccharomyces cerevisiae* have identified 14 eEF1A residues phosphorylated under various conditions. Here, a series of eEF1A mutations at these proposed sites were created and the effects on eEF1A activity were analyzed. The eEF1A-S53D and eEF1A-T430D phosphomimetic mutant strains were inviable, while corresponding alanine mutants survived but displayed defects in growth and protein synthesis. The activity of an eEF1A-S289D mutant was significantly reduced in the absence of the guanine nucleotide exchange factor eEF1Bα and could be restored by an exchange-deficient form of the protein, suggesting that eEF1Bα promotes eEF1A activity by a mechanism other than nucleotide exchange. Our data show that several of the phosphorylation sites identified by high-throughput analysis are critical for eEF1A function.

Keywords: elongation factor; guanine nucleotide exchange factor; phosphorylation; translation; yeast

Regulated translation is recognized as an important determinant of gene expression in many biological processes including development, synaptic plasticity, and stress response [1]. Work in the area of translational control has focused primarily on the regulation of the initiation phase of protein synthesis; however, regulation of translation elongation allows additional fine-tuning of protein synthesis [2]. Oxidative stress [3], heat shock, proteotoxic stress, and passage through mitosis all influence translation elongation [4–6].

In most eukaryotes, translation elongation is catalyzed by two soluble factors, eukaryotic elongation factor 1 (eEF1) and eEF2 (reviewed in Ref. [7]). eEF1 is composed of eEF1A, a classic G protein, and eEF1B, its guanine nucleotide exchange factor (GEF) complex. eEF1A, the homolog of prokaryotic EF-Tu, binds aminoacyl-tRNA (aa-tRNA) in its GTP bound form and delivers it to the A-site of the ribosome [8,9]. Stimulation of the GTPase activity of eEF1A by the ribosome results in the release of eEF1A:GDP. eEF1B is composed of two subunits in yeast: eEF1Bα, an essential factor that catalyzes nucleotide exchange [10], and eEF1Bγ which has a modest stimulatory effect on the exchange reaction [11]. Metazoan eEF1B contains a third subunit eEF1Bδ which can also catalyze nucleotide exchange [12,13]. Despite a lack of homology at the sequence level, eEF1Bα is the functional equivalent of EF-Ts, the GEF for EF-Tu. Following peptide bond formation, eEF2, a single polypeptide catalyzes the movement of the peptidyl-tRNA from

Abbreviations
aa-tRNA, aminoacyl-tRNA; eEF1, elongation factor 1; GEF, guanine nucleotide exchange factor; TCA, trichloroacetic acid.

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the A-site to the P-site of the ribosome thus allowing multiple rounds of amino acid incorporation. Fungi and likely other lower eukaryotes require a third factor for translation elongation, eEF3 [14–16]. While the exact function of this factor is unknown, eEF3 is proposed to facilitate deacylated-tRNA release from the ribosomal E-site [17].

One way to regulate the elongation phase of translation is to modulate the activity of the soluble factors that facilitate this step of protein synthesis. It has been shown that the activity of several translation elongation factors can be affected by post-translational modification. One of the best characterized examples is the phosphorylation of eEF2 which is observed in both yeast and higher eukaryotes. Phosphorylation by eEF2-kinase (eEF2K) inhibits eEF2's ability to bind to the ribosome and catalyze translocation [18,19]. Phosphorylation of eEF2 has been observed during nutrient deprivation [20] and in neurons [21,22]. While less well-defined, multiple studies have suggested that phosphorylation of members of the mammalian eEF1 complex can also regulate translation elongation. Both stimulatory and inhibitory effects of phosphorylation have been reported. For example, stimulation of mammalian cell proliferation with either insulin or phorbol esters results in a rapid induction of protein synthesis that is correlated with an increase in phosphorylation of the eEF1 complex on the eEF1A, eEF1Bα, and eEF1Bδ subunits and eEF1 purified from treated cells are more active in elongation assays [23,24]. Alternatively, a decrease in ribosomal transit time is associated with eEF1A phosphorylation upon glutamate stimulation of glial cells [25] while phosphorylation of eEF1A by type I TGF-β receptor on Ser300 decreases its ability to bind aa-tRNA and is correlated with overall inhibition of protein synthesis [26]. In addition, phosphorylation of eEF1Bδ by cyclin-dependent kinase 1 in mitosis reduces its interaction with eEF1A and is also associated with a decrease in elongation [6]. Several *in vitro* studies have shown that mammalian eEF1A can be phosphorylated by a number of different kinases; however, either the sites of phosphorylation or the effect of the phosphorylation event on eEF1A activity is unclear [27–33]. Phosphorylation of EF-Tu in prokaryotes has also been observed in response to nutrient deprivation resulting in a decrease in its activity in translation [34–37].

Perhaps not surprisingly due to its abundance, studies of global protein phosphorylation have identified multiple potential phosphorylation sites on yeast eEF1A [38–40]; however, the phosphorylation of eEF1A at these sites has not been studied systematically. In order to address the role of phosphorylation in the regulation of eEF1A, a mutational and proteomic analysis of eEF1A phosphorylation was performed in *Saccharomyces cerevisiae*. Analysis of the post-translational modification of immunopurified eEF1A did not detect phosphorylation at any of the predicted sites under the growth conditions studied; however, mutation of these previously identified residues demonstrated several that are important for the activity of eEF1A.

Experimental procedures

Yeast strains and growth conditions

Yeast cells were grown in either YEPD media (1% Bacto-yeast extract, 2% Bacto-tryptone, 2% dextrose) or defined synthetic complete (C) media (2% dextrose) lacking the indicated amino acids. For spotting assays, 10-fold serial dilutions of yeast cultures starting at an A_{600} of 3 were pinned on the indicated media. Growth was analyzed on YEPD plates grown at 17 °C, 30 °C, and 37 °C as well as on plates containing 0.8 м NaCl, 1.2 м sorbitol, 25 mм menadione, and 1.0 $\mu g \cdot mL^{-1}$ sulfometuron methyl. Growth curves were generated by diluting exponentially growing cultures to an A_{600} of 0.1 in a 96-well plate and measuring the optical density at the indicated time points.

Mutations in eEF1A were generated by QuickChange (Agilent Technologies, Santa Clara, CA, USA) site-directed mutagenesis of plasmid TKB929 according to the manufacturer's instructions. Plasmids expressing mutant eEF1A were transformed into strain TKY1714 using the lithium acetate method. Transformants were plated on synthetic complete medium containing 5-fluoroorotic acid (5-FOA) to identify loss of the WT *TEF1 URA3* plasmid.

Plasmids TKB929 (eEF1A-WT), TKB1216 (eEF1A-S53A), TKB1218 (eEF1A-S289A), or TKB1219 (eEF1A-S289D) were transformed into TKY631. Transformants were plated on synthetic complete medium containing 5-FOA to identify loss of the *TEF5 URA3* plasmid. Strains TKY1761-63 were transformed with the plasmids pRS315 (vector), TKB105 (eEF1Bα-WT), or TKB526 (eEF1Bα-K205A) [41]. All strains were pinned on YEPD as described above (Table 1).

Translation assays

^{35}[S] methionine incorporation assays were performed with the indicated yeast strains grown to mid-log phase in complete medium lacking methionine media at 30 °C. Assays were performed as previously described with each time point sampled in triplicate [42].

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference
TKY631	*MATα ura3-52 lys2-81 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tef5::KanMX pTEF5 URA3*	[61]
TKY1714	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2ΔpTEF1 URA3 CEN*	This work
TKY1717	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN*	[62]
TKY1718	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN (S53A)*	This work
TKY1719	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN (S289A)*	This work
TKY1720	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN (S289D)*	This work
TKY1729	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN (S394A)*	This work
TKY1730	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN (S394D)*	This work
TKY1731	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN (T430A)*	This work
TKY1732	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN (T82A)*	This work
TKY1733	*MATα ura3-52 leu2-3, 112 trp1-Δ1 lys2-20 MET2 his4-713 tef1::LEU2 tef2Δ pTEF1 TRP1 CEN (T82D)*	This work
TKY1761	*MATα ura3-52 lys2-81 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tef5::KanMX pTEF1 TRP1 CEN*	This work
TKY1762	*MATα ura3-52 lys2-81 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tef5::KanMX pTEF1 TRP1 CEN (S289A)*	This work
TKY1763	*MATα ura3-52 lys2-81 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 tef5::KanMX pTEF1 TRP1 CEN (S289D)*	This work
TKY1765	*MATα ura3-52 lys2-81 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 1 tef5::KanMX pTEF1 TRP1 CEN (S53A)*	This work

Sensitivity to translation inhibitors was assessed using exponentially growing strains in liquid YEPD media that were diluted to an A_{600} of 0.6, and 200 µL was spread on YEPD plates. Sterile disks were placed on the plates, and 10 µL of 1 mM cycloheximide or 800 $mg·mL^{-1}$ paromomycin was pipetted onto the disk. Plates were incubated at 30 °C for 2 days at which time the diameter of the zone of growth inhibition was measured.

eEF1A purification

WT and mutant eEF1A proteins were purified from yeast strains as described previously [43]. Briefly, yeast cells expressing either WT or mutant eEF1A as the only form were frozen in liquid nitrogen and lysed by grinding using a 6870 Freezer/Mill (SPEX SamplePrep, Metuchen, NJ, USA). Cell powder was dissolved in lysis buffer (50 mM Tris-Cl, pH 7.5, 50 mM NH_4Cl, 5 mM $MgCl_2$, 0.1 mM EDTA, pH 8.0, 10% glycerol, 1 mM DTT, and 0.2 mM PMSF). Glycerol was added to a final concentration of 25%, and cleared lysate was passed over Q-Sepharose and SP-Sepharose columns in tandem. The columns were equilibrated in buffer A (20 mM Tris-Cl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, pH 8.0, 25% glycerol, 1 mM DTT, and 0.2 mM PMSF). eEF1A was eluted from the SP-Sepharose column using a linear gradient with buffer B (20 mM Tris-Cl, pH 7.5, 500 mM KCl, 0.1 mM EDTA, pH 8.0, 25% glycerol, 1 mM DTT, and 0.2 mM PMSF). eEF1A containing fractions were pooled, concentrated, and loaded onto a HiLoad 16/60 Superdex 200 gel filtration column equilibrated in buffer C (20 mM Tris-Cl, pH 7.4, 200 mM KCl, 0.1 mM EDTA, pH 8.0, 25% glycerol, and 1 mM DTT).

Immunoprecipitation assays

Whole cell lysates were prepared from 10 mL of exponentially growing yeast in YEPD media using lysis buffer (50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1% Triton X-100, and 1 mM PMSF) and vortexing with glass beads. Fifty microgram of extract was incubated with 20 µL Protein A/G beads (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 h at 4 °C then centrifuged for 5 min to preclear the lysate. Lysates were then incubated with rabbit polyclonal antiserum to full length *S. cerevisiae* eEF1A (Kinzy Laboratory, Piscataway, NJ, USA) for 1 h at 4 °C, and the complex was precipitated by incubation with Protein A/G beads (1 h, 4 °C). Control reactions contained 10 µg of mouse IgG antibody (Millipore, Burlington, MA, USA). Immunoprecipitates were washed four times with lysis buffer, eluted using Laemmli buffer, and separated by SDS/PAGE. Membranes were blotted with rabbit polyclonal antiserum to full length *S. cerevisiae* eEF1Bα (Kinzy Laboratory) (1 : 5000) or eEF1A (1 : 10 000), and the secondary antibody (1 : 2000) was VeriBlot for IP Detection Reagent (Abcam, Cambridge, UK).

Aminoacylation of phenylalanyl-tRNA

Total *S. cerevisiae* $tRNA^{Phe}$ was aminoacylated in a 100 µL reaction containing 36.5 µM $tRNA^{Phe}$, 0.12

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A_{280} units of crude *S. cerevisiae* tRNA synthetase prep, 1.8 μCi of [^{14}C] phenylalanine (PerkinElmer, Waltham, MA, USA), 100 mM Tris-HCl, pH 8.0, 5 mM ATP, 50 mM magnesium acetate, 2.5 mM EDTA, and 3 mM β-mercaptoethanol. The reactions were incubated for 20 min at 37 °C. Potassium acetate was added to a final concentration of 0.2 mM, and the reactions were sequentially extracted with phenol (pH 4) and chloroform:isoamyl alcohol (24 : 1). The reactions were ethanol precipitated and resuspended in 5 mM sodium acetate pH 4.8.

Poly(U)-directed poly(Phe) synthesis

Polyphenylalanine synthesis was performed in 50 μL reactions containing 10 pmol eEF1A, 6 pmol 80S ribosomes, 8 pmol of eEF2, 6.5 pmol of eEF3, 0.2 A_{260} units of poly(U) RNA, 60 pmol of [^{14}C] Phe-tRNA^{Phe}, 20 mM Tris, pH 7.5, 100 mM KCl, 7 mM magnesium acetate, 2 mM GTP, 2.1 mM creatine phosphate, 80 μg·mL^{-1} creatine kinase, and 1 mM DTT. Reactions were incubated at 37 °C for 5 min followed by trichloroacetic acid (TCA) precipitation. All proteins and ribosomes were purified in the laboratory using standard protocols. Samples were applied to Whatman GF/C 25-mm filters prewet in 5% TCA and washed three times with 5 mL 5% TCA and once with 5 mL 95% ethanol. Filters were air-dried, and incorporation was determined by liquid scintillation counting.

Sample preparation for mass spectrometry

Since the potential signal for eEF1A phosphorylation is unknown, cells for analysis were grown under six different experimental conditions. These included exponential growth in YEPD medium, heat shock at 37 °C for 30 min in YEPD, exponential growth in complete medium, osmotic stress (complete media plus 0.8 M NaCl for 15 min), and amino acid starvation (2 μg·mL^{-1} sulfometuron methyl for 2 h or 30 mM 3-amino-1,2,4-triazole for 2 h). After each treatment, the yeast cells were frozen as pellets in liquid nitrogen. All yeast pellets were combined and lysed by grinding using a 6870 Freezer/Mill (SPEX SamplePrep). Cell powder was dissolved in 50 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 50 mM NH_4Cl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, and MS-SAFE protease and phosphatase inhibitor (Sigma, St. Louis, MO, USA). The lysate was cleared by centrifugation, and 1 mg of cell lysate was used for each immunoprecipitation. Rabbit antiserum to *S. cerevisiae* eEF1A was affinity-purified and cross-linked to agarose beads using the Pierce Crosslink Immunoprecipitation kit (Waltham, MA, USA) as per the manufacturer's instructions. Immunoprecipitations were performed as recommended except that the wash buffer was 25 mM HEPES pH 7.4, 300 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, and MS-SAFE protease and phosphatase inhibitor. Eluted eEF1A in solution was analyzed by the Mass Spectrometry and Proteomic Resource Laboratory at Harvard University.

Mass spectrometry and analysis

Each sample was submitted for single LC-MS/MS experiment that was performed on a LTQ Orbitrap Elite (Thermo Fisher, Waltham, MA, USA) equipped with Waters (Milford, MA, USA) NanoAcquity HPLC pump Peptides were separated onto a 100 μm inner diameter microcapillary trapping column packed first with approximately 5 cm of C18 Reprosil resin (5 μm, 100 Å, Dr. Maisch GmbH, Ammerbuch, Germany) followed by an analytical column ~ 20 cm of Reprosil resin (1.8 μm, 200 Å, Dr. Maisch GmbH). Separation was achieved through applying a gradient from 5% to 27% ACN in 0.1% formic acid over 90 min at 200 nL min^{-1}. Electrospray ionization was enabled through applying a voltage of 1.8 kV using a homemade electrode junction at the end of the microcapillary column and sprayed from fused silica pico tips (New Objective, Littleton, MA, USA). The LTQ Orbitrap Elite was operated in the data-dependent mode for the mass spectrometry methods. The mass spectrometry survey scan was performed in the Orbitrap in the range of 395–1800 m/z at a resolution of 6×10^4, followed by the selection of the 20 most intense ions (TOP20) for CID-MS2 fragmentation in the ion trap using a precursor isolation width window of 2 m/z, AGC setting of 10 000, and a maximum ion accumulation of 200 ms. Singly charged ion species were not subjected to CID fragmentation. Normalized collision energy was set to 35 V and an activation time of 10 ms, AGC was set to 50 000, the maximum ion time was 200 ms. Ions in a 10 p.p.m. m/z window around ions selected for MS2 were excluded from further selection for fragmentation for 60 s.

Raw data were submitted for analysis in PROTEOME DISCOVERER 2.1.0.81 (Thermo Scientific) software. Assignment of MS/MS spectra was performed using the Sequest HT algorithm by searching the data against a protein sequence database including all entries from the Human Uniprot database (SwissProt 16 768 and TrEMBL 62 460 total of 79 228 protein forms, 2015) and other known contaminants such as human keratins and common laboratory contaminants. Sequest HT searches were performed using a 20 p.p.m. precursor ion tolerance and requiring each

Table 2. Proposed sites of eEF1A phosphorylation in *Saccharomyces cerevisiae*. A plus sign indicates that the residue is identical in human eEF1A1 while a plus sign in parentheses indicates a conservative substitution. A minus sign designates a nonconserved residue.

Residue	Conserved	Reference
S6	(+)	[39]
S18	+	[39]
T38	+	[63]
S53	+	[38]
T72	+	[39]
T82	+	[39]
S157	+	[64]
S163	–	[39,40]
T259	+	[38,40]
S289	+	[40,63,65,66]
S314	+	[63]
S394	+	[38,40]
S414	+	[39]
T430	+	[39,40]

peptide N-/C termini to adhere with trypsin protease specificity while allowing up to two missed cleavages. Cysteine carbamidomethyl (+57.021) was set as static modifications while methionine oxidation (+15.99492 Da) was set as variable modification. MS2 spectra assignment false discovery rate of 1% on protein level was achieved by applying the target-decoy database search. Filtering was performed using a Percolator (64 bit version, reference [6]). For quantification, a 0.02 m/z window centered on the theoretical m/z value of each of the six reporter ions and the intensity of the signal closest to the theoretical m/z value was recorded. Reporter ion intensities were exported in result file of PROTEOME DISCOVERER 2.1 search engine as excel tables. All fold changes were analyzed after normalization between samples based on total unique peptides ion signal.

Results

A search of the *Saccharomyces* Genome Database [SGD Project. http://www.yeastgenome.org/locus/S000000322/protein (June 2013)] and iPTMnet [44] yielded 14 potential serine or threonine phosphorylation sites in yeast eEF1A identified through large-scale studies of protein phosphorylation [38–40]. These sites are distributed throughout all three domains of eEF1A (Table 2). Of these sites, five residues were chosen for mutational analysis based on conservation with mammalian eEF1A and location within the protein. These included Ser53, Thr82, Ser289, Ser394, and Thr430 (Fig. 1). Each amino acid was mutated to alanine and aspartic acid which represent a nonphosphorylatable and a phosphomimetic residue, respectively. These mutants were transformed into yeast lacking the chromosomal *TEF1* and *TEF2* genes encoding eEF1A to determine if they can serve as the only form of eEF1A by plasmid shuffling and the growth rates of the strains viable when expressing only the mutant protein were determined (Table 3). Strains expressing aspartic acid mutations of either Ser53 or Thr430 were inviable while mutation of the same residues to alanine resulted in moderate growth defects. Mutation of either Thr82 or Ser289 decreased growth when changed to aspartic acid but not alanine and both mutants of Ser394 grew at the same rate as cells expressing WT eEF1A.

In order to determine whether the proposed phosphorylation sites of eEF1A were important for the response to cellular stress, all viable mutant strains were screened for growth under a range of conditions including osmotic stress, oxidative stress, amino acid deprivation, and temperature sensitivity (see Experimental procedures). The majority of mutant strains showed no additional growth defects under these conditions (data not shown); however, the strain expressing eEF1A-S289D exhibited temperature-sensitive growth at 37 °C and a modest salt sensitivity which was not observed when the same residue was mutated to alanine (Fig. 2). Thus, while these data show some of these residues to be important in overall function these phosphomimetics and nonphosphorylatable modifications do not show an altered stress response.

A parallel approach was taken to attempt to directly examine the phosphorylation of *S. cerevisiae* eEF1A by mass spectrometry. Since the conditions under which eEF1A might be phosphorylated in yeast are unknown, eEF1A was immunoprecipitated from a pooled whole cell lysate obtained from cells in exponential growth (both rich and defined media) or exposed to heat shock, osmotic stress, or two types of amino acid starvation (for details see Experimental procedures). The immunopurifed eEF1A was subjected to mass spectrometric analysis after digestion with either trypsin or Glu-C and Lys-C. As expected, eEF1A was the most predominant protein identified in the immunoprecipitation sample, and sequence coverage was 94% or better depending on which enzyme was used. While all known methylation sites were detected in this analysis, no phosphorylation was detectable on any of the serine or threonines represented in the databases. Furthermore, no additional phosphorylation sites were identified.

Despite the lack of evidence of phosphorylation under the conditions tested, the growth defects observed in several of the mutant strains suggest that

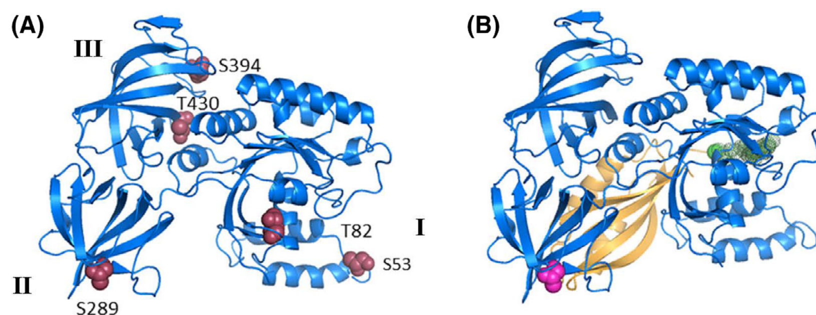

Fig. 1. Localization of proposed phosphorylation sites analyzed on the crystal structure of *Saccharomyces cerevisiae* eEF1A bound to a C-terminal fragment of eEF1Bα (PDB 1IJF; [55]). (A) Conserved, potential phosphorylation sites (red) are found in all three domains of eEF1A (blue). (B) Position of residue S289 (magenta) of eEF1A (blue) is shown in relation to eEF1Bα (yellow). Mg^{2+} (green dot) GDP (gray mesh).

Table 3. Effect of eEF1A mutations on growth and sensitivity to protein synthesis inhibitors. Growth and antibiotic sensitivity of proposed mutants of eEF1A phosphorylation sites. Doubling times (in min) were determined for each strain in YEPD media at 30 °C. Values represent the average of two experiments performed in triplicate including the standard deviation. Antibiotic sensitivity was determined by measuring the diameter of inhibition of growth (in mm) around a filter disk containing 10 µL of either 1 mM cycloheximide or 800 mg·mL^{-1} paromomycin. Cells were plated on YEPD and grown at 30 °C. Diameters represent the average of three experiments including standard deviation.

		Antibiotic sensitivity (mm)	
eEF1A	Growth rate (min)	Cyclo	Paro
WT	112 ± 6	22 ± 1	10 ± 1
S53			
A	137 ± 5	29 ± 2	8 ± 1
D	Inviable	n.a.	n.a.
T82			
A	114 ± 7	22 ± 2	12 ± 1
D	140 ± 5	27 ± 1	10 ± 1
S289			
A	116 ± 5	25 ± 1	14 ± 2
D	128 ± 8	25 ± 1	14 ± 2
S394			
A	112 ± 6	23 ± 1	10 ± 2
D	115 ± 6	24 ± 3	11 ± 1
T430			
A	146 ± 4	29 ± 2	7 ± 0
D	Inviable	n.a.	n.a.

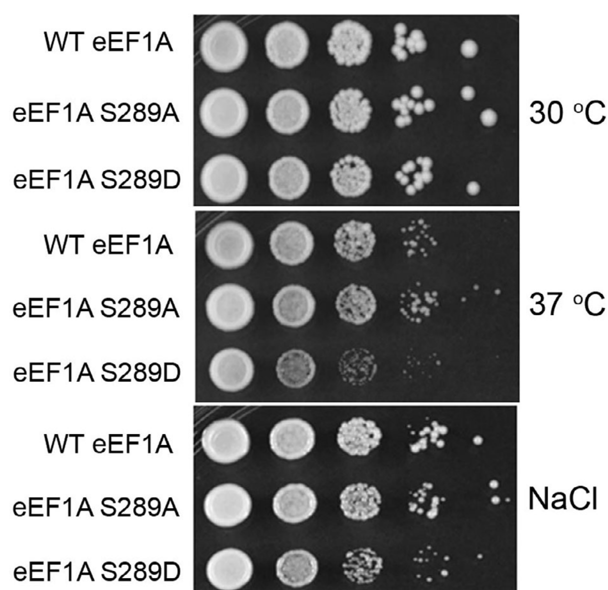

Fig. 2. Cells expressing eEF1A-S289D are temperature and salt-sensitive. Strains expressing WT (TKY1717) or S289A (TKY1719) or S289D (TKY1720) mutant forms of eEF1A were grown to an A_{600} of 3 in YEPD. 10-fold serial dilutions were spotted onto YEPD media (top two panels) or YEPD containing 0.8 M NaCl (bottom panel). Plates were incubated at 30 °C or 37 °C as indicated for 2 days.

these mutations compromise the function of eEF1A. To determine whether these mutations alter the translation function of eEF1A, the sensitivity of the mutant strains to two protein synthesis inhibitors, cycloheximide, which inhibits translation elongation and paromomycin, an aminoglycosidic antibiotic which induces translational misreading, was determined by a growth inhibition assay (Table 3). Mutant strains expressing eEF1A-S53A and eEF1A-T82D were more sensitive to cycloheximide than a strain expressing WT eEF1A while strains expressing either the Ala or Asp mutation of Ser289 were more sensitive to paromomycin. The strain expressing eEF1A-T430A was the only strain which showed altered sensitivity to both drugs being both more sensitive to cycloheximide and resistant to paromomycin. Strains expressing either eEF1A-S394A or S394D were not more sensitive than not that wild type to either of the inhibitors tested and were not analyzed further. These results suggest that several of

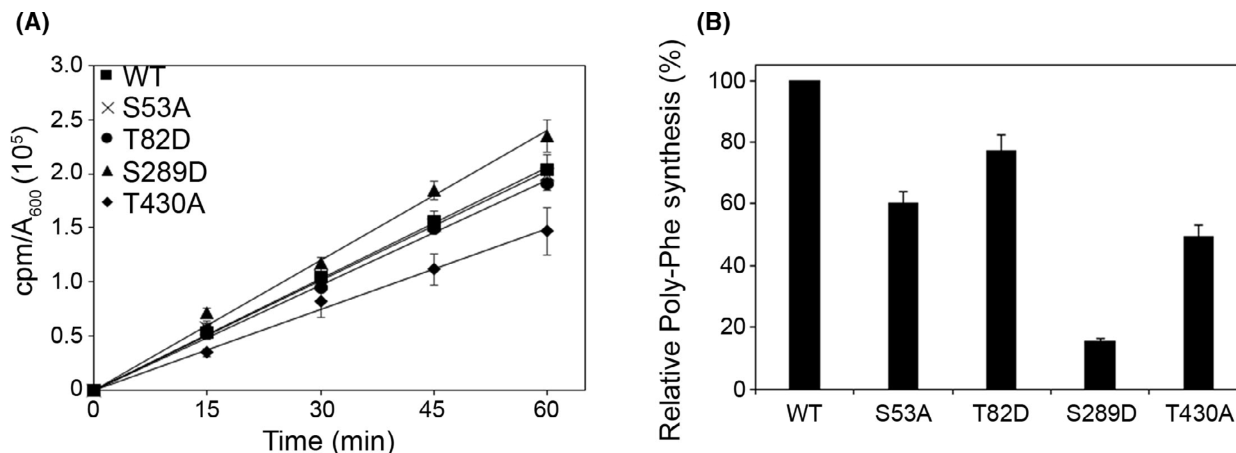

Fig. 3. eEF1A-S289D show protein synthesis defects *in vitro* but not *in vivo*. (A) *Saccharomyces cerevisiae* strains expressing (TKY1717) or the S53A (TKY1718), T82D (TKY1733), S289D (TKY1720) and T430A (TKY1731) mutant forms of eEF1A were grown to log phase in C-Met at 30 °C. ^{35}[S] methionine was added, and total protein synthesis was measured by TCA precipitation at the indicated time points. Incorporation (counts per min) is expressed per A_{600} unit. Error bars represent standard error. (B) Wild type and mutant eEF1A from strains as in figure were purified from yeast and assayed in a polyU directed polyphenylalanine synthesis assay. The amount of polyphenylalanine produced in each reaction was measured following TCA precipitation. The activity of the mutant proteins is expressed as a percentage of the amount of polyphenylalanine synthesized by WT eEF1A. Error bars represent standard error.

the eEF1A mutant strains have defects in translation and/or fidelity that are exacerbated by exposure to the protein synthesis inhibitors.

To determine whether defects in growth and antibiotic sensitivity directly correlated with changes in translation, total protein synthesis was measured by ^{35}[S] methionine incorporation in the strains expressing eEF1A-S53A, T82D, S289D, or T430A mutants that showed the largest growth defects. Strains expressing WT eEF1A, eEF1A-S53A, and eEF1A-T82D had similar rates of protein synthesis (Fig. 3A) suggesting that any translation defects present in these strains are modest and do not impact the overall rate of protein synthesis during exponential growth. A strain expressing the eEF1A-S289D mutant showed a slight increase in total translation during the course of the experiment while the eEF1A-T430A expressing strain was the only mutant strain to have a significant decrease (28%) in overall protein synthesis. In order to investigate mechanisms through which each of these mutations alters the function of eEF1A, the mutant proteins were purified and analyzed in an *in vitro* initiation-independent assay of translation elongation. All four mutant proteins tested exhibited a decrease in the amount of polyU directed polyphenylalanine synthesized, ranging from 16% to 78% of that produced by the WT protein (Fig. 3B). Interestingly, eEF1A-S289D was the most defective of all the mutants despite its rather modest effects on growth and slight increase in protein synthesis *in vivo*. To rule out that this lack of activity was due to misfolding *in vitro*, CD spectra were obtained for the purified WT, S289A, and S289D proteins. No significant differences in overall conformation or thermostability were detected (data not shown).

Ser289 of eEF1A is located near the hydrophobic pocket of eEF1A which interacts directly with eEF1Bα (Fig. 1B) [45]. It is possible that an altered interaction between these two proteins could be responsible for the mild paromomycin sensitivity and growth defect observed in the eEF1A-S289D strain. To address this question, eEF1A was immunoprecipitated from strains expressing either WT eEF1A, eEF1A-S289A, or eEF1A-S289D, and the resulting immunoprecipitates were immunoblotted with antibodies raised against eEF1Bα (Fig. 4). Essentially, equal amounts of eEF1Bα were found in complex with eEF1A in both the WT and S289A expressing strains. However, the eEF1A-S289D strain showed a significant decrease in the amount of eEF1Bα in complex with the mutant eEF1A.

One explanation for the lower activity of eEF1A-S289D *in vitro* compared to its apparent cellular activity could be the absence of its exchange factor, eEF1Bα. Since eEF1A has similar affinities for both GDP and GTP [46] and GTP is not limiting, the polyphenylalanine synthesis assay does not require the presence of the eEF1Bα. Previous studies have shown that while the gene which encodes eEF1Bα, *TEF5*, is essential, its requirement can be bypassed by the

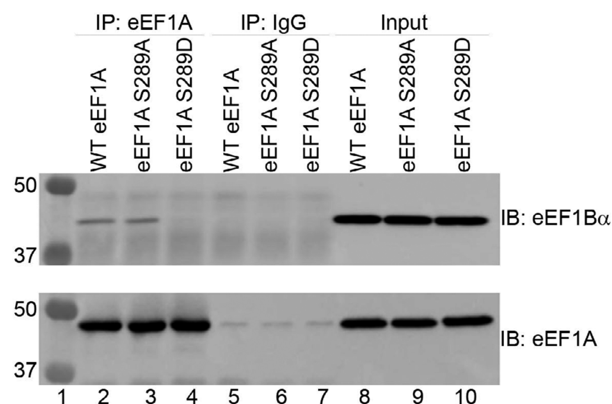

Fig. 4. The interaction between eEF1A-S289D and eEF1Bα is significantly reduced. Cell lysates from strains expressing either WT or S289 mutant forms of eEF1A as in Fig. 2 were incubated with control IgG (lanes 5–7) or and a polyclonal antibody to eEF1A (lanes 2–4). Immunoprecipitates were run on SDS/PAGE gels and immunoblotted with antiserum to eEF1Bα (upper panel) or eEF1A (lower panel). Input (lanes 8–10) represents 4% of the cell lysate used in the immunoprecipitation.

presence of an extra, plasmid borne copy of eEF1A [47]. In order to examine the activity of eEF1A-S289D in the absence of eEF1Bα, plasmids expressing either WT or mutant eEF1A (S289A, S289D, or S53A) were transformed into a yeast strain lacking the chromosomal copy of *TEF5* which is maintained by the presence of a *TEF5 URA3* plasmid. Following transformation, cells that were able to spontaneously lose the *TEF5 URA3* plasmid were selected by growth on 5-FOA (Fig. 5A). Strains lacking the chromosomal *TEF5* gene and containing the two chromosomal genes encoding eEF1A are viable due to the presence of a *TEF5 URA3* plasmid In the presence of a *TRP1,* plasmid strains expressing WT eEF1A or any of the mutant eEF1As were able to grow on 5-FOA indicating replacement of the need for the *TEF5* encoded eEF1Bα protein. Expression of either eEF1A-S289A or eEF1A-S53A was able to rescue growth of the *tef5Δ* strain to the same extent as WT eEF1A (Fig. 5A). However, the strain expressing eEF1A-S289D and lacking eEF1Bα showed a significant decrease in growth (Fig. 5B). This result indicates that eEF1A-S289D has reduced activity in the absence of eEF1Bα. In order to determine whether eEF1A-S289D required the nucleotide exchange activity of eEF1Bα, a nucleotide exchange-deficient form of eEF1Bα was tested for its ability to rescue growth of the eEF1A-S289D expressing *tef5Δ* strain. A plasmid expressing eEF1Bα-K205A which has 13-fold lower rate of nucleotide exchange [41] was transformed into the strains and growth analyzed by a spotting assay. Expression of eEF1Bα-K205A increased the growth rate of *tef5Δ* strains overexpressing either WT eEF1A or eEF1A-S289A. Expression of this exchange-deficient mutant form of eEF1Bα was also able to rescue growth of the eEF1A-S289D expressing *tef5Δ* strain (Fig. 5C). This observation suggests that eEF1Bα stimulates eEF1A activity in a manner independent of its nucleotide exchange activity.

Discussion

Precise regulation of the energy-intensive process of protein synthesis is an important part of nutrient-dependent cell growth and the cellular stress response pathway. Phosphorylation of components of the protein synthesis machinery plays a key role in this process, and work from a number of laboratories suggests that phosphorylation of both EF-Tu in prokaryotes and eEF1A in metazoans can modulate its activity [34,48]. Despite evidence from studies of the global phosphoproteome which suggest that *S. cerevisiae* eEF1A is also phosphorylated, mass spectrometry did not detect any eEF1A specific phosphopeptides in the immunoprecipitated yeast protein. While these results suggest that phosphorylation of eEF1A in *S. cerevisiae* is not a significant method by which its activity is regulated, this result does not preclude this possibility. Most notably, eEF1A may not be significantly phosphorylated under the conditions examined, or perhaps this is masked due to the high level of other post-translational modifications present on eEF1A.

Mutants made to probe the effects of residues identified as potentially phosphorylated showed the importance of some of these sites for the function of eEF1A. Threonine 430, highly conserved from bacteria to humans, is expected to be involved in tRNA binding based on the crystal structure of the EF-Tu·GTP·aa-tRNA complex [49] and the observation that phosphorylation of this residue of EF-Tu inhibits ternary complex formation [34]. In yeast, mutation of this residue to aspartic acid resulted in an eEF1A mutant protein that was unable to support growth of *S. cerevisiae* while its mutation to alanine reduced eEF1A activity both *in vivo* and *in vitro*. A previous study demonstrated that a T430C mutation inhibited growth; however, it did not affect tRNA binding or *in vitro* elongation activity of the mutant protein [43]. These observations highlight the importance of this residue for the activity of eEF1A. Three kinases have been identified in mammals that are capable of phosphorylating this residue *in vitro* including protein kinase Cδ [28], the Rho-associated coiled-coil kinase-2 (ROCK2) [30], and the PAS (Pre-Arnt Sim) domain kinase,

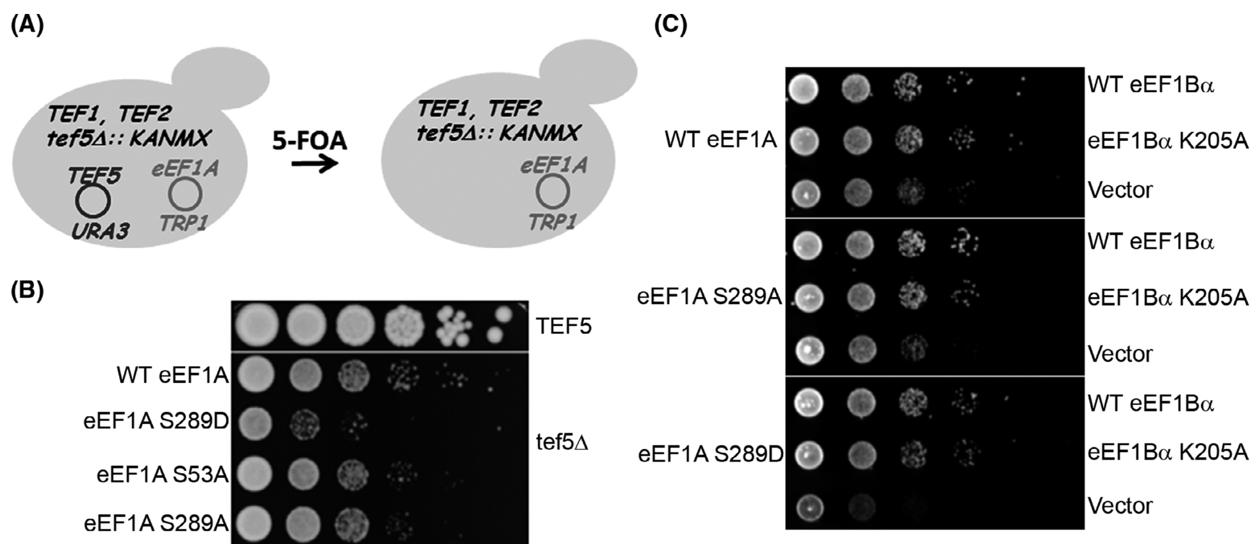

Fig. 5. The function of eEF1A-S289D is compromised in the absence of eEF1Bα encoded by *TEF5*. (A) Schematic representation of the plasmid shuffle experiment in which overexpression of eEF1A compensates for the loss of eEF1Bα. (B) The *tef5Δ* strains lacking eEF1Bα and overexpressing a form of eEF1A from plasmids pTKB929 (eEF1A-WT), pTKB1216 (eEF1A-S53A), pTKB1218 (eEF1A-S289A), or pTKB1219 (eEF1A-S289D) were grown to an A_{600} of 3 in YEPD. 10-fold serial dilutions of the indicated strains were pinned on YEPD plates and grown at 30 °C for 3 days. (C) The strains utilized in B. were transformed with either an empty vector, a plasmid expressing WT eEF1Bα, or a plasmid expressing the nucleotide exchange-deficient mutant, eEF1Bα-K205A and maintained on C-Trp-Leu medium. Pinning was performed as in B, and plates were grown for 2 days at 30 °C.

PASKIN [32]; however, the conditions under which these kinases could modify eEF1A *in vivo* is unknown. Serine 53 is conserved in mammals and its mutation to aspartic acid also rendered the protein unable to support cell viability as has been previously reported for a mutation of this residue to glutamic acid [50]. Mutation of Ser53 to alanine reduced its activity both *in vivo* and *in vitro*. Phosphorylation of Ser53 by protein kinase C β1 has been reported but the physiological conditions where this modification could occur and its effect is also unknown [51]. Interestingly, this residue is also the site of glucosylation by the *Legionella pneumophila* glucosyltransferases and its modification is associated with a decrease in protein synthesis [52]. The location of Ser53 near the GTP-binding domain of eEF1A suggests that mutation and/or modification of this residue may impact the nucleotide-binding activity but this remains to be established. Threonine 82 in the nucleotide-binding domain of yeast eEF1A is also conserved in both prokaryotes and humans and mutation of this residue to aspartic acid had modest effects on cell growth and protein synthesis. No kinases have been reported to phosphorylate this residue to date.

The eEF1A-S289D protein was unique among the eEF1A mutants analyzed in that it could support nearly wild type growth *in vivo* but was significantly inhibited in *in vitro* elongation assays. Experiments using a strain lacking eEF1Bα demonstrated that this protein has a unique dependence on the presence of its GEF for activity. This is an interesting parallel to the finding of phosphorylation of the mammalian GEF eEF1Bδ by cyclin-dependent kinase 1 [6]. The ability to rescue the slow growth phenotype of eEF1Bα-deficient, eEF1A-S289D expressing cells with a nucleotide exchange-deficient form of eEF1Bα further suggests that eEF1Bα has other roles in promoting eEF1A activity than simply facilitating nucleotide exchange. Interestingly, Domain II of eEF1A is also a proposed site of tRNA binding based on the EF-Tu·GTP·aa-tRNA structure [49] and crosslinking studies in eukaryotes [53]. Overlay of Domain II of eEF1A with Domain II of aa-tRNA bound EF-Tu suggests that Phe163 of eEF1Bα occupies the same space as the 3′ terminal A base of the tRNA [45]. Therefore, it has been proposed that the eEF1A·GTP·eEF1B complex may facilitate the interaction of eEF1A with aa-tRNA by altering the conformation of Domain II of eEF1A [45]. Kinetic studies of ternary complex formation using *Escherichia coli* elongation factors support this hypothesis as it was observed that EF-Ts directly facilitates tRNA binding by EF-Tu [54].

The co-crystal structure of *S. cerevisiae* eEF1A bound to the catalytically active C-terminal fragment

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of eEF1Bα identified two major binding interfaces in Domains I and II of eEF1A which differs significantly from the interaction of EF-Tu and EF-Ts [45,55,56]. The interaction between Lys205 of eEF1Bα with Domain I of eEF1A disrupts the Mg^{2+} binding pocket releasing bound GDP, while interaction of Phe163 with a hydrophobic pocket in Domain II of eEF1A is important for stable binding of the two factors [57]. The loss of interaction between the eEF1A-S289D mutant and eEF1Bα in cell extracts supports the role of Domain II in this interaction. Although phosphorylation of this residue was not detected under the conditions examined and no potential kinases have been identified to date, phosphorylation of Ser289 of eEF1A would likely affect its interaction with its exchange factor.

eEF1A is a highly post-translationally modified protein. In addition to phosphorylation, it has also been reported to be methylated, acetylated, AMPylated, S-nitrosylated, and ubiquitinylated [58]. eEF1A also contains unique modifications of glutamate residues including ethanolamine phosphoglycerol in mammals [59] and glutaminylation in yeast [60]. How these multiple modifications combine to influence the activity of eEF1A in protein synthesis or its other cellular functions in response to cellular cues remains to be determined.

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Conflict of interest

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions

MKM and TGK designed the overall work, MKM, DH and PS performed the experiments, MKM, DH, PS and TGK analyzed the results and contributed to writing the manuscript. MKM and TGK finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

Data accessibility

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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