Published in "Journal of Biological Chemistry292(39):16014–16023, 2017"
which should be cited to refer to this work.

Protein glutaminylation is a yeast-specific posttranslational modification of elongation factor 1A

Thomas Jank[‡1], Yury Belyi[§¶], Christophe Wirth[‖], Sabine Rospert[‖], Zehan Hu[‡‡§§¶¶], Jörn Dengjel[‡‡§§¶¶], Tina Tzivelekidis[‡2], Gregers Rom Andersen[‖‖], Carola Hunte[‖**], Andreas Schlosser[***], and Klaus Aktories[‡**§§3]**

*From the ‡Institute for Experimental and Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Freiburg, 79104 Freiburg, Germany, the §Gamaleya Research Centre, Moscow 123098, Russia, the ¶Bioclinicum, Moscow 123098, Russia, the ‖Institute for Biochemistry and Molecular Biology, ZBMZ, Faculty of Medicine, University of Freiburg, 79104 Freiburg, Germany, the **BIOSS Centre for Biological Signalling Studies, University of Freiburg, 79106 Freiburg, Germany, the ‡‡Department of Dermatology, Faculty of Medicine, University of Freiburg, 79106 Freiburg, Germany, the §§Freiburg Institute for Advanced Studies (FRIAS), University of Freiburg, 79104 Freiburg, Germany, the ¶¶Department of Biology, University of Fribourg, Chemin du Musée 10, 1700 Fribourg, Switzerland, the ‖‖Department of Molecular Biology and Genetics, Center for Structural Biology, Aarhus University, DK8000 Aarhus, Denmark, and the ***Rudolf Virchow Center for Experimental Biomedicine, University of Würzburg, 97080 Würzburg, Germany*

http://doc.rero.ch

Ribosomal translation factors are fundamental for protein synthesis and highly conserved in all kingdoms of life. The essential eukaryotic elongation factor 1A (eEF1A) delivers aminoacyl tRNAs to the A-site of the translating 80S ribosome. Several studies have revealed that eEF1A is posttranslationally modified. Using MS analysis, site-directed mutagenesis, and X-ray structural data analysis of *Saccharomyces cerevisiae* eEF1A, we identified a posttranslational modification in which the α amino group of mono-L-glutamine is covalently linked to the side chain of glutamate 45 in eEF1A. The MS analysis suggested that all eEF1A molecules are modified by this glutaminylation and that this posttranslational modification occurs at all stages of yeast growth. The mutational studies revealed that this glutaminylation is not essential for the normal functions of eEF1A in *S. cerevisiae*. However, eEF1A glutaminylation slightly reduced growth under antibiotic-induced translational stress conditions. Moreover, we identified the same posttranslational modification in eEF1A from *Schizosaccharomyces pombe* but not in various other eukaryotic organisms tested despite strict conservation of the Glu^{45} residue among these organisms. We therefore conclude that eEF1A glutaminylation is a yeast-specific posttranslational modification that appears to influence protein translation.

This work was supported by the Deutsche Forschungsgemeinschaft Collaborative Research Center 746 (to T. J., K. A., S. R., and C. H.), Deutsche Forschungsgemeinschaft AK6/22–1 (to K. A.), and the Excellence Initiative of the German Federal and State Governments (cluster of excellence 294) BIOSS (to K. A. and C. H.). The authors declare that they have no conflicts of interest with the contents of this article.
This article contains supplemental Figs. 1–5, Table 1, and References.
The atomic coordinates and structure factors (code 5o8w) have been deposited in the Protein Data Bank (http://wwpdb.org/).
[1] To whom correspondence may be addressed: Albert-Ludwigs-Universität Freiburg, Medizinische Fakultät, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albertstraße 25, 79104 Freiburg, Germany. Tel.: 49-761-203-5301; Fax: 49-761-203-5311; E-mail: thomas.jank@pharmakol.uni-freiburg.de.
[2] Present address: Roche Diagnostics GmbH, 68305 Mannheim, Germany.
[3] To whom correspondence may be addressed: Albert-Ludwigs-Universität Freiburg, Medizinische Fakultät, Institut für Experimentelle und Klinische Pharmakologie und Toxikologie, Albertstraße 25, 79104 Freiburg, Germany. Tel.: 49-761-203-5301; Fax: 49-761-203-5311; E-mail: klaus.aktories@pharmakol.uni-freiburg.de.

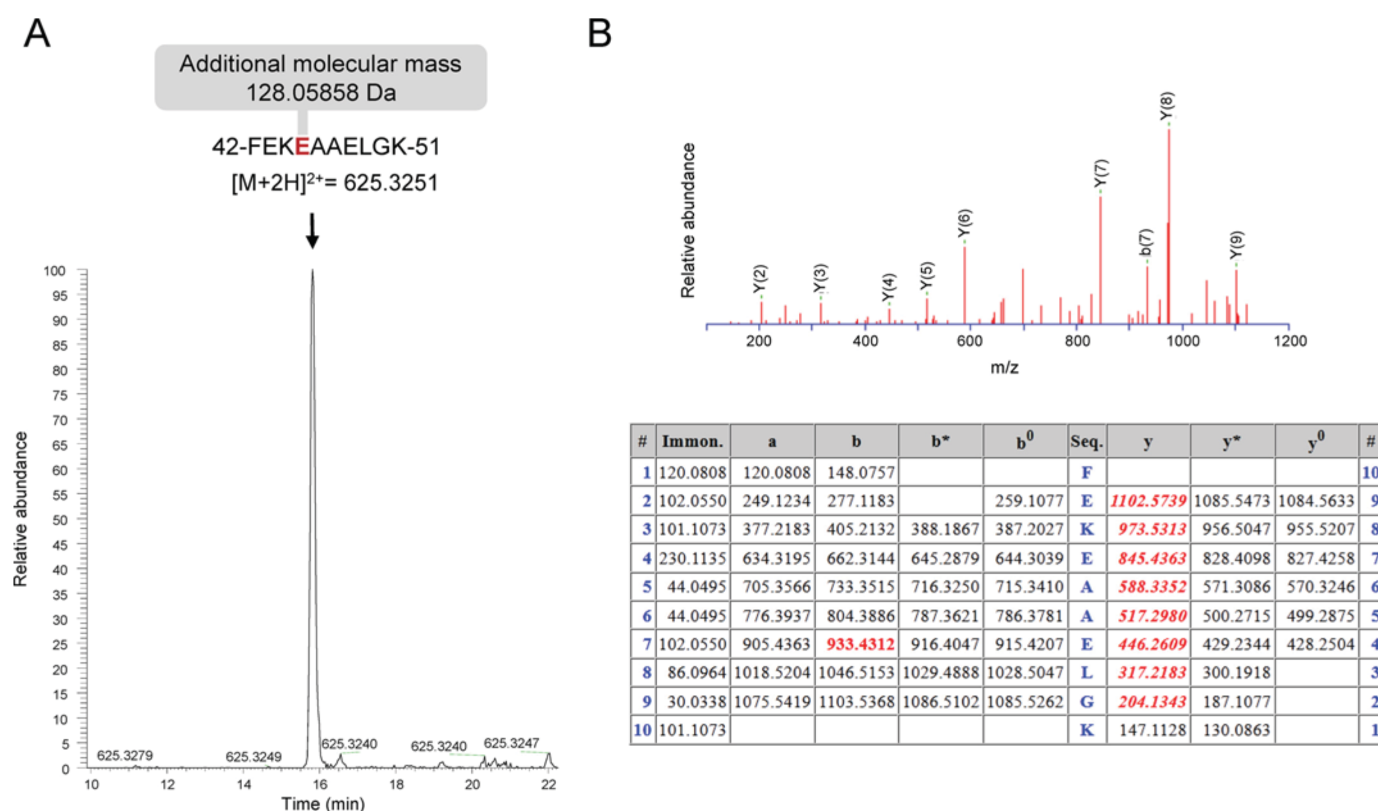
One of the most conserved biological processes is ribosomal protein synthesis, comprising initiation, elongation, termination, and recycling steps. Each step is dependent on specific translation factors. Eukaryotic elongation factor 1A (eEF1A),[4] a large GTPase that is one of the most abundant cytosolic proteins, is important for the binding, stabilization, and delivery of aminoacylated tRNA to the translating ribosome. Correct codon–anticodon pairing of the aminoacyl–tRNA with the mRNA at the ribosomal A-site triggers ribosome-dependent hydrolysis of GTP and leads to dissociation of eEF1A from the ribosome. eEF1A is reactivated by nucleotide exchange factor eEF1B (1) and then able to reassociate with charged tRNA to start a new translation cycle. Besides its essential role in mRNA translation, eEF1A participates in many other cellular functions and is reportedly involved in actin bundling, nuclear export (2), signal transduction (3), apoptosis, proteasomal degradation, and tumorigenesis (4, 5).

Crystal structures of archaeal, mammalian, and yeast eEF1A (partially in complex with subunits of eEF1B) showed that the elongation factor consists of three domains (I–III) (6–8). Domain I is the GTP-binding domain and resembles GTPases of the Ras family. Domains II and III are likely to act as a rigid functional unit and are involved in aminoacyl–tRNA binding. These domains were also reported to be implicated in the interaction with cytoskeletal proteins (9, 10).

eEF1A is extensively posttranslationally modified by lysyl acetylation, methylation (11), ubiquitination, nitrosylation, glutathionylation, phosphorylation (12), C-terminal methyl esterification (13), and the attachment of ethanolamine phosphoglycerol (EPG) (14). Most posttranslational modifications of eEF1A occur in domains II and III, whereas fewer modifications are found in the enzymatic GTPase domain. However, domain I of eEF1A is posttranslationally modified by *Legionella pneumophila* glucosyltransferases Lgt1–3, which attach glu-

[4] The abbreviations used are: eEF1A, eukaryotic elongation factor 1A; EPG, ethanolamine phosphoglycerol; CID, collision-induced dissociation; aa, aminoacyl; ETD, electron transfer dissociation.

http://doc.rero.ch



#	Immon.	a	b	b*	b⁰	Seq.	y	y*	y⁰	#
1	120.0808	120.0808	148.0757			F				10
2	102.0550	249.1234	277.1183		259.1077	E	1102.5739	1085.5473	1084.5633	9
3	101.1073	377.2183	405.2132	388.1867	387.2027	K	973.5313	956.5047	955.5207	8
4	230.1135	634.3195	662.3144	645.2879	644.3039	E	845.4363	828.4098	827.4258	7
5	44.0495	705.3566	733.3515	716.3250	715.3410	A	588.3352	571.3086	570.3246	6
6	44.0495	776.3937	804.3886	787.3621	786.3781	A	517.2980	500.2715	499.2875	5
7	102.0550	905.4363	933.4312	916.4047	915.4207	E	446.2609	429.2344	428.2504	4
8	86.0964	1018.5204	1046.5153	1029.4888	1028.5047	L	317.2183	300.1918		3
9	30.0338	1075.5419	1103.5368	1086.5102	1085.5262	G	204.1343	187.1077		2
10	101.1073					K	147.1128	130.0863		1

Figure 1. eEF1A isolated from *S. cerevisiae* is posttranslationally modified in the GTPase domain at Glu⁴⁵. *A*, extracted ion chromatogram showing that the tryptic peptide 42-FEKEAAELGK-51 ($[M+2H]^{2+} = 625.3251$) of yeast EF1A is covalently modified by the attachment of a mass of 128.059 Da. *B*, MS-MS fragmentation analysis revealed that the tryptic peptide 42-FEKEAAELGK-51 of yeast EF1A is modified at a side chain of Glu^{45}. *Immon.*, immonium ion; *Seq.*, sequence.

cose onto Ser^{53} of yeast and mammalian eEF1A (15, 16). Although toxin-induced modification of eEF1A results in inhibition of protein synthesis in yeast and mammalian cells, the roles and functional consequences of most endogenous posttranslational modification of eEF1A are still not clear (17).

eEF1A belongs to the most conserved proteins across all kingdoms of life (18). However, in contrast to the prokaryotic orthologue, eukaryotic and archaeal EF1A contain an additional subdomain, the helix A*–loop–helix A′ region (amino acids 36–69). This double helix insert is part of the switch I region within the GTPase domain. Extensive structural alterations of this region during GDP/GTP exchange and during interaction with the ribosome suggest a pivotal role of this additional helix–loop–helix region in eukaryotic organisms (7).

Here we describe, for the first time, a novel type of posttranslational modification, the glutaminylation of a glutamate residue, that occurs within the helix A*–loop–helix A′ region of yeast eEF1A. We show that the glutamine residue is attached via its α amino group to the side chain carboxyl group of Glu^{45} within eEF1A. Glutaminylation of eEF1A was detected in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Experiments with *S. cerevisiae* expressing Glu^{45} eEF1A mutants instead of the wild-type elongation factor indicate that glutaminylation slightly enhances growth defects under translational stress conditions.

Results

Posttranslational modification of the switch I region in eEF1A

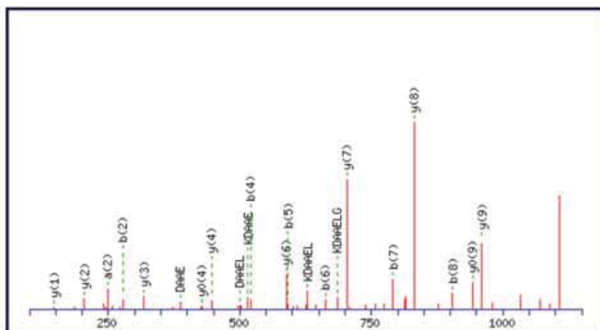
We set out to specifically analyze the helix A*–loop–helix A′ region in EF1A for unconventional posttranslational modifications. eEF1A was purified from *S. cerevisiae* using affinity chromatography with His_6-tagged guanine nucleotide exchange factor eEF1B (19). MS analysis revealed that the tryptic peptide 42-FEKEAAELGK-51 $[M+2H]^{2+} = 625.3251$ was shifted to a higher mass by 128.05858 Da ± 2 ppm (Fig. 1*A*). The additional mass suggested a modification with an organic molecule with the sum formula $C_5H_8N_2O_2$, which is exactly the mass of the amino acid glutamine reduced by an H_2O molecule. Further CID MS-MS fragmentation analysis showed that the additional mass was clearly attached to the side chain of Glu^{45}(Fig. 1*B*). The attachment of a single glutamine to proteins has so far not been reported. We termed this novel type of posttranslational modification "glutaminylation."

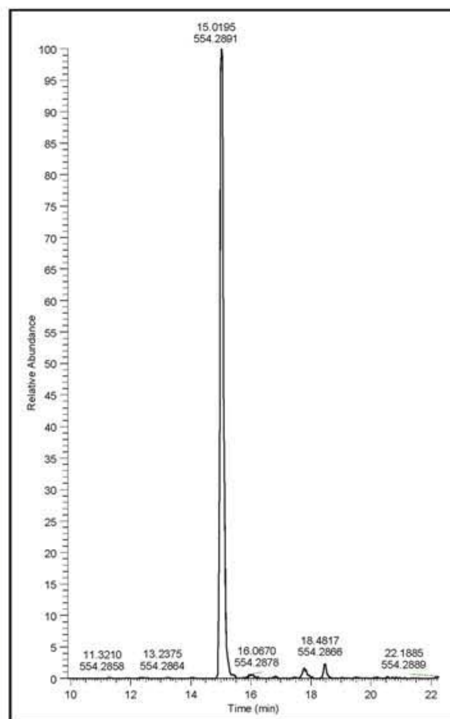
Confirmation of eEF1A glutaminylation at Glu⁴⁵ by site-directed mutagenesis

To confirm the glutaminylation of Glu^{45} in eEF1A, we performed MS-MS analysis with the site-directed mutants eEF1A E45A and eEF1A E45D expressed in and isolated from *S. cerevisiae* (Fig. 2). Mutation of Glu^{45} to alanine prevented modification by glutamine. Similarly, the mutant eEF1A E45D with shortened side-chain carbonyl was not modified. Thus, glutaminylation seems to be highly specific for glutamate at position 45. Notably, using wild-type eEF1A, we were not able to identify unmodified eEF1A in yeast cells. Also, at different cell stages of yeast growth, eEF1A was always completely glutaminylated (supplemental Fig. 1).

A

eEF1A E45D



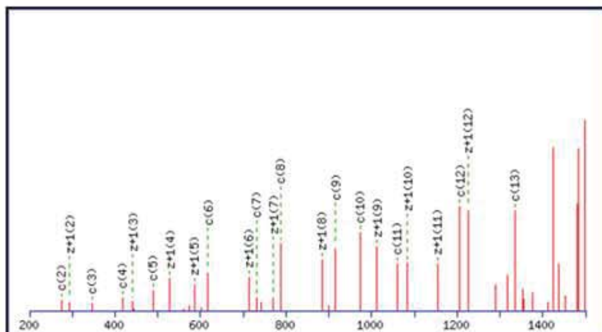


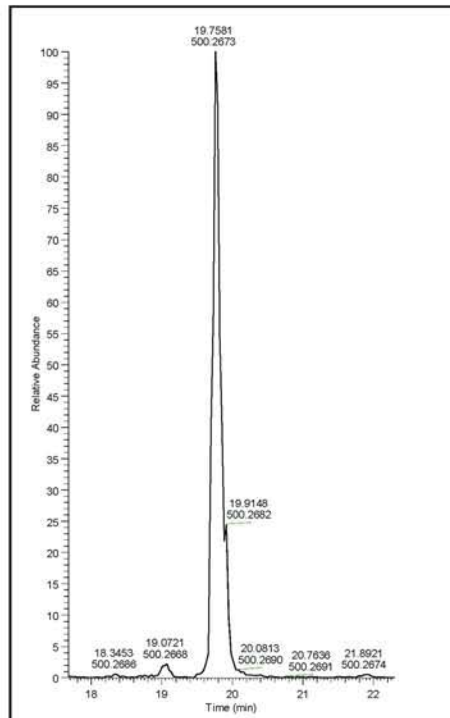
Monoisotopic mass of neutral peptide Mr(calc): 1106.5608
Ions Score: 86 Expect: 2.4e-010
Matches : 24/95 fragment ions using 29 most intense peaks (help)

#	Immon.	a	b	b*	b^0	Seq.	y	y*	y^0	#
1	120.0808	120.0808	148.0757			F				10
2	102.0550	249.1234	277.1183		259.1077	E	960.4997	943.4731	942.4891	9
3	101.1073	377.2183	405.2132	388.1867	387.2027	K	831.4571	814.4305	813.4465	8
4	88.0393	492.2453	520.2402	503.2136	502.2296	D	703.3621	686.3355	685.3515	7
5	44.0495	563.2824	591.2773	574.2508	573.2667	A	588.3352	571.3086	570.3246	6
6	44.0495	634.3195	662.3144	645.2879	644.3039	A	517.2980	500.2715	499.2875	5
7	102.0550	763.3621	791.3570	774.3305	773.3464	E	446.2609	429.2344	428.2504	4
8	86.0964	876.4462	904.4411	887.4145	886.4305	L	317.2183	300.1918		3
9	30.0338	933.4676	961.4625	944.4360	943.4520	G	204.1343	187.1077		2
10	101.1073					K	147.1128	130.0863		1

B

eEF1A E45A





Monoisotopic mass of neutral peptide Mr(calc): 1497.7827
Ions Score: 108 Expect: 2.8e-012
Matches : 23/39 fragment ions using 35 most intense peaks (help)

#	c	Seq.	z+1	z+2	#
1	147.0764	E			14
2	275.1714	K	1353.7287	1354.7365	13
3	346.2085	A	1225.6337	1226.6416	12
4	417.2456	A	1154.5966	1155.6045	11
5	488.2827	A	1083.5595	1084.5673	10
6	617.3253	E	1012.5224	1013.5302	9
7	730.4094	L	883.4798	884.4876	8
8	787.4308	G	770.3957	771.4036	7
9	915.5258	K	713.3743	714.3821	6
10	972.5473	G	585.2793	586.2871	5
11	1059.5793	S	528.2579	529.2657	4
12	1206.6477	F	441.2258	442.2336	3
13	1334.7427	K	294.1574	295.1652	2
14		Y	166.0624	167.0703	1

http://doc.rero.ch

http://doc.rero.ch

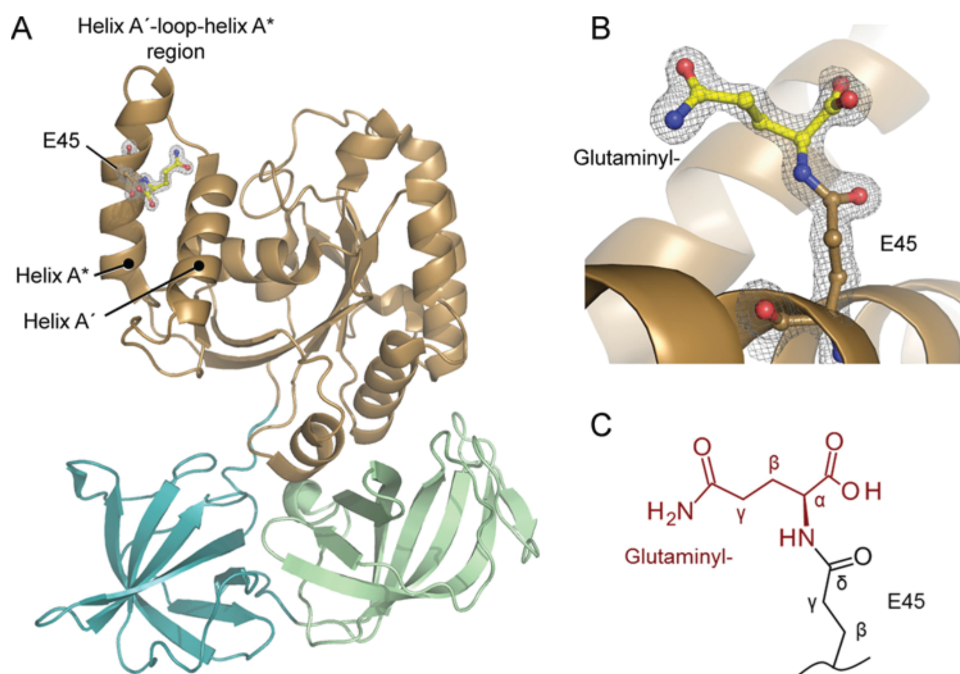


Figure 3. Crystal structure of glutaminylated yeast eEF1A. *A*, the crystal structure of posttranslationally modified eEF1A from the eEF1A–eEF1B complex. The helix A*–loop–helix A′ region of the GTPase domain (*brown*) of eEF1A is marked. The modified Glu[45] is shown as *sticks* and *balls*. Domain II and domain III are shown in *cyan* and *green*, respectively. eEF1B was omitted for clarity. Original structure factors were deduced from Ref. 8. *B*, electron density map $2mF_o - dF_c$ (*gray*), contoured at a level of 1.7 σ in the vicinity of the L-glutaminyl moiety (*yellow*) covalently attached via its α amino group to Glu[45] of yeast eEF1A. *C*, chemical structure of the covalent linkage of the glutaminyl moiety (*red*) to Glu[45].

L-Glutamine is linked to Glu[45] via the α amino group

To analyze glutaminylation in molecular detail and clarify how the glutaminyl moiety is attached, we used the 1.67-Å-resolution crystal structure of *S. cerevisiae* eEF1A in complex with the C-terminal catalytic domain of the exchange factor eEF1B (PDB code 1F60 (8)), which is the highest-resolution structure available for this protein in the Protein Data Bank. An unassigned electron density close to residue Glu[45] permitted addition of a glutaminyl moiety in a defined orientation (Fig. 3, *A* and *B*). The α amino group of the glutamine is covalently linked to the δ C atom of the carboxylic group of the Glu[45] side chain. This is a peptide bond–like connection with a characteristic planar geometry and a short C-N bond. The bond formation results in the loss of one oxygen atom of the carboxylic group from Glu[45] and equates to the release of a water molecule (Fig. 3*C*). The modification extends the glutamate side chain as a branched moiety, with the side chain and the carboxylic group of glutamine both exposed to the surface of the GTPase domain of eEF1A. Structure refinement comparing the fit of L- and D-glutamine enantiomers clearly indicated the attachment of L-glutamine. Multiple posttranslational modifications were described previously for eEF1A (11–14). Of those, monomethylation of Lys[30] and trimethylation of Lys[79] (11) could also be assigned to electron density features, and these modifications were included in the final refined structure (PDB code 5O8W) (supplemental Fig. 2, *A* and *B*). The attachment of a glutamate-derived glutamyl is a mechanism known for several proteins, *e.g.* in polyglutamylation of α and β tubulin (20), γ-glutamylation in glutathione metabolism, or xenobiotics detoxification (21). In contrast, the attachment of a single glutamine via its α amino group has not been reported previously.

Mouse, bovine, fish, insect, and archaeal eEF1A are not glutaminylated at Glu[45]

Glu[45] is highly conserved within the helix A*–loop–helix A′ region of eukaryotic eEF1A and also present in several archaeal EF1A molecules (Fig. 4). To determine whether glutaminylation of Glu[45] is also conserved, we isolated elongation factors from fission yeast (*S. pombe*), archaea (*Haloferax volcanii*), zebrafish (*Danio rerio*) ZF4 fibroblasts, bovine (*Bos taurus*) liver, mouse (*Mus musculus*) brain and liver, and wax moth larvae (*Galleria mellonella*) homogenate and analyzed the proteins by LC/MS-MS. Identified peptidic fragments were well-assigned and clearly indicated that glutaminylation at Glu[45] was present in eEF1A from fission yeast (*S. pombe*) but not present in elongation factors isolated from any of the other organisms (Fig. 5 and data not shown). In addition, we analyzed the crystal structures of eEF1A from rabbit (PDB code 4C0S (7), *Aeropyrum pernix* (PDB codes 3WXM (22) and 3VMF (23)), and *Sulfolobus acidocaldarius* (PDB codes 1SKQ (24) and 1JNY (6)). In

Figure 2. Confirmation of eEF1A modification by site-directed mutagenesis of Glu[45] in *S. cerevisiae*. *A*, MS-MS fragmentation analysis by collision-induced dissociation (CID) of the eEF1A E45D mutant expressed and purified from *S. cerevisiae* genetically deprived of wild-type eEF1A. A representative MS-MS fragmentation pattern (*left panel*) and extracted ion chromatogram (*right panel*) are shown. Posttranslational modification of the tryptic peptide 42-FEKDAAELGK-51 of eEF1A was not observed. *B*, electron transfer dissociation (ETD) MS-MS analysis of the eEF1A E45A mutant expressed and purified from *S. cerevisiae* genetically deprived of wild-type eEF1A. Posttranslational modification of the chymotryptic peptide 43-EKAAAELGKGSFKY-56 was not observed. A representative MS-MS fragmentation pattern (*left panel*) and extracted ion chromatogram (*right panel*) are shown.

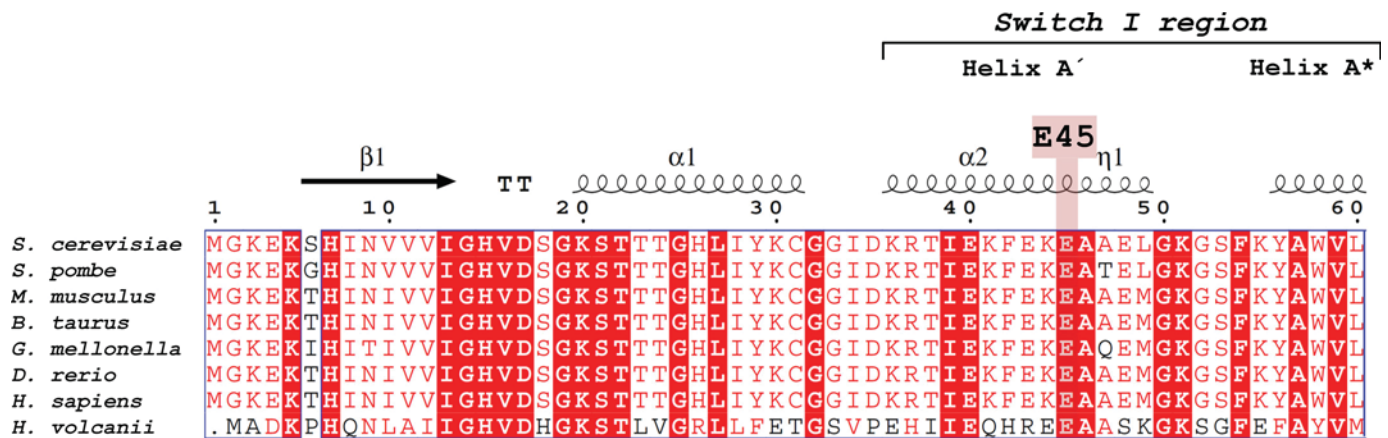


Figure 4. Glu^{45} in translation elongation factor 1A is highly conserved from archaea to human. Shown is the amino acid sequence alignment of the N terminus of eEF1A of various organisms. The alignment was prepared with ClustalW with eEF1A from *S. cerevisiae* (accession no. P02994), *S. pombe* (accession no. P0CT53), *M. musculus* (accession no. P10126), *B. taurus* (accession no. P68103), *G. mellonella* (accession no. Q8MWN6), *D. rerio* (accession no. Q92005), *Homo sapiens* (accession no. P68104), and *H. volcanii* (accession no. D4GWR0). Secondary structural elements were deduced from *S. cerevisiae* eEF1A (PDB code 1IJF). The N-terminal part of the switch I region, which is missing in prokaryotic EF-Tu, is *highlighted*.

http://doc.rero.ch

agreement with our MS data, unassigned electron density around Glu^{45} or equivalent residues was not detectable in any of the three structures containing mammalian or archaeal eEF1A. Thus, glutaminylation of eEF1A seems to be restricted to yeast.

Glutaminylation of eEF1A increases yeast growth defects under translational stress conditions

To analyze the biological effects of Glu^{45} glutaminylation of eEF1A, we performed growth assays under various cell stress conditions with *S. cerevisiae* strains in which wild-type eEF1A was substituted by eEF1A-E45A, -E45D, or -E45K (supplemental Fig. 3*A*). Yeast cells expressing the mutant versions of eEF1A were viable and did not display growth defects under a variety of stress conditions, including temperature stress, osmotic stress, or endoplasmic reticulum stress in the presence of benomyl, caffeine, or polymyxin (supplemental Fig. 3*B*).

Interestingly, the response of the tested eEF1A mutants toward translation-specific stress conditions induced by the antibiotics Geneticin, paromomycin, and, to a lesser extent, anisomycin showed that modification at Glu^{45} by glutaminylation (eEF1A Glu^{45}-glut) slightly enhanced growth defects compared with mutant eEF1A E45D that was not modified by attachment of glutamine (Fig. 6). Moreover, introduction of a local positive charge and bulky side chain, as in the E45K mutant, increased the susceptibility of the mutant yeast strain toward translation-specific antibiotics. These data suggest that glutaminylation of eEF1A at the critical position Glu^{45} might regulate translation under specific growth conditions.

Glycosylation of Ser^{53} of eEF1A by the Legionella effector Lgt3 is not influenced by glutaminylation

The results of the previous experiments with translational inhibitors suggested that site-directed mutation (eEF1A E45K) or glutaminylation of Glu^{45} might alter the conformation of the helix A* loop region of the switch I region of eEF1A. Glu^{45} is structurally located close to Ser^{53}, the specific modification site of the *L. pneumophila* toxin effector glucosyltransferases Lgt1, 2, and 3 (15, 16). To analyze whether glutaminylation of Glu^{45} results in structural alterations of the helix A*–loop– helix A′ region unfavorable for Lgt3-catalyzed glucosylation, we compared the initial velocities of glycosylation of non-glutaminylated (eEF1A purified from mouse liver) *versus* glutaminylated eEF1A (purified from *S. cerevisiae*) (supplemental Fig. 4). Additionally, we compared the glucosylation of the ternary complex (eEF1A, GTP, and Phe-tRNA^{Phe}) constituting the *bona fide* substrate of Lgt3. We found that the initial glucosylation rate of glutaminylated and non-glutaminylated eEF1A within the ternary complex was similar, showing that Glu^{45} glutaminylation does not influence modification of the elongation factor at Ser^{53} by Lgt3.

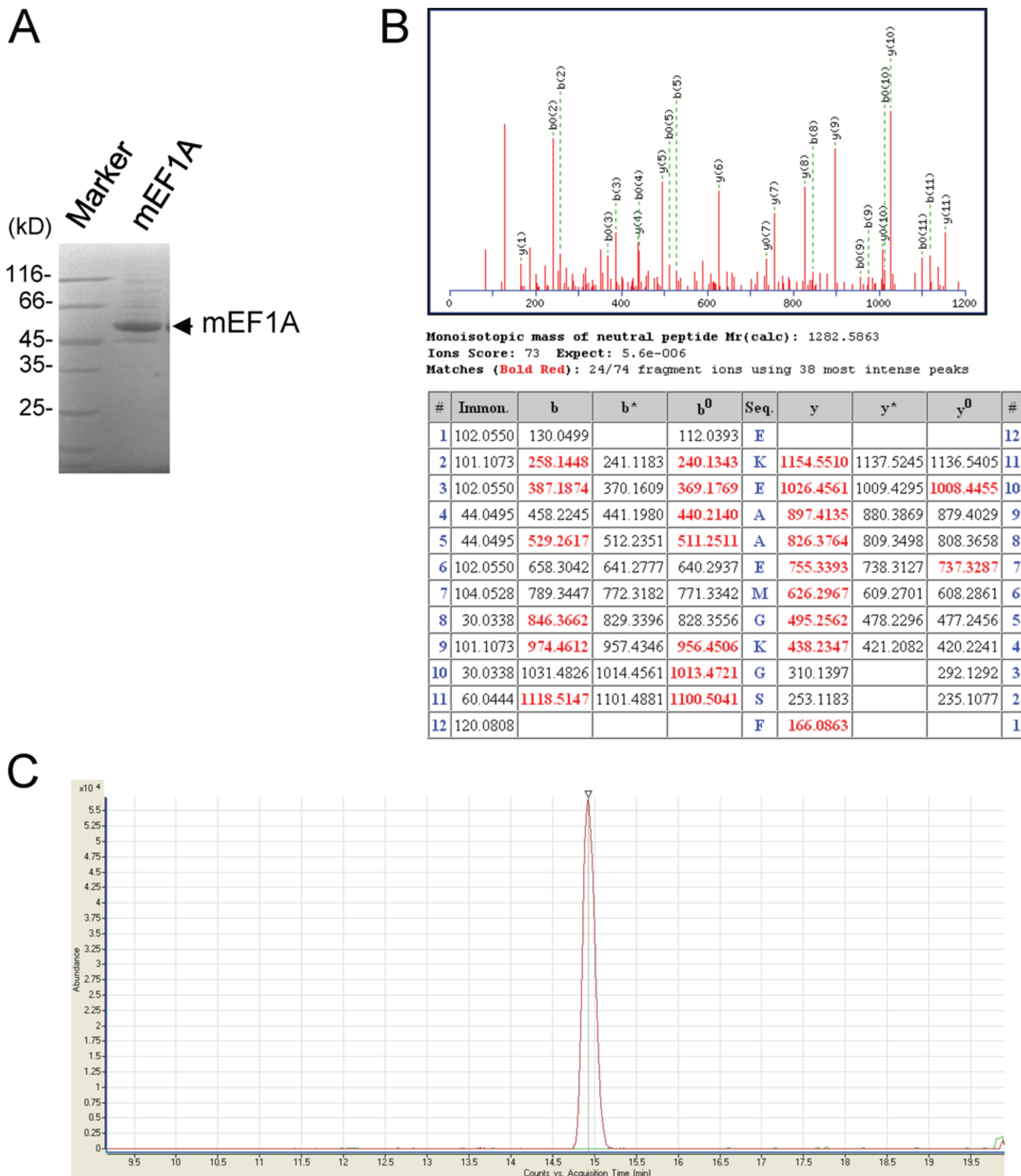
Discussion

Here we show, by using tandem mass spectrometric analysis, site-directed mutagenesis, and structural data that eEF1A from yeast is modified by the attachment of a single glutamine moiety to amino acid Glu^{45} within the GTPase domain. Structural data reveal covalent linkage of L-glutamine via the α amino group.

Although glutamylation and polyglutamylation (the attachment of glutamic acids) are well-known posttranslational modifications (*e.g.* modification of α- and β-tubulin (20)) catalyzed by γ-glutamyltransferase (21), the posttranslational modification of proteins by attachment of glutamine (glutaminylation) via the α amino group has not been reported previously.

The chemical principle of the novel posttranslational modification reaction reported here appears to be similar to the reaction catalyzed by glutaminyl-tRNA synthetase (Gln-RS) during the charging of tRNA at the 3′ acceptor stem region, which results in the same covalent linkage of a glutaminyl moiety (25). Several aminoacyl-tRNA synthetase (aa-RS) paralogs with unknown functions exist in various species, including yeast. The class II lysyl-tRNA synthetase paralog GenX/PoxA/YjeA was shown to attach a lysine residue to another lysine of *Escherichia coli* elongation factor P (26, 27). This lysine modification resembles the glutaminylation reaction of eEF1A. Thus, one may speculate that glutaminylation of eEF1A might also be catalyzed by a glutaminyl-tRNA synthetase paralog. Notably, the

http://doc.rero.ch



#	Immon.	b	b*	b0	Seq.	y	y*	y0	#
1	102.0550	130.0499		112.0393	E				12
2	101.1073	258.1448	241.1183	240.1343	K	1154.5510	1137.5245	1136.5405	11
3	102.0550	387.1874	370.1609	369.1769	E	1026.4561	1009.4295	1008.4455	10
4	44.0495	458.2245	441.1980	440.2140	A	897.4135	880.3869	879.4029	9
5	44.0495	529.2617	512.2351	511.2511	A	826.3764	809.3498	808.3658	8
6	102.0550	658.3042	641.2777	640.2937	E	755.3393	738.3127	737.3287	7
7	104.0528	789.3447	772.3182	771.3342	M	626.2967	609.2701	608.2861	6
8	30.0338	846.3662	829.3396	828.3556	G	495.2562	478.2296	477.2456	5
9	101.1073	974.4612	957.4346	956.4506	K	438.2347	421.2082	420.2241	4
10	30.0338	1031.4826	1014.4561	1013.4721	G	310.1397		292.1292	3
11	60.0444	1118.5147	1101.4881	1100.5041	S	253.1183		235.1077	2
12	120.0808				F	166.0863			1

Figure 5. Mouse elongation factor 1A is not glutaminylated at Glu[45]. *A*, SDS-PAGE of eEF1A affinity-purified from mouse liver (mEF1A), which was used for mass spectrometric analysis. *B*, CID MS-MS revealed no modification of Glu[45] in the chymotryptic peptide 43-EKEFAAEMGKGSF-54 (precursor *m/z* = 642.3 (2+)). *Immon.*, immonium ion; *Seq.*, sequence. *C*, a representative LC/MS-MS extracted ion chromatogram of the peptide 43-EKEAAEMGKGSF-54 peak at *m/z* = 642.3 (2+) from mouse eEF1A.

site of glutaminylation in eEF1A is located close to the 3′ aminoacyl acceptor stem of the eEF1A-bound aa-tRNA (supplemental Fig. 5).

So far, it is unclear whether glutaminylation in yeast is a transient and reversible modification. Despite extensive efforts, we were not able to identify unmodified eEF1A in yeast cells. Anal-

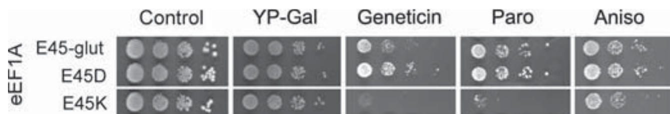


Figure 6. Glutaminylation or mutation of eEF1A Glu^{45} to lysine increased growth defect under translational stress conditions in yeast. Growth phenotypes of *S. cerevisiae* strains expressing wild-type eEF1A Glu^{45}-glut, eEF1A E45D, or eEF1A E45K. Serial dilutions of the corresponding *S. cerevisiae* variants were spotted onto YPGal (2% galactose) or YPD agar supplemented with different stress agents (50 ng/ml Geneticin, 350 μg/ml paromomycin (*Paro*), or 5 μg/ml anisomycin (*Aniso*)) and cultivated for 72 h at 30 °C.

yses performed at different cell stages of yeast growth exclusively resulted in detection of completely glutaminylated eEF1A (supplemental Fig. 1). By contrast, we were not able to detect glutaminylation of mammalian, fish, or insect eEF1A. The site of glutaminylation and the nature of the acceptor amino acid is highly specific. We observed that amino acid point mutations of the glutaminyl attachment site (Glu^{45}) of yeast eEF1A prevented glutaminylation, even when glutamate was replaced by negatively charged aspartic acid with a C1 atom-shortened side chain. This demonstrates that the attachment of glutamine is strictly specific for Glu^{45}.

Besides glutaminylation of eEF1A, there are three other "unusual" posttranslational modifications also found in eukaryotic ribosome-associated factors (28): EPG modification of Glu^{301} and Glu^{374} in eEF1A, hypusine modification of eukaryotic initiation factor 5A (eIF5A), and diphthamide modification of eukaryotic elongation factor 2 (eEF2) (29). The precise functional roles of these modifications are not well-understood. Although the diphthamide modification might play a role in translation fidelity (30), the function of hypusine and EPG modifications are still enigmatic (28). Expression of a glutaminyl-deficient mutant of eEF1A (E45A or E45D) in *S. cerevisiae*, after deletion of endogenous eEF1A, demonstrated that attachment of glutamine to eEF1A is not essential for growth. Surprisingly, translation stress conditions induced by Geneticin, paromomycin, or anisomycin showed growth retardation of native glutaminylated eEF1A (local neutral charge) or eEF1A with an introduced bulkier side chain of lysine at position Glu^{45} (local positive charge) in comparison with the E45D mutant (local negative charge). Thus, in the eEF1A E45K mutant, as in the Glu^{45}-glutaminylated eEF1A, the effect of the bulky and positively charged side chain might influence the interaction with the ribosomal factor-binding site and, therefore, translation efficiency. These data suggest that, in yeast, glutaminylation might have a regulatory function in protein synthesis. Previously, we reported that Ser^{53} of eEF1A is modified by glucosylation catalyzed by the *L. pneumophila* effectors Lgt1–3 (15, 16). We found that Lgt-induced glucosylation did not differ with glutaminylated and non-glutaminylated eEF1A, which is a further indication that modification of Glu^{45} did not cause drastic structural changes of the molecule.

Recently, the mammalian elongation complex structure with eEF1A bound to the 80S ribosome has been reported (31). In this complex, the conserved Glu^{45} is oriented close to the sarcin–ricin loop. The sarcin–ricin loop is suggested to stimulate the GTPase activity of eEF1A after codon–anticodon recognition at the A-site of the ribosome (31, 32). Analysis of the described mammalian 80S ribosome complex reveals that glutaminylation of amino acid Glu^{45} of eEF1A would be accommodated in this complex. On the other hand, it is conceivable that the glutaminylation is involved in binding of the eEF1A ternary complex to the factor-binding site of the ribosome and, therefore, has the potential to influence translation (supplemental Fig. 5).

Taken together, here we describe a novel posttranslational modification of yeast eEF1A by glutaminylation. In all organisms, the genomic content is restricted to a specific protein repertoire. Fine-tuning of protein functions (*e.g.* in signaling events) and/or functional extensions are achieved by a large array of posttranslational modifications, resulting in additional diversification of the proteome. Translation elongation factors especially appear to be substrates of an extended spectrum of posttranslational modifications not observed in other proteins. Our findings add another type of posttranslational modification to this spectrum. It remains to be clarified whether glutaminylation is restricted to eEF1A or also observed in other proteins. Identification of the responsible enzyme will be an essential requirement for future detailed characterization of this novel posttranslational modification.

Experimental procedures

Strains, vectors, and materials

E. coli DH10B and TG1 were used for cloning and *E. coli* BL21 (DE3) for recombinant protein production (Invitrogen). Haploid *S. cerevisiae* MH272–3fα (ura3, leu2, his3, trp1, ade2) or diploid MH272–3fα/a (ura3/ura3, leu2/leu2, his3/his3, trp1/trp1, ade2/ade2) was used for yeast genetic studies (33). The following commercial *E. coli* and *S. cerevis*iae/*E. coli* shuttle vectors were used: pET28a, pET11 (Novagen, Madison, WI), and pRS313[His3] (34). For yeast transformation, standard genetic techniques were applied (35). All sequences of the corresponding plasmids and site-directed mutations were confirmed by sequencing (GATC Inc., Konstanz, Germany). DNA-modifying enzymes, *Pfu* DNA polymerase, 5-fluoroorotic acid, and kits for plasmid DNA and PCR product isolation were purchased from Fermentas (St. Leon-Rot, Germany). UDP-[^{14}C]glucose was from American Radiolabeled Chemicals (St. Louis, MO). The components of liquid medium for *S. cerevisiae* and *E. coli* were from Difco (BD Biosciences) and Carl Roth GmbH (Karlsruhe, Germany), respectively. *S. cerevisiae* was cultivated in YPD (1% yeast extract, 2% peptone, and 2% glucose) or minimal (0.67% yeast nitrogen base without amino acids and with ammonium sulfate, 2% glucose or 2% galactose, and the corresponding supplement) medium at 30 °C. *S. pombe* was cultivated in YES medium (0.5% yeast extract, 3% glucose, and 0.02% (of each) adenine, leucine, histidine, and uracil). Glucose, galactose, uracil, adenine, histidine, tryptophan, lysine, and leucine were from Sigma. The yeast DNA isolation kit was from Pierce (Thermo Fisher Scientific, Bonn, Germany). Isopropyl-β-D-thiogalactopyranoside was from Roth. RNase inhibitor was from Promega (Mannheim, Germany). All other reagents were of analytical grade and purchased from commercial sources.

http://doc.rero.ch

Protein purification

The cloning, recombinant expression and purification of Lgt3 (gene *lgt3*/lpg1488) from *L. pneumophila* strain Philadelphia-1 was described previously (36). For recombinant eEF1B (37) and Lgt3 protein production, the corresponding genes were induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside at 22 °C overnight on a shaker. Bacterial cells were collected by centrifugation and lysed by French press on ice. The proteins were purified by nickel affinity chromatography using HisTrap columns (GE Healthcare) connected to an ÄKTA purifier (GE Healthcare) and stored in 10% glycerol/TBS solution at −80 °C. Purification of native EF1A from *S. cerevisiae*, *S. pombe*, *H. volcanii*, zebrafish ZF4 cells, bovine liver tissue, mouse brain or liver, or total *G. mellonella* homogenate was performed by a method based on eEF1A interaction with His-tagged eEF1Bα as described previously (37).

Cloning and mutagenesis of S. cerevisiae eEF1A

The coding sequence of yeast eEF1A with ∼500-nt upstream and downstream regions was amplified from *S. cerevisiae* chromosomal DNA using primers 5′-CATATCACATAGGATCCCAACAGGCG and 5′-CATTACAGATAGCGTCGACCAAAGTATT (engineered restriction endonuclease sites are underlined). The PCR product was digested with BamHI/SalI restriction endonucleases and ligated into similarly digested pRS313. Site-directed mutagenesis was performed as described previously using the QuikChange (Stratagene) technology with the oligonucleotides 5′-GTTCGAAAAGGATGCCGCTGAATTAG/5′-CTAATTCAGCGGCATCCTTTTCGAAC, 5′-GTTCGAAAAGAAAGCCGCTGAATTAG/5′-CTAATTCAGCGGCTTTCTTTTCGAAC and 5′-GTTCGAAAAGGCAGCCGCTGAATTAG/5′-CTAATTCAGCGGCTGCCTTTTCGAAC to generate eEF1A with the E45D, E45K, and E45A substitutions, respectively.

Engineering of S. cerevisiae strains with eEF1A mutations

Construction of *S. cerevisiae* variant with inactivated chromosomal copies of eEF1A and containing wild-type eEF1A on an Ura3 marker–containing plasmid was described previously (38). This strain was transformed individually with the pRS313-based plasmids coding for eEF1A wild-type, E45D, E45K, and E45A and passed over 5-fluoroorotic acid–containing agar plates (39) to remove the initial Ura3 marker–carrying plasmid coding for wild-type eEF1A.

Yeast growth assay

To estimate yeast growth phenotypes, engineered *S. cerevisiae* cells were titrated 10-fold from the starting value of A_{600} = 1.0. From each dilution, an aliquot of 5 μl of suspension was dropped onto SD agar supplemented with the corresponding marker substances. Where indicated, additional stress agents were included (20 mM DTT, 2% galactose, 50 ng/ml Geneticin, 350 μg/ml paromomycin, 5 μg/ml anisomycin, 1 M sorbitol, 1 M NaCl, 1 M KCl, 10 μg/ml benomyl, 0.15% caffeine, or 200 μg/ml polymyxin), or the pH level was adjusted correspondingly. Petri plates were incubated for 3–5 days at 30 °C (or at the temperature mentioned in the figure legends) before photography. In comparison with liquid culture experiments, assays on solid medium led to results that were more conclusive.

Glucosyltransferase assay

Glucosylation was performed with 140 nM recombinant His-tagged Lgt3 and eEF1A or the eEF1A-GTP-Phe-aatRNA^{Phe} ternary complex (3 μM) in a total volume of 20 μl. Production of Phe-tRNA^{Phe} and ternary eEF1A complex formation was performed as described previously (36). The standard glycosylation reaction was performed at 30 °C for the indicated times in 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM $MnCl_2$, and 10 μM UDP-[^{14}C]glucose. The reaction was stopped by addition of SDS sample buffer and heating at 95 °C for 5 min. Subsequently, the samples were subjected to polyacrylamide gel electrophoresis (40). Proteins were stained with Coomassie Brilliant Blue R250, and radiolabeled bands were analyzed by phosphorimaging and quantified using ImageQuant 5.2 (GE Healthcare) and Sigma Plot.

LC/MS-MS analysis

For in-gel digestion, the excised gel bands were destained with 30% acetonitrile, shrunk with 100% acetonitrile, and dried in a vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany). Digests with trypsin were performed at 37 °C and digests with chymotrypsin at 25 °C overnight in 0.1 M NH_4HCO_3 (pH 8). About 0.1 μg of protease was used for one gel band. Peptides were extracted from the gel slices with 5% formic acid. LC/MS-MS analyses were performed on a Q-TOF (quadrupole time-of-flight) mass spectrometer (Agilent 6520, Agilent Technologies) and on an ion trap mass spectrometer (Agilent 6340, Agilent Technologies) equipped with an ETD source. Both instruments were coupled to a 1200 Agilent nanoflow system via a HPLC chip cube electrospray ionization interface. Peptides were separated on an HPLC chip with an analytical column of 75-μm inner diameter and 150-mm length and a 40-nl trap column, both packed with Zorbax 300SB C-18 (5-μm particle size). Peptides were eluted with a linear acetonitrile gradient with 1%/min at a flow rate of 300 nl/min (starting with 3% acetonitrile). The Q-TOF was operated in the 2-GHz extended dynamic range mode. MS-MS analyses were performed using data-dependent acquisition mode. After an MS scan (2 spectra/s), a maximum of three peptides were selected for MS-MS (2 spectra/s). Singly charged precursor ions were excluded from selection. Internal calibration was applied. The ETD ion trap was operated in data-dependent acquisition mode. After an MS scan (standard enhanced mode), a maximum of three peptides were selected for MS-MS (standard enhanced mode). The ICC control for the survey scan was set to 350,000, and the maximum accumulation time was set to 300 ms. The accumulation time for fluoranthene was set to 4 ms (according to an ICC of 500,000–600,000), and the ETD reaction was set to 100 ms. Resonance excitation (smart decomposition) was used for doubly charged peptides. Mascot Distiller 2.3 was used for raw data processing and for generating peak lists, essentially with standard settings for the Agilent Q-TOF or Agilent ion trap, respectively. Mascot Server 2.3 was used for database searching with the following parameters: peptide

http://doc.rero.ch

mass tolerance, 20 ppm (Q-TOF)/1.1 Da (ion trap); MS-MS mass tolerance, 0.05 Da (Q-TOF)/0.3 Da (ion trap); enzyme, "trypsin" or "chymotrypsin" with two uncleaved sites allowed; variable modifications, carbamidomethyl (C), Gln-> pyroGlu (N-term. Q), oxidation (M), and hexose (ST). For protein identification, a custom database was used.

X-ray structure analysis and refinement

The vicinity of Glu[45] in the 1.67-Å-resolution X-ray structure of eEF1A from *S. cerevisiae* (PDB code 1F60 (8)) was inspected for unassigned electron density, which was calculated using TLS (translation, libration, screw motion) refinement in phenix.refine (41) with coordinates and structure factors retrieved from the Protein Data Bank. The $mF_o - dF_c$ difference electron density map clearly indicated the position and orientation of the posttranslational modification. Water molecules in the proximity of Glu[45] were removed, and the glutaminylation was manually inserted as covalent attachment to Glu[45] in a peptide bond–like manner between the α amino group of glutamine and the δ C atom of the Glu[45] side chain. The structure was refined employing restraints for a peptide-like bond according to Ref. 42. Specifically, refinement targets for atomic distances were set to 1.336 Å (N_{Gln}-$C\delta_{E45}$), 1.229 Å ($C\delta_{E45}$-$O\epsilon_{E45}$), 1.459 Å ($C\alpha_{Gln}$-N_{Gln}), and 1.525 Å ($C\delta_{E45}$-$C\gamma_{E45}$). Refinement targets for the angles were set as follows: 121.7° ($C\alpha_{Gln}$-N_{Gln}-$C\delta_{E45}$), 122.7° (N_{Gln}-$C\delta_{E45}$-$O\epsilon_{E45}$), 120.1° ($O\epsilon_{E45}$-$C\delta\gamma_{E45}$), and 117.2° (N_{Gln}-$C\delta_{E45}$-$C\gamma_{E45}$). In addition, planarity restraints were used for atoms $C\alpha_{Gln}$, N_{Gln}, $C\delta_{E45}$, $C\gamma_{E45}$, and $O\epsilon_{E45}$. After refinement, these distances and the plane were identical to the ideal values. The quality of the model and fit to the electron density were compared for L- and D-glutamine, and the ideally fitting L-enantiomer was included in the final structure. Water molecules were inserted, and model building was completed using COOT (43). This included insertion of three polyethylene glycol moieties and two additional posttranslational modifications an Nϵ-monomethylation of Lys[30] and Nϵ,Nϵ,Nϵ-trimethylation of Lys[79]. Electron density was visible for an additional short α-helical peptide that was likely to be part of the C terminus of eEF1A, as fragmentary electron density at 1σ and continuous electron density at 0.5σ connected that peptide to the C terminus of eEF1A. However, the connecting loop was not ordered enough to allow refinement. The structure was refined using phenix.refine with TLS to a crystallographic R and R_{free} of 15.6% and 18.4%, respectively. The quality of the structure was checked using MolProbity (44). There was no Ramachandran outlier, and 97.44% of the residues were in the favored regions. Data refinement statistics are summarized in supplemental Table 1. The figures were prepared using the PyMOL molecular graphics system (version 1.5.0.4, Schrödinger, LLC).

Author contributions—T. J. designed and conducted experiments, analyzed the data, and wrote the paper. A. S., Z. H., and J. D. performed mass spectrometric analyses. C. W., C. H., and G. R. A. conducted structural analyses. Y. B. and S. R. performed yeast experiments. T. T. performed glycosylation experiments. K. A. designed the study, analyzed the data, and wrote the paper. All authors discussed the results and commented on the manuscript.

Acknowledgments—We thank Peter Gebhardt for technical assistance and Edith Fitzke for assistance with analysis of the yeast mutant strains. H. volcanii cultures and zebrafish ZF4 cells were provided by Sonja-Verena Albers (Molecular Biology of Archaea, University of Freiburg) and Wolfgang Driever (Developmental Biology, University of Freiburg), respectively.

References

1. Andersen, G. R., Nissen, P., and Nyborg, J. (2003) Elongation factors in protein biosynthesis. *Trends Biochem. Sci.* **28,** 434–441
2. Khacho, M., Mekhail, K., Pilon-Larose, K., Pause, A., Côté, J., and Lee, S. (2008) eEF1A is a novel component of the mammalian nuclear protein export machinery. *Mol. Biol. Cell* **19,** 5296–5308
3. Panasyuk, G., Nemazanyy, I., Filonenko, V., Negrutskii, B., and El'skaya, A. V. (2008) A2 isoform of mammalian translation factor eEF1A displays increased tyrosine phosphorylation and ability to interact with different signalling molecules. *Int. J. Biochem. Cell Biol.* **40,** 63–71
4. Mateyak, M. K., and Kinzy, T. G. (2010) eEF1A: thinking outside the ribosome. *J. Biol. Chem.* **285,** 21209–21213
5. Lamberti, A., Caraglia, M., Longo, O., Marra, M., Abbruzzese, A., and Arcari, P. (2004) The translation elongation factor 1A in tumorigenesis, signal transduction and apoptosis: review article. *Amino Acids* **26,** 443–448
6. Vitagliano, L., Masullo, M., Sica, F., Zagari, A., and Bocchini, V. (2001) The crystal structure of *Sulfolobus solfataricus* elongation factor 1α in complex with GDP reveals novel features in nucleotide binding and exchange. *EMBO J.* **20,** 5305–5311
7. Crepin, T., Shalak, V. F., Yaremchuk, A. D., Vlasenko, D. O., McCarthy, A., Negrutskii, B. S., Tukalo, M. A., and El'skaya, A. V. (2014) Mammalian translation elongation factor eEF1A2: X-ray structure and new features of GDP/GTP exchange mechanism in higher eukaryotes. *Nucleic Acids Res.* **42,** 12939–12948
8. Andersen, G. R., Pedersen, L., Valente, L., Chatterjee, I., Kinzy, T. G., Kjeldgaard, M., and Nyborg, J. (2000) Structural basis for nucleotide exchange and competition with tRNA in the yeast elongation factor complex eEF1A:eEF1Bα. *Mol. Cell* **6,** 1261–1266
9. Gross, S. R., and Kinzy, T. G. (2005) Translation elongation factor 1A is essential for regulation of the actin cytoskeleton and cell morphology. *Nat. Struct. Mol. Biol.* **12,** 772–778
10. Gross, S. R., and Kinzy, T. G. (2007) Improper organization of the actin cytoskeleton affects protein synthesis at initiation. *Mol. Cell. Biol.* **27,** 1974–1989
11. Cavallius, J., Zoll, W., Chakraburtty, K., and Merrick, W. C. (1993) Characterization of yeast EF-1α: non-conservation of post-translational modifications. *Biochim. Biophys. Acta* **1163,** 75–80
12. Traugh, J. A. (2001) Insulin, phorbol ester and serum regulate the elongation phase of protein synthesis. *Prog. Mol. Subcell. Biol.* **26,** 33–48
13. Zobel-Thropp, P., Yang, M. C., Machado, L., and Clarke, S. (2000) A novel post-translational modification of yeast elongation factor 1A: methylesterification at the C terminus. *J. Biol. Chem.* **275,** 37150–37158
14. Whiteheart, S. W., Shenbagamurthi, P., Chen, L., Cotter, R. J., and Hart, G. W. (1989) Murine elongation factor 1 α (EF-1 α) is posttranslationally modified by novel amide-linked ethanolamine-phosphoglycerol moieties: addition of ethanolamine-phosphoglycerol to specific glutamic acid residues on EF-1 α. *J. Biol. Chem.* **264,** 14334–14341
15. Belyi, Y., Niggeweg, R., Opitz, B., Vogelsgesang, M., Hippenstiel, S., Wilm, M., and Aktories, K. (2006) *Legionella pneumophila* glucosyltransferase inhibits host elongation factor 1A. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 16953–16958
16. Belyi, Y., Tabakova, I., Stahl, M., and Aktories, K. (2008) Lgt: a family of cytotoxic glucosyltransferases produced by *Legionella pneumophila*. *J. Bacteriol* **190,** 3026–3035
17. Ejiri, S. (2002) Moonlighting functions of polypeptide elongation factor 1: from actin bundling to zinc finger protein R1-associated nuclear localization. *Biosci. Biotechnol Biochem.* **66,** 1–21

http://doc.rero.ch

18. Gerstberger, S., Hafner, M., Ascano, M., and Tuschl, T. (2014) Evolutionary conservation and expression of human RNA-binding proteins and their role in human genetic disease. *Adv. Exp. Med. Biol.* **825,** 1–55
19. Andersen, G. R., Valente, L., Pedersen, L., Kinzy, T. G., and Nyborg, J. (2001) Crystal structures of nucleotide exchange intermediates in the eEF1A-eEF1Bα complex. *Nat. Struct. Biol* **8,** 531–534
20. Janke, C., Rogowski, K., and van Dijk, J. (2008) Polyglutamylation: a fine-regulator of protein function? Protein modifications: beyond the usual suspects' review series. *EMBO Rep.* **9,** 636–641
21. Whitfield, J. B. (2001) γ Glutamyl transferase. *Crit. Rev. Clin. Lab. Sci.* **38,** 263–355
22. Kobayashi, K., Kikuno, I., Kuroha, K., Saito, K., Ito, K., Ishitani, R., Inada, T., and Nureki, O. (2010) Structural basis for mRNA surveillance by archaeal Pelota and GTP-bound EF1α complex. *Proc. Natl. Acad. Sci. U.S.A.* **107,** 17575–17579
23. Kobayashi, K., Saito, K., Ishitani, R., Ito, K., and Nureki, O. (2012) Structural basis for translation termination by archaeal RF1 and GTP-bound EF1α complex. *Nucleic Acids Res.* **40,** 9319–9328
24. Vitagliano, L., Ruggiero, A., Masullo, M., Cantiello, P., Arcari, P., and Zagari, A. (2004) The crystal structure of *Sulfolobus solfataricus* elongation factor 1α in complex with magnesium and GDP. *Biochemistry* **43,** 6630–6636
25. Freist, W., Gauss, D. H., Ibba, M., and Söll, D. (1997) Glutaminyl-tRNA synthetase. *Biol. Chem.* **378,** 1103–1117
26. Yanagisawa, T., Sumida, T., Ishii, R., Takemoto, C., and Yokoyama, S. (2010) A paralog of lysyl-tRNA synthetase aminoacylates a conserved lysine residue in translation elongation factor P. *Nat. Struct. Mol. Biol.* **17,** 1136–1143
27. Roy, H., Zou, S. B., Bullwinkle, T. J., Wolfe, B. S., Gilreath, M. S., Forsyth, C. J., Navarre, W. W., and Ibba, M. (2011) The tRNA synthetase paralog PoxA modifies elongation factor-P with (R)-β-lysine. *Nat. Chem. Biol.* **7,** 667–669
28. Greganova, E., Altmann, M., and Bütikofer, P. (2011) Unique modifications of translation elongation factors. *FEBS J.* **278,** 2613–2624
29. Mittal, N., Subramanian, G., Bütikofer, P., and Madhubala, R. (2013) Unique posttranslational modifications in eukaryotic translation factors and their roles in protozoan parasite viability and pathogenesis. *Mol. Biochem. Parasitol.* **187,** 21–31
30. Liu, S., Bachran, C., Gupta, P., Miller-Randolph, S., Wang, H., Crown, D., Zhang, Y., Wein, A. N., Singh, R., Fattah, R., and Leppla, S. H. (2012) Diphthamide modification on eukaryotic elongation factor 2 is needed to assure fidelity of mRNA translation and mouse development. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 13817–13822
31. Shao, S., Murray, J., Brown, A., Taunton, J., Ramakrishnan, V., and Hegde, R. S. (2016) Decoding mammalian ribosome-mRNA states by translational GTPase complexes. *Cell* **167,** 1229–1240.e15
32. Voorhees, R. M., and Ramakrishnan, V. (2013) Structural basis of the translational elongation cycle. *Annu. Rev. Biochem.* **82,** 203–236
33. Gietz, R. D., and Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six-base pair restriction sites. *Gene* **74,** 527–534
34. Sikorski, R. S., and Hieter, P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122,** 19–27
35. Sherman, F. (1991) Getting started with yeast. *Methods Enzymol.* **194,** 3–21
36. Tzivelekidis, T., Jank, T., Pohl, C., Schlosser, A., Rospert, S., Knudsen, C. R., Rodnina, M. V., Belyi, Y., and Aktories, K. (2011) Aminoacyl-tRNA-charged eukaryotic elongation factor 1A is the *bona fide* substrate for *Legionella pneumophila* effector glucosyltransferases. *PLoS ONE* **6,** e29525
37. Pedersen, L., Andersen, G. R., Knudsen, C. R., Kinzy, T. G., and Nyborg, J. (2001) Crystallization of the yeast elongation factor complex eEF1A-eEF1B α. *Acta Crystallogr. D Biol. Crystallogr.* **57,** 159–161
38. Belyi, Y., Tartakovskaya, D., Tais, A., Fitzke, E., Tzivelekidis, T., Jank, T., Rospert, S., and Aktories, K. (2012) Elongation factor 1A is the target of growth inhibition in yeast caused by *Legionella pneumophila* glucosyltransferase Lgt1. *J. Biol. Chem.* **287,** 26029–26037
39. Boeke, J. D., Trueheart, J., Natsoulis, G., and Fink, G. R. (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* **154,** 164–175
40. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685
41. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., *et al.* (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66,** 213–221
42. Engh, R. A., and Huber, R. (2001) in *International Tables for Crystallography*, Vol. *F* (Rossmann, M. G., and Arnold, E., eds.) pp. 382–392, Kluwer Academic Publishers, Dordrecht, The Netherlands
43. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60,** 2126–2132
44. Chen, V. B., Arendall, W. B., 3rd, Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S., and Richardson, D. C. (2010) MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallogr. D Biol. Crystallogr.* **66,** 12–21

http://doc.rero.ch

SUPPLEMENTAL DATA

Protein glutaminylation is a yeast-specific posttranslational modification of elongation factor 1A

Thomas Jank[1,*], Yury Belyi[2,3], Christophe Wirth[4], Sabine Rospert[4,5], Zehan Hu[6,7,8], Jörn Dengjel[6,7,8], Tina Tzivelekidis[9], Gregers Rom Andersen[10], Carola Hunte[4,5], Andreas Schlosser[11], Klaus Aktories[1,5,7,*]

http://doc.rero.ch

CONTENT:

Table S1

Supplemental Figures 1-5

Supplemental References

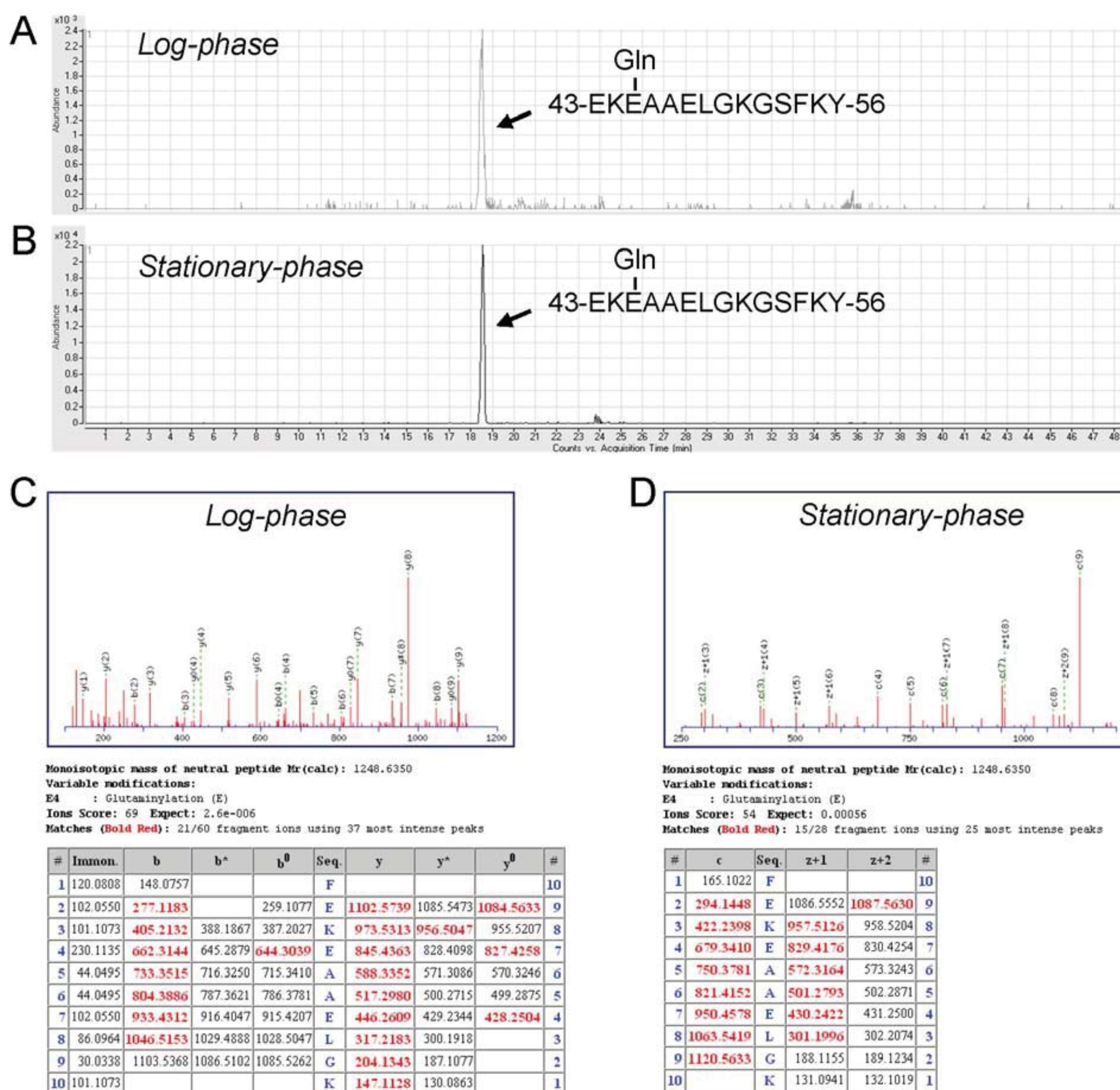
Table S1: Refinement statistics of the eEF1A-eEF1Bα complex.

Resolution (Å)	20.0 – 1.67
Reflections	
Refinement	63528
Test set	3217
Number of atoms	
Protein (including PTM)	4271
Water	725
Ligands	30
R / R_{free}	15.58 / 18.44
R.m.s deviations	
Bonds (Å)	0.009
Angles (°)	0.97
Ramachandran plot (%)	
Favored	97.44
Allowed	2.56
Disallowed	0.00

Note: Data were collected and processed by Andersen *et al.* (1) and Pedersen *et al.* (2).

http://doc.rero.ch

http://doc.rero.ch

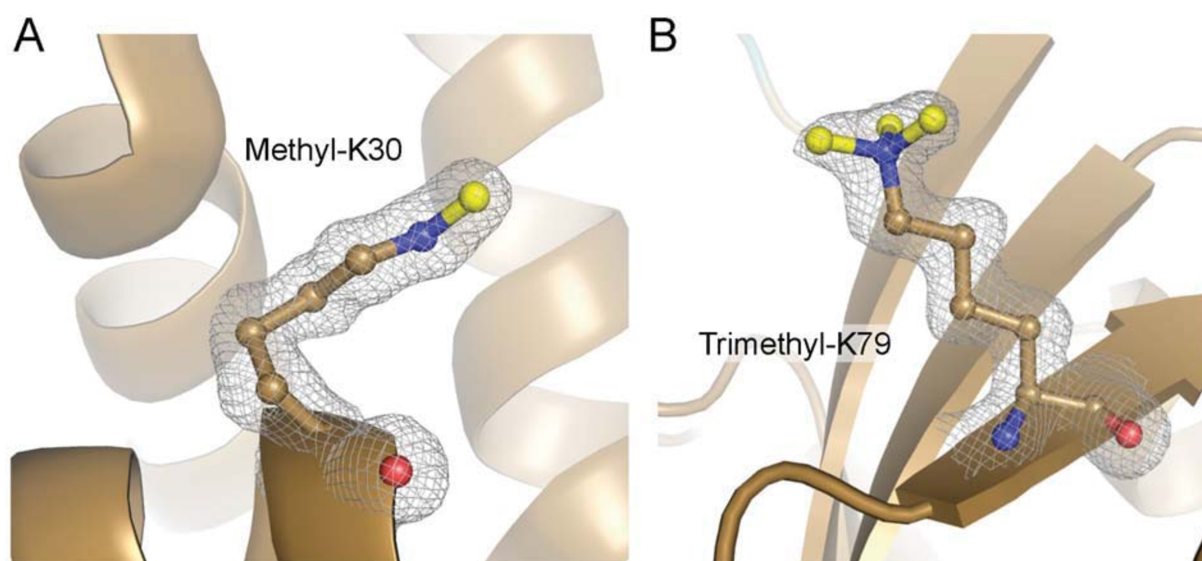


#	Immon.	b	b*	b⁰	Seq.	y	y*	y⁰	#
1	120.0808	148.0757			F				10
2	102.0550	277.1183		259.1077	E	1102.5739	1085.5473	1084.5633	9
3	101.1073	405.2132	388.1867	387.2027	K	973.5313	956.5047	955.5207	8
4	230.1135	662.3144	645.2879	644.3039	E	845.4363	828.4098	827.4258	7
5	44.0495	733.3515	716.3250	715.3410	A	588.3352	571.3086	570.3246	6
6	44.0495	804.3886	787.3621	786.3781	A	517.2980	500.2715	499.2875	5
7	102.0550	933.4312	916.4047	915.4207	E	446.2609	429.2344	428.2504	4
8	86.0964	1046.5153	1029.4888	1028.5047	L	317.2183	300.1918		3
9	30.0338	1103.5368	1086.5102	1085.5262	G	204.1343	187.1077		2
10	101.1073				K	147.1128	130.0863		1

#	c	Seq.	z+1	z+2	#
1	165.1022	F			10
2	294.1448	E	1086.5552	1087.5630	9
3	422.2398	K	957.5126	958.5204	8
4	679.3410	E	829.4176	830.4254	7
5	750.3781	A	572.3164	573.3243	6
6	821.4152	A	501.2793	502.2871	5
7	950.4578	E	430.2422	431.2500	4
8	1063.5419	L	301.1996	302.2074	3
9	1120.5633	G	188.1155	189.1234	2
10		K	131.0941	132.1019	1

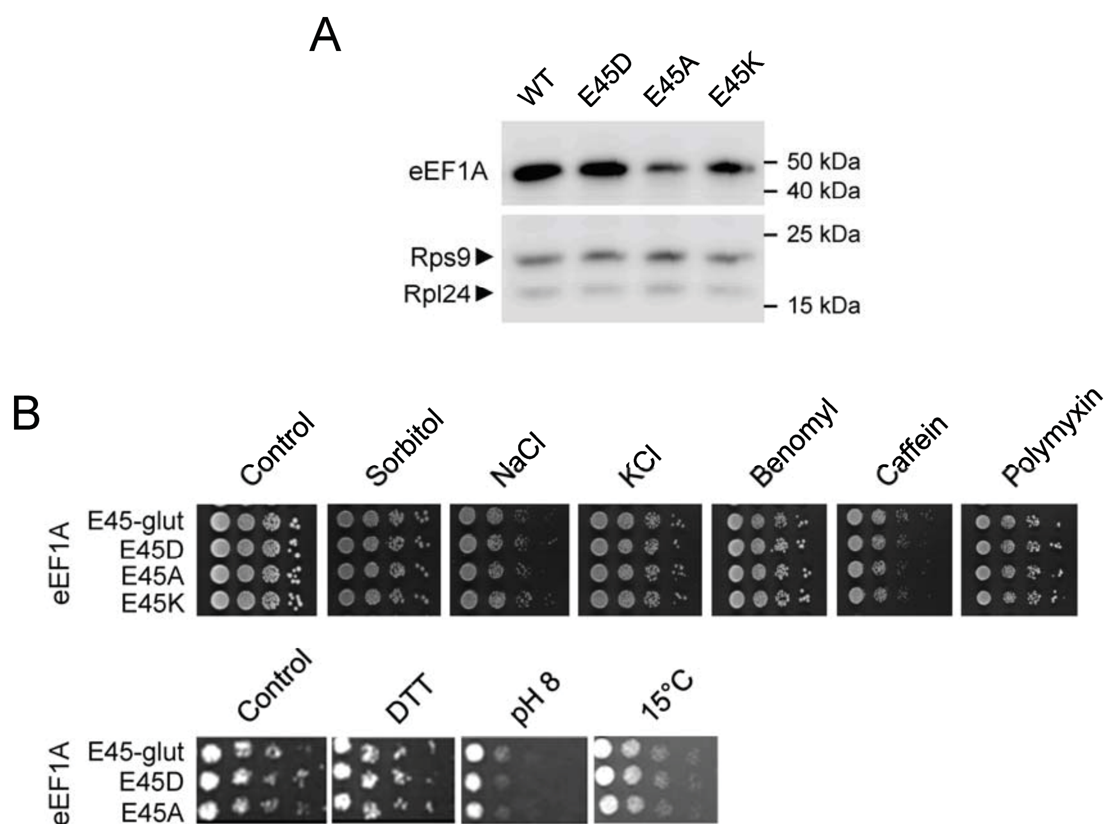
Suppl. Fig. 1. Glutaminylation of yeast eEF1A is not depending on a specific growth phase. Extracted ion chromatogram of the chymotryptic peptide 43-EKEAAELGKGSFKY-56 of eEF1A (precursor m/z = 421.97 (4+)) isolated from *S. cerevisiae* grown in logarithmic (A) or stationary phase (B). The MS/MS data revealed quantitative modification of E45. The unmodified peptide could not be detected. Collision-induced dissociation MS/MS analysis of the tryptic peptide 42-FEKEAAELGK-51 of yeast eEF1A (precursor m/z = 642.3 (2+)) in logarithmic (C) or stationary growth phase (D) is shown.

S-3



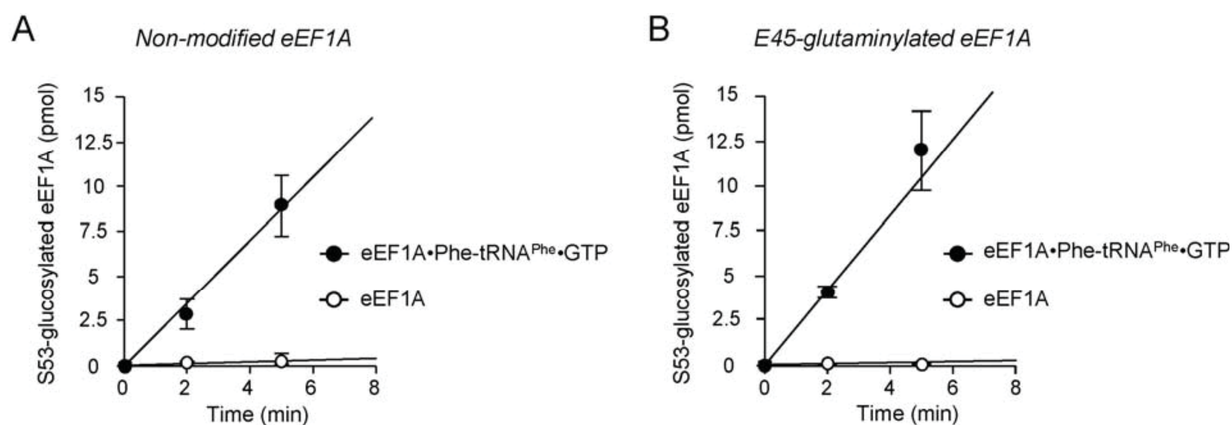
Suppl. Fig. 2. Mono- and trimethylation sites in the eEF1A structure (PDB 5O8W). Nε-monomethylated K30 (A) and Nε,Nε,Nε-trimethylated K79 (B) of the eEF1A structure are shown in stick-and-ball presentation with surrounding $2mF_o$-dF_c electron density map (grey) contoured at a level of 1.0 σ.

http://doc.rero.ch



Suppl. Fig. 3. *S. cerevisiae* expressing eEF1A E45 mutants were not influenced by various stress conditions. (A) Amount of engineered eEF1A in different variants of *S. cerevisiae*. Yeast were grown in YPD broth to an $OD_{600} = 0.5$, lyzed and tested in Western blotting with anti-eEF1A serum and with the sera against ribosomal proteins Rps9/Rpl24 as loading control. Please note that the concentration of eEF1A E45A is slightly reduced probably due to its instability or lower production. (B) Growth phenotypes of *S. cerevisiae* strains containing eEF1A with E45 substitutions. Serial dilutions of the corresponding yeast variants were spotted onto YPD agar supplemented with different stress agents (1 M sorbitol, 1 M NaCl, 1 M KCl, 10 µg/ml benomyl, 0.15% caffeine, 200 µg/ml polymyxin, 20 mM DTT, or pH 8) and cultivated for 72 h at 30°C or 15°C.

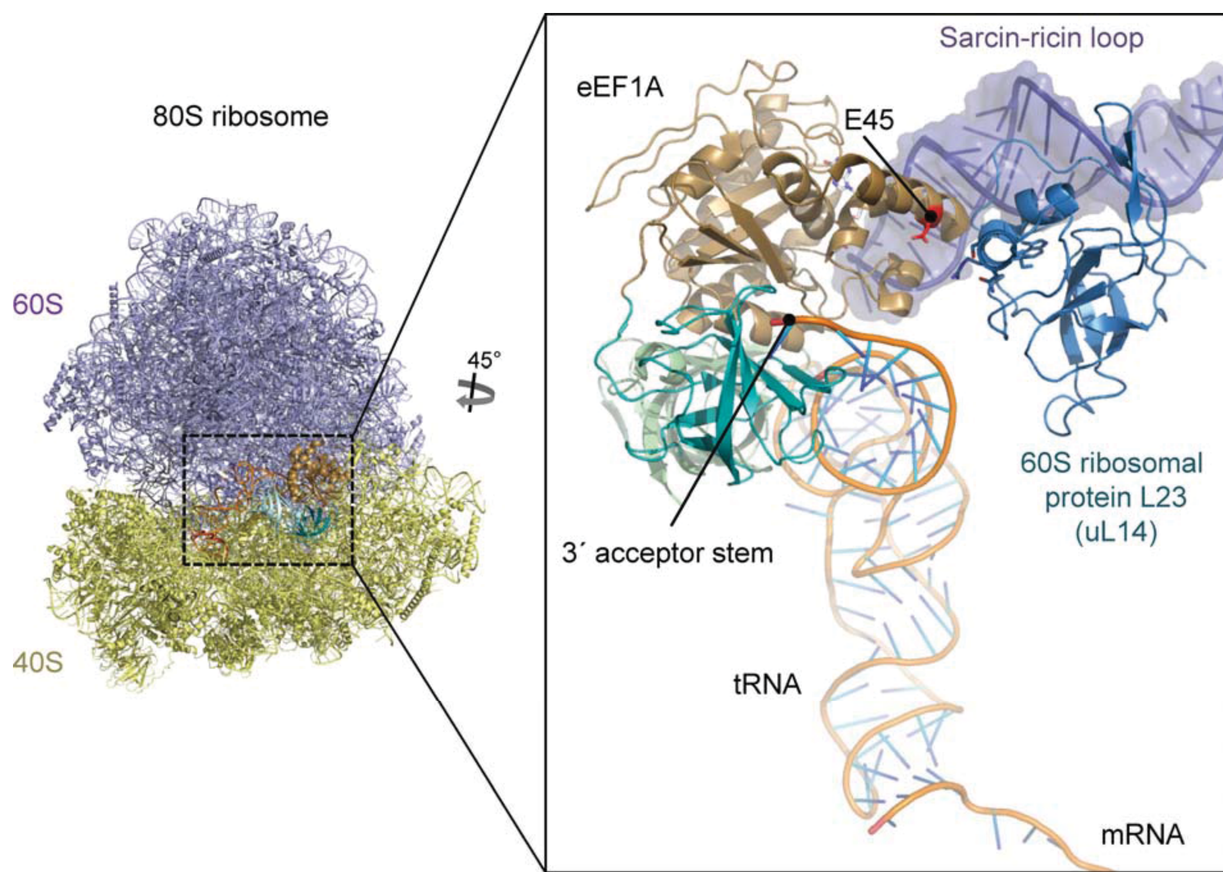
http://doc.rero.ch



Suppl. Fig. 4. Glucosylation of S53 of eEF1A in the ternary complex is not affected by glutaminylation. Kinetics of ^{14}C-glucosylation of non-glutaminylated eEF1A (A) and glutaminylated eEF1A (B) by *Legionella* glucosyltransferase Lgt3 in the absence (*open circles*) or presence of Phe-tRNA^{Phe} and GTP (*filled circles*). The amount of eEF1A glucosylation is shown as the mean (+/- SD) of at least three independent experiments.

S-6

http://doc.rero.ch



Suppl. Fig. 5. Location of E45 in rabbit eEF1A bound to the eukaryotic ribosome. Cryo-electron microscopy structure of the (80S) mammalian elongation complex comprising the large (60S, *violet*) and the small (40S, *yellow*) ribosomal subunits (PDB 5LZS) (3). tRNAs at the P and E site are shown in grey, mRNA is shown in dark red. Insert: The structure of eEF1A at the A-site of the ribosome is shown with E45 (*red, stick-and-ball representation*) located between the tRNA (*orange*) and the ribosomal L23 protein (*blue*). The sarcin-ricin loop is shown in violet.

http://doc.rero.ch

SUPPLEMENTAL REFERENCES:

1. Andersen, G. R., Pedersen, L., Valente, L., Chatterjee, I., Kinzy, T. G., Kjeldgaard, M., and Nyborg, J. (2000) Structural basis for nucleotide exchange and competition with tRNA in the yeast elongation factor complex eEF1A:eEF1Balpha. *Mol. Cell* **6**, 1261-1266

2. Pedersen, L., Andersen, G. R., Knudsen, C. R., Kinzy, T. G., and Nyborg, J. (2001) Crystallization of the yeast elongation factor complex eEF1A-eEF1B alpha. *Acta Crystallogr. D. Biol. Crystallogr* **57**, 159-161

3. Shao, S., Murray, J., Brown, A., Taunton, J., Ramakrishnan, V., and Hegde, R. S. (2016) Decoding mammalian ribosome-mRNA states by translational GTPase complexes. *Cell* **167**, 1229-1240 e1215

http://doc.rero.ch

Protein glutaminylation is a yeast-specific posttranslational modification of elongation factor 1A

Thomas Jank, Yury Belyi, Christophe Wirth, Sabine Rospert, Zehan Hu, Jörn Dengjel, Tina Tzivelekidis, Gregers Rom Andersen, Carola Hunte, Andreas Schlosser and Klaus Aktories

J. Biol. Chem. 2017, 292:16014-16023.
doi: 10.1074/jbc.M117.801035 originally published online August 11, 2017

Access the most updated version of this article at doi: 10.1074/jbc.M117.801035

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2017/08/11/M117.801035.DC1

This article cites 43 references, 13 of which can be accessed free at
http://www.jbc.org/content/292/39/16014.full.html#ref-list-1

http://doc.rero.ch