SUPPLEMENTARY INFORMATION

Initiation Factor eIF2γ Promotes eIF2–GTP–Met-tRNA$_i^{Met}$ Ternary Complex Binding to the 40S Ribosome

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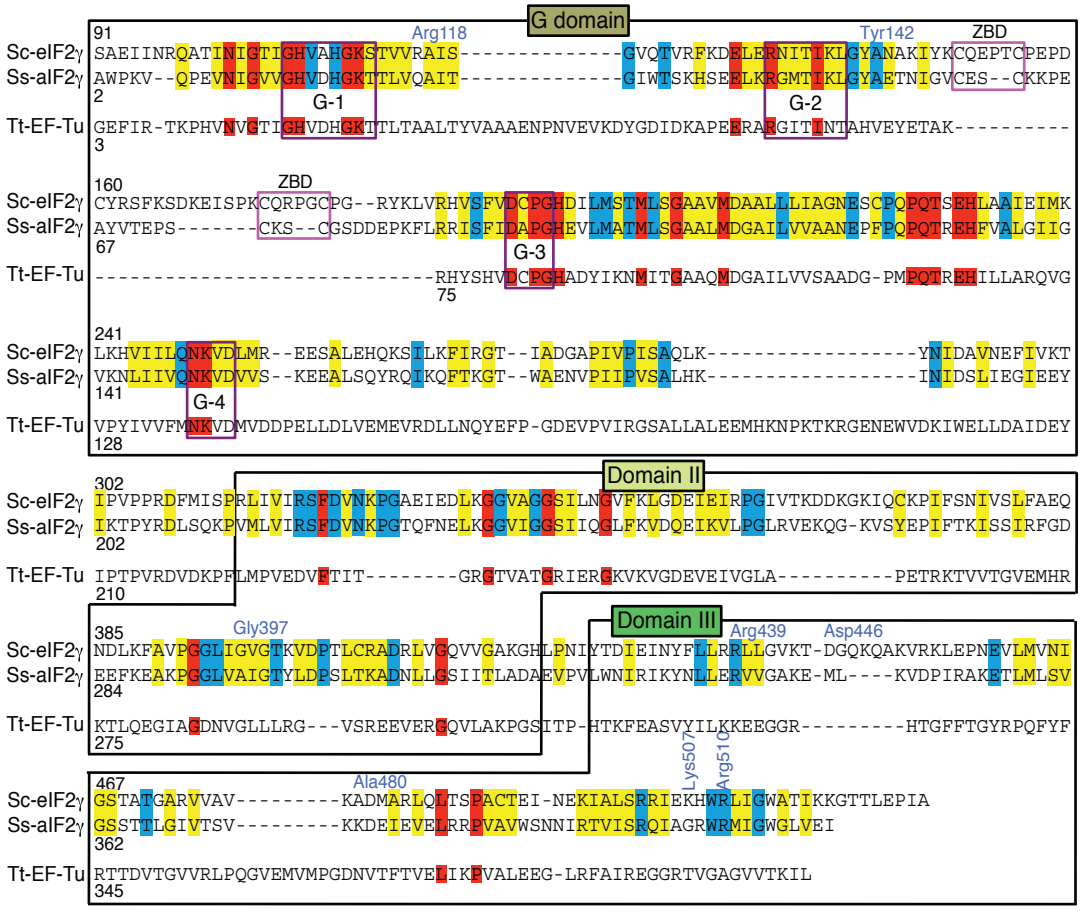
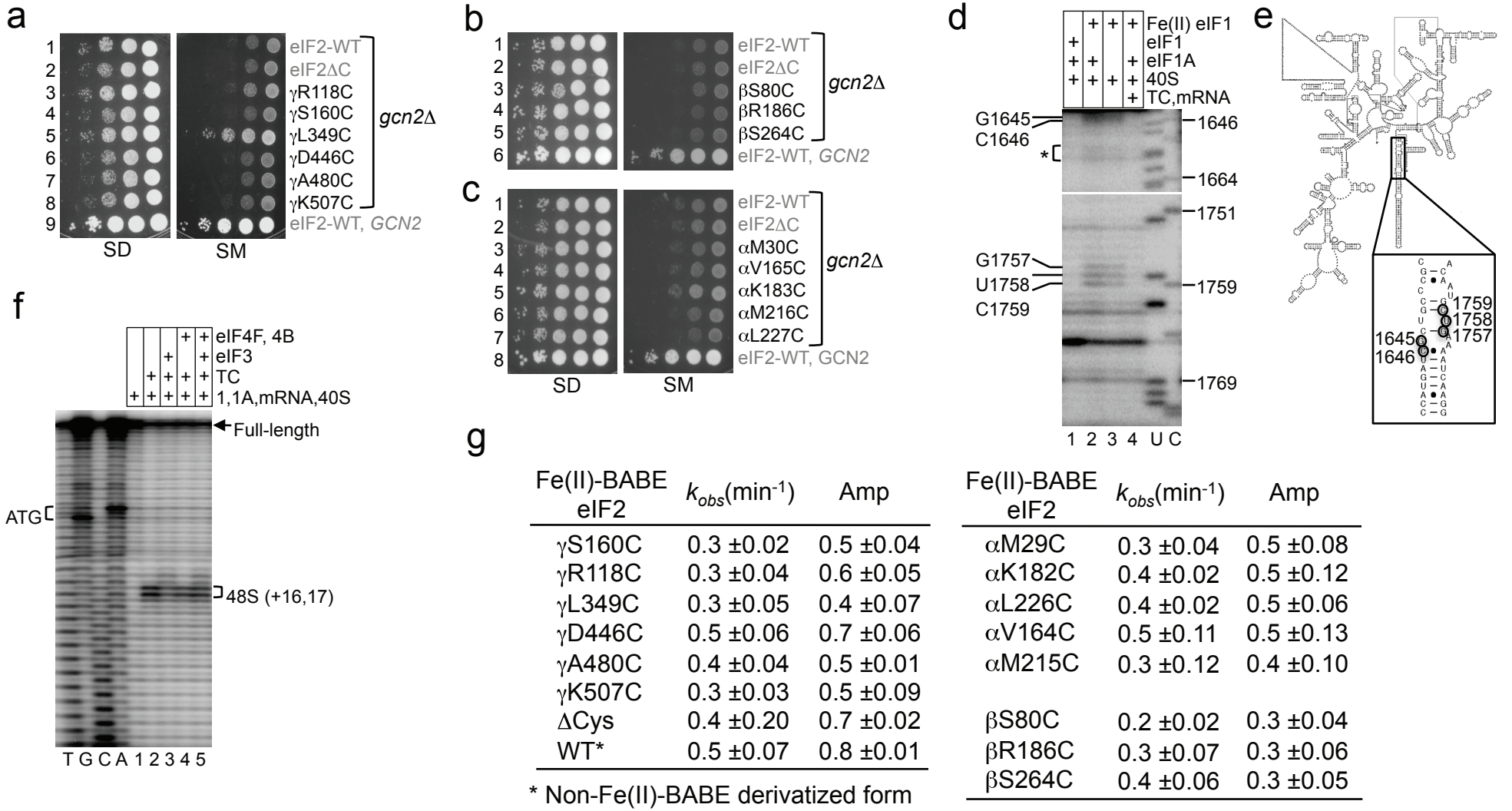


Supplementary Figure 1. Sequence alignment of eIF2γ (*S. cerevisiae*, Sc), aIF2γ (*S. solfataricus*, Ss), and EF-Tu (*T. thermophilus*, Tt).

Residues in red are identical in all three proteins; residues in blue are conserved only in eIF2γ and aIF2γ; and residues in yellow are similar (conservative replacement) in all aIF2(eIF2) proteins. G-domain, domain II and domain III are boxed and labeled, as are the G domain motifs G-1 to G-4 involved in GTP binding and the two zinc binding domain elements (ZBD). Residues of interest in this study are labeled in blue and depicted above the sequence alignment.



g

Fe(II)-BABE eIF2	k_{obs}(min^{-1})	Amp
γS160C	0.3 ±0.02	0.5 ±0.04
γR118C	0.3 ±0.04	0.6 ±0.05
γL349C	0.3 ±0.05	0.4 ±0.07
γD446C	0.5 ±0.06	0.7 ±0.06
γA480C	0.4 ±0.04	0.5 ±0.01
γK507C	0.3 ±0.03	0.5 ±0.09
ΔCys	0.4 ±0.20	0.7 ±0.02
WT*	0.5 ±0.07	0.8 ±0.01

Fe(II)-BABE eIF2	k_{obs}(min^{-1})	Amp
αM29C	0.3 ±0.04	0.5 ±0.08
αK182C	0.4 ±0.02	0.5 ±0.12
αL226C	0.4 ±0.02	0.5 ±0.06
αV164C	0.5 ±0.11	0.5 ±0.13
αM215C	0.3 ±0.12	0.4 ±0.10
βS80C	0.2 ±0.02	0.3 ±0.04
βR186C	0.3 ±0.07	0.3 ±0.06
βS264C	0.4 ±0.06	0.3 ±0.05

* Non-Fe(II)-BABE derivatized form

Supplementary Figure 2. Analysis of eIF2 Cys mutants and Reconstituted Translation System Reagents

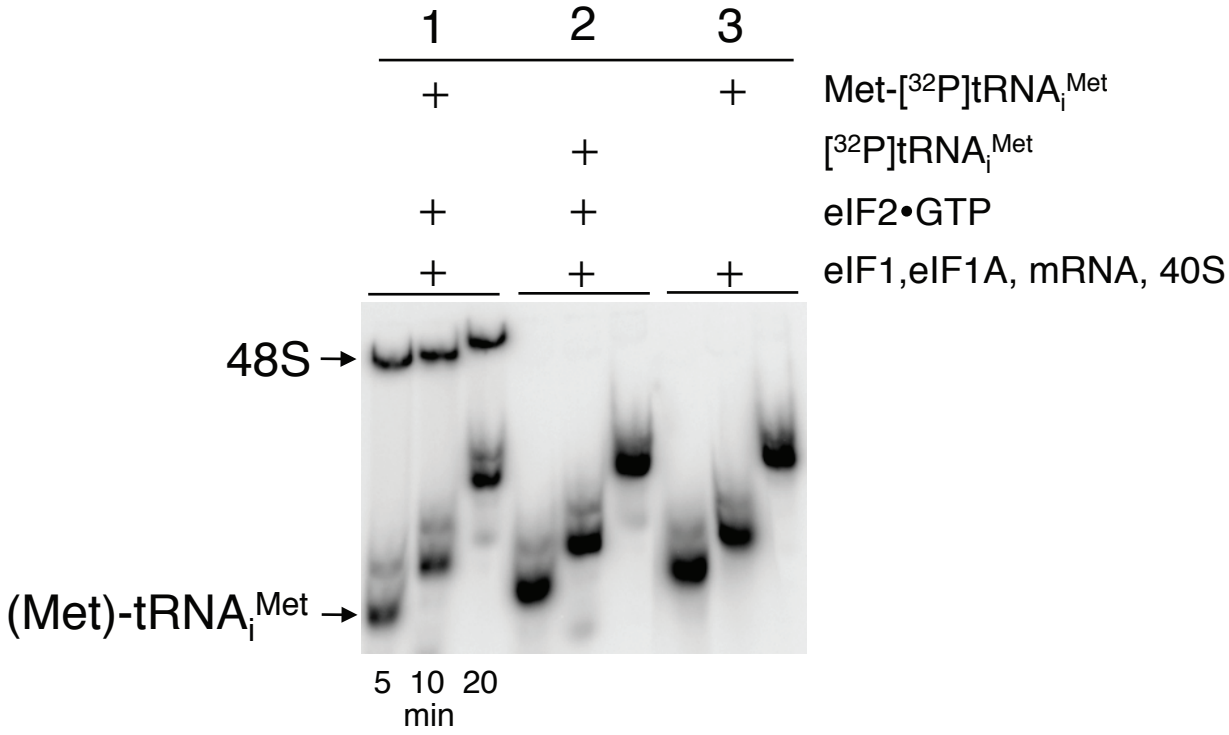
(**a–c**) Test of growth and *GCN4* translational control in yeast cells expressing eIF2 Cys mutants. Derivatives of the *gcn2Δ* yeast strain J551 carrying a high copy-number plasmids expressing wild type eIF2, eIF2ΔC, or the indicated eIF2 subunit mutants were grown to saturation, and 4 µl of serial dilutions (of OD_{600} = 1.0, 0.1, 0.01, 0.001, and 0.0001) were spotted on minimal medium with essential nutrients (SD) or SD medium containing 0.3 µg/ml sulfometuron methyl (SM), and incubated at 30 ˚C for 3 days. J531, the *GCN2*$^{+}$ parent of strain J551, was included as a positive control for growth on SM medium.

(**d**) Primer extension analysis of 18S rRNA helix h44 cleavages by Fe(II)-BABE linked to the native Cys residues in eIF1. Various initiation complexes were assembled using the indicated eIF1, Fe(II)-BABE-eIF1, eIF1A, eIF2 TCs, mRNA and 40S subunits. Hydroxyl radical cleavage products were resolved on 5% (w/v) denatured polyacrylamide gels, and cleavage sites on 18S rRNA were determined by comparison with samples containing unmodified eIF1 (lane 1). Sites of enhanced cleavage with eIF1-Fe(II)-BABE are labeled on the left. Lanes marked “U” and “C” present 18S rRNA sequencing reactions using reverse transcriptase and the indicated dideoxynucleotide; the numbering of helix h44 residues is shown on the right. The lack of rRNA cleavage in lane 4 is consistent with the release of eIF1 from 48S complexes following AUG codon selection. * denotes helix h44 cleavages that were not reproducible and are not shown on the 18S rRNA secondary structure map in panel **e**.

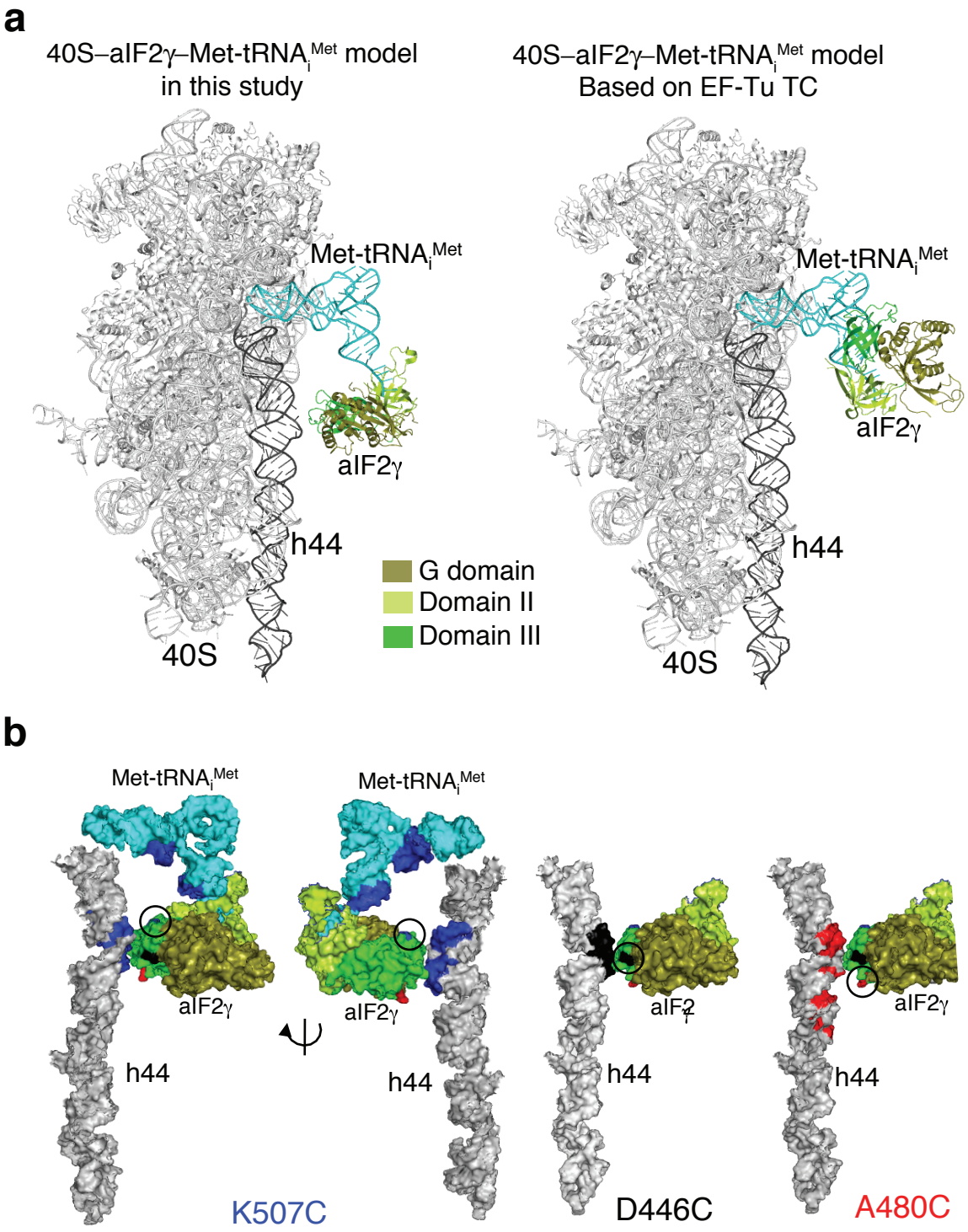
(**e**) Sites of directed hydroxyl radical cleavage by Fe(II)-BABE-eIF1 mapped on the secondary structure of yeast 18S rRNA. Residues circled and numbered in the inset show sites of cleavage near the top of helix h44.

(**f**) Primer extension (toeprinting) analysis of 48S preinitiation complexes on the model mRNA. The first four lanes are dideoxy-sequencing reactions. Lanes 1-5 all contain mRNA, 40S subunits, eIF1 and eIF1A; other factors including eIF2–GDPNP–Met-tRNA$_i^{Met}$ TCs are added as indicated. The location of the AUG start codon is shown on the left. The toeprints at +16 and +17 indicate that the 48S complexes are bound at the AUG start codon.

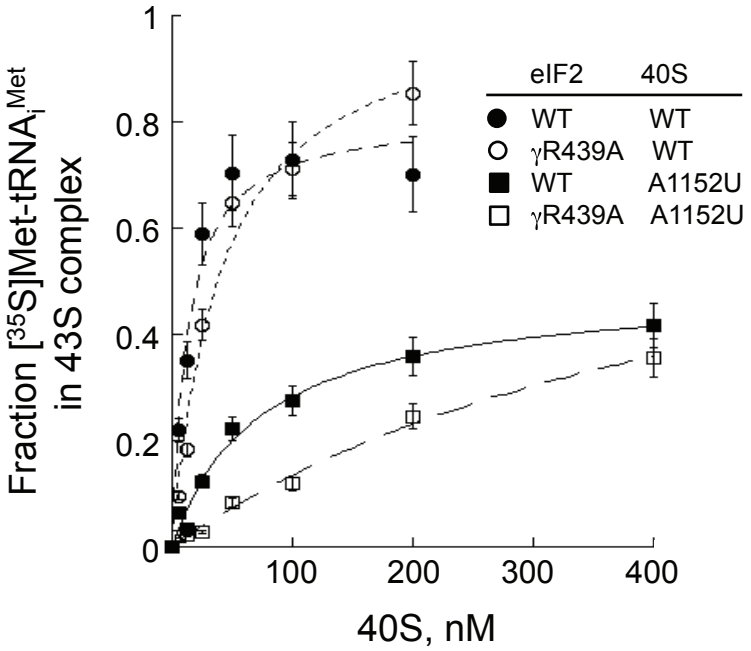
(**g**) Observed rate constants and amplitudes from 48S complex formation assays shown in Figure 2**c**. Errors are s.d. from two independent measurements.



Supplementary Figure 3. 48S Complex Formation Assay with Met-[^{32}P]tRNA$_i^{Met}$. Preformed eIF2–GDPNP–Met-[^{32}P]tRNA$_i^{Met}$ TCs (panel **1**), or reactions containing [^{32}P]tRNA$_i^{Met}$ (panel **2**) or lacking eIF2 (panel **3**) were incubated with eIF1, eIF1A, model mRNA, and 40S subunits. Reactions were incubated at 26 ˚C for 5, 10 or 20 min, as indicated, and then separated on a native gel. Only reactions containing eIF2 and Met-[^{32}P]tRNA$_i^{Met}$ yielded 48S complexes.



Supplementary Figure 4. 40S–aIF2γ–Met-tRNA$_i^{Met}$ models.
(**a**) Comparison of our proposed 40S–aIF2γ–Met-tRNA$_i^{Met}$ complex model (left) in which aIF2γ domain III contacts 18S rRNA helix h44 with the same complex modeled on the EF-Tu TC (right) in which aIF2γ domain III contacts the T-stem of the tRNA. In the EF-Tu based 43S complex model (right), aIF2γ domain III is remote from 18S rRNA helix h44.
(**b**) Docking of aIF2γ on 18S rRNA helix h44 based on hydroxyl radical cleavage results. Figures are surface representations of the docking model shown in Figures 4**c–d**. Locations of aIF2γ residues corresponding to eIF2γ-K507 (blue), eIF2γ-D446 (black) and eIF2γ-A480 (red) are colored and circled, and the locations of rRNA and tRNA$_i^{Met}$ cleavages generated by Fe(II)-BABE linked to these sites are shaded the corresponding color.



Supplementary Figure 5. Fitting curves for determination of K_d values shown in Figure 6e.

Preformed eIF2–GDPNP–[^{35}S]Met-tRNA$_i^{Met}$ TCs containing either wild type or the γR439A mutant of eIF2 were incubated with eIF1, eIF1A, and the indicated amounts of either wild type or 18S rRNA-A1152U mutant 40S subunits. Reactions were incubated for either 15 (WT 40S) or 60 (A1152U mutant 40S) min and then separated on native gels. The fraction of [^{35}S]Met-tRNA$_i^{Met}$ bound to 43S complexes was measured; each point represents the mean of three independent experiments and error bars indicate the s.d.

Supplementary Table 1. Plasmids used in this study

Plasmid	Description	Source
p920	*LEU2, CEN/ARS, SUI3* in pRS315	T. Dever
pC92	*LEU2, CEN/ARS*, eIF2(*SUI2, SUI3, GCD11*) in pRS315	T. Dever
pC171	*LEU2, CEN/ARS, SUI2* in pRS315	1
pC2805	*LEU2*, 2μ, eIF2ΔC (*SUI2-ΔC, SUI3-ΔC, His_8-GCD11-ΔC*) in YEplac181	this study
pC2825	*SUI3-ΔC-S80C* in pC2805	this study
pC2826	*SUI3-ΔC-R186C* in pC2805	this study
pC2827	*SUI3-ΔC-S264C* in pC2805	this study
pC2829	*His_8-GCD11-ΔC-S160C* in pC2805	this study
pC2832	*His_8-GCD11-ΔC-D446C* in pC2805	this study
pC2833	*His_8-GCD11-ΔC-K507C* in pC2805	this study
pC2834	*SUI2-ΔC-M29C* in pC2805	this study
pC2835	*SUI2-ΔC-V164C* in pC2805	this study
pC2860	*LEU2, CEN4, His_8-GCD11* in YCplac111	2
pC2861	*LEU2,* 2μ*, His_8-GCD11* in YEplac181	2
pC2836	*SUI2-ΔC-M215C* in pC2805	this study
pC3570	*GCD11-ΔC-L349C* in pC2805	this study
pC3571	*GCD11-ΔC-A480C* in pC2805	this study
pC3644	*GCD11-ΔC-R118C* in pC2805	this study

pC3650	*SUI2-ΔC-K182C* in pC2805	this study
pC3651	*SUI2-ΔC-L226C* in pC2805	this study
pC3652	*GCD11-R439A* in pC92	this study
pC3653	*GCD11-R510H* in pC92	this study
pC3654	*GCD11-R503A* in pC92	this study
TF3	*T. thermophilus tuf1* (EF-Tu) in pET3a (N-term His_6-TEV)	3
pC3739	EF-Tu-T394C in TF3	this study

References

1. Dey, M. et al. PKR and GCN2 kinases and guanine nucleotide exchange factor eukaryotic translation initiation factor 2B (eIF2B) recognize overlapping surfaces on eIF2α. *Mol Cell Biol* **25**, 3063-3075 (2005).
2. Alone, P.V., Cao, C. & Dever, T.E. Translation initiation factor 2γ mutant alters start codon selection independent of Met-tRNA binding. *Mol Cell Biol* **28**, 6877-88 (2008).
3. Sanderson, L.E. & Uhlenbeck, O.C. Directed mutagenesis identifies amino acid residues involved in elongation factor Tu binding to yeast Phe-$tRNA^{Phe}$. *J Mol Biol* **368**, 119-30 (2007).

SUPPLEMENTARY METHODS

Plasmid Construction. Mutations designed to remove Cys residues were initially constructed in separate plasmids for each eIF2 subunit: pC171[*SUI2*(eIF2α), *LEU2*][1], p920[*SUI3*(eIF2β), *LEU2*] (in pRS315), and pC2860[*His$_8$-GCD11*(His$_8$-eIF2γ), *LEU2*][2]. Whereas the 4 Cys residues involved in binding zinc in both eIF2β and eIF2γ were not mutated, a total of 12 Cys residues in eIF2α plus eIF2β and eIF2γ were mutated as shown in Figure 2**a**. The Cys-less *SUI2* gene on a 2.5-Kb *Bam*HI fragment and the Cys-lite versions of *SUI3* on a 1.8-Kb *Hind*III fragment and *His$_8$-GCD11* on a 2.6-Kb *Sac*I-*Pst*I fragment, each under the control of its native promoter, were subcloned together in the high copy-number *LEU2* vector YEplac181[3] generating the plasmid pC2805[*SUI2-ΔC*, *SUI3-ΔC*, *His$_8$-GCD11-ΔC*, *LEU2*]. Using a QuikChange site-directed mutagenesis kit (Stratagene), pC2805 was mutated by introducing single Cys residues at various surface exposed sites on all three subunits of eIF2 as shown in Figure 2**b**. The DNA sequence of all genes and the presence of the desired mutations were verified by DNA sequencing.

Construction of eIF2 Deletion Strain. The heterozygous *gcd11Δ*::*kanMX4*/*GCD11* (eIF2γ) diploid strain in the BY4743 background was obtained from the yeast genome deletion collection. After replacing the *KanMX4* marker with a *NAT* (nourseothricin) resistance marker using the plasmid p4339[4], generating *gcd11Δ*::*NAT*/*GCD11*, one of the two *SUI3* (eIF2β) genes was

disrupted by *KanMX4*. Finally, one of the *SUI2* (eIF2α) genes in the diploid strain was disrupted using a *hisG::URA3::hisG* cassette[5]. The resulting strain was then printed on medium containing 5-fluoroorotic acid (5-FOA) to select for cells in which the *URA3* gene was lost by recombination between the flanking *hisG* repeats. The resulting *gcd11Δ*::*NAT*/*GCD11 sui3Δ::KanMX4/SUI3 sui2Δ::hisG/SUI2* heterozygous diploid strain was transformed with the high copy-number *URA3, SUI2, SUI3, GCD11* plasmid p1780[6]. The resulting strain was sporulated and NAT^+, Kan^+, Ura^+ haploid products were isolated. Candidate haploid strains were tested using PCR for the presence of the *sui2Δ::hisG* deletion. These candidate strains were then tested for growth on 5-FOA medium. While haploid strains carrying the WT *SUI2* gene were able to grow on 5-FOA medium, strains carrying the *sui2Δ::hisG* allele failed to grow on 5-FOA medium and were selected for further analysis. Following PCR analyses to confirm disruption of the chromosomal *SUI2, SUI3* and *GCD11* genes, the strain was further modified to facilitate eIF2 purification. First, plasmid p1780 was replaced with the plasmid pC92[*SUI2, SUI3, GCD11, LEU2*] by plasmid shuffling and then the *GCN2* gene was disrupted using the *gcn2::hisG::URA3::hisG* cassette in plasmid p3328 as described above to insure that eIF2α was not phosphorylated. Next, a *HIS3* marker was used to disrupt the *PEP4* gene encoding proteinase A in order to increase the stability of eIF2 during purification. Following reintroduction of p1780, and loss of pC92, the genotype of the final strain (J551) is: *MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sui2Δ::hisG sui3Δ::KanMX4 gcd11Δ::NAT gcn2Δ::hisG pep4::HIS3*, p1780 [2μ *LEU2*, *SUI2, SUI3, GCD11*].

For yeast growth assays, transformants were grown to saturation, and 4 µl of serial dilutions (of OD_{600} = 1.0, 0.1, 0.01, 0.001, and 0.0001) were spotted on appropriate medium and incubated at 30 °C for 3 days.

Protein Purification. In order to purify eIF2ΔC or its single Cys mutants, derivatives of the high copy-number *LEU2* plasmid pC2805 were introduced into J551 by plasmid shuffling[7], and the resulting strains were grown in YPD medium. To purify eIF2 complexes that retain the native Cys residues, but are mutated at other residues in eIF2γ, derivatives of the high copy-number *LEU2* plasmid pC2861 carrying WT or mutant versions of *His_8-GCD11*, encoding a N-terminal His_8-tagged version of eIF2γ, was introduced into J551. The resulting strains were grown in synthetic complete (SC) liquid medium containing all amino acids and required nutrients except uracil and leucine. The eIF2 was purified using a protocol described by Acker et al.[8], with some modifications. A 10 L culture of yeast was grown to OD_{600} = 5 to 8, cells were harvested, and then broken using liquid nitrogen and a Waring Blender[8]. The cell lysate was then precipitated by adding 75% (w/v) ammonium sulfate, and the resulting protein pellet was dissolved in 200 ml of Ni-10 buffer (20 mM Tris [pH 7.5], 500 mM KCl, 0.1 mM $MgCl_2$, 10 mM imidazole, 10% (v/v) glycerol, 10 mM GDP, 5 mM 2-mercaptoethanol, and standard protease inhibitors [1X Roche Complete EDTA-free protease inhibitor cocktail, 1 mg per L leupeptin, 1mg per L pepstatin and 0.5 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF)]). Following addition of 10 ml Ni-NTA resin (Qiagen), the mixture was stirred at 4 °C

for 1 h. The resin was then washed with 30 ml of Ni-10 buffer, and bound proteins were eluted in 20 ml of Ni-500 buffer (Ni-10 buffer containing 500 mM of imidazole). The protein was then dialyzed overnight at 4 °C against H-100 buffer (20 mM Tris [pH 7.5], 100 mM KCl, 0.1 mM $MgCl_2$, 10 mM of GDP, 10% (v/v) glycerol and 2 mM DTT). The eIF2 was further purified by chromatography on a 5 ml HiTrap Heparin column (GE Healthcare) and a 5 ml HiTrap Q HP column (GE Healthcare) using 60 ml linear gradients of 0.1 M to 1 M KCl in H-100 buffer with a 2 ml per min flow rate. Fractions were analyzed by SDS-PAGE and fractions containing eIF2 were then pooled and dialyzed in 20 mM HEPES [pH 7.5], 100 mM potassium acetate, 0.1 mM magnesium acetate, 2 mM DTT and 10% (v/v) glycerol. Protein solutions were concentrated (to ~20 μM) by filtration using a microcon YM-50 (Amicon), and then aliquots were flash frozen in liquid N_2 and stored at -80 °C.

Initiation factors eIF1 and eIF1A were expressed in *E. coli* BL21(DE3) CodonPlus cells (Stratagene) and purified using the IMPACT Protein Purification System (New England Biolabs), based on the procedure described previously[8]. Briefly, freshly transformed *E. coli* cells containing the plasmid pTYB2-eIF1 (or pTYB2-eIF1A) were grown in 2 L LB medium (+ 100 mg per L ampicillin) to OD_{600} = 0.5. Recombinant protein expression was induced by addition of 0.5 mM IPTG and the culture was incubated with shaking at 25 °C for 4 h. Following harvesting the cell pellet was resuspended in 30 ml intein lysis buffer (20 mM Tris [pH 7.5], 0.5 M KCl, 0.1% (v/v) Triton X-100, 1 mM EDTA, 1 tablet of protease inhibitor cocktail (Roche), and 0.5 mM AEBSF). The cells were broken by sonication (10

cycles of 30 sec pulse followed by 30 sec cooling) and then cell debris and unbroken cells were removed by centrifugation at 25,000 x *g* for 20 min. Following equilibration of 1 ml of chitin resin in 10 ml lysis buffer in a mini-column, the resin was gently mixed with the cell lysate for 1 h at 4 °C. Following drainage of the column, the resin was washed with 30 ml intein wash buffer (20 mM Tris [pH 7.5], 1 M KCl, 0.1% (v/v) Triton X-100, 1 mM EDTA), and then with 10 ml intein cleavage buffer (20 mM Tris [pH 8.0], 0.5 M KCl, 1 mM EDTA). Finally, 3 ml of intein cleavage buffer containing 75 mM DTT was applied to the column, 1 ml of the buffer was allowed to flow through, and then the ends of the column were sealed. Following incubation overnight at room temperature to allow intein cleavage and protein release from the resin, column fractions were collected, pooled, and then dialyzed against 20 mM HEPES [pH 7.5], 100 mM KOAc, 2 mM DTT, and 10% (v/v) glycerol. Aliquots of purified proteins were flash frozen in liquid N_2 and stored at -80 °C.

WT and mutant forms of *T. thermophilus* His_6-EF-Tu were expressed in *E. coli* BL21(DE3) CodonPlus cells (Stratagene) using derivatives of the plasmid TF3 (pET3a-*tuf1*; obtained from Olke Uhlenbeck) and purified as described previously[9] with minor modification. *E. coli* transformants were grown at 37 °C in 500 ml LB (+ 100 µg per ml ampicillin) medium in a 2-L flask to A_{600} = 0.5. Following addition of 0.5 mM IPTG to induce recombinant protein expression, cells were incubated at 25 °C for 4 h with shaking. The cells were harvested, and the cell pellet was resuspended in 30 ml buffer A (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 5% (v/v) glycerol, 10 mM imidazole, 5 mM b-mercarptoethanol, 100

mM GDP, 1 tablet of protease inhibitor cocktail (Roche), and 0.5 mM AEBSF). Cells were broken by sonication (10 cycles of 30 sec pulse followed by 30 sec cooling), and then cell debris and unbroken cells were removed by centrifugation at 25,000 *g* for 20 min. The cleared lysate was gently mixed with 1 ml Ni-NTA resin (Qiagen) for 1 h at 4 °C. After washing the resin with 30 ml buffer A containing 1 M NaCl and 0.5% (v/v) Tween 20, proteins were eluted by a step gradient of imidazole in buffer A (0.1 to 1 M, in 0.1 M steps). Fractions containing EF-Tu were pooled and dialyzed against buffer A containing 50% (v/v) glycerol. Aliquots of purified EF-Tu were flash frozen in liquid N_2 and stored at -80 °C.

40S ribosomal subunits were purified from the yeast strain F353 (*MAT*α *ura3-52 trp1 leu2-*Δ*1 his3-*Δ*200 pep4::HIS3 prb1-*Δ*6R can1*) as described previously[10]. For hydroxyl radical cleavage experiments, the 40S subunits obtained from sucrose gradients were pelleted and then dissolved in ribosome storage buffer without sucrose and DTT (20 mM HEPES [pH 7.4], 100 mM potassium acetate, and 2.5 mM magnesium acetate).

Fe(II)BABE Derivatization. Prior to Fe(II) derivatization, eIF2 (or eIF1) was reduced by incubating in the presence of 2 mM DTT for 30 min at 4 °C. The proteins were then dialyzed for 6 h in 1 L modification buffer (30 mM HEPES [pH 7.5], 500 mM KCl). Proteins were derivatized with Fe(II)-BABE (Dojindo, Rockville, MD) by incubating 10 - 20 μM eIF2 or eIF1 with 1 mM Fe(II)-BABE in modification buffer for 30 min at 30 °C (typical reaction volume = 50 μl). Unincorporated Fe(II)BABE was removed by filtration using Microcon YM-50

(Amicon) microconcentrators, and the protein was washed four times in 500 ml cleavage buffer (20 mM HEPES [pH 7.5], 100 mM KCl, and 3 mM $MgCl_2$). Aliquots of the derivatized proteins [5 to 20 μM] were stored at -80 °C.

Initiator tRNA Preparation and Hydroxyl Radical Cleavage. Yeast initiator $tRNA_i^{Met}$ was transcribed *in vitro* using the T7 RNA polymerase and template system described previously[11]. Briefly, the plasmid pUC19-IMT, encoding yeast $tRNA_i^{Met}$ under the control of a phage T7 promoter, was digested with *Bst*NI to yield a 3′ CCA end. T7 transcription reactions, typically 2 ml, containing 40 mM Tris [pH 8.0], 30 mM $MgCl_2$, 2 mM spermidine, 0.1% (v/v) Triton X-100, 10 mM DTT, 50 mg per ml bovine serum albumin, 2.5 mM NTPs, 20 mM AMP, 100 μg of *Bst*N1-digested plasmid template and 200 units of T7 RNA polymerase (Roche) were incubated overnight at 37 °C. After extracting the reaction twice by phenol [pH 8.0] and once by chloroform, the RNA was precipitated with ethanol. Finally, the $tRNA_i^{Met}$ was purified by 12% (w/v) denaturing polyacrylamide gel electrophoresis (PAGE) as described by Acker et al.[8].

For examination of hydroxyl radical cleavages in Met-$tRNA_i^{Met}$, 48S complexes were assembled using Met-[5′-^{32}P]$tRNA_i^{Met}$. To make Met-[5′-^{32}P]$tRNA_i^{Met}$, $tRNA_i^{Met}$ was dephosphorylated using calf intestinal phosphatase (Roche), subjected to phenol–chloroform extraction and ethanol precipitation, and then 5′-end labeled using T4 polynucleotide kinase (Roche) and [γ-^{32}P]ATP (Perkin Elmer). The [5′-^{32}P]$tRNA_i^{Met}$ was aminoacylated with methionine using yeast methionyl-tRNA synthetase as described previously[8]. Incorporation of Met-

[5′-^{32}P]tRNA$_i^{Met}$ into 48S complexes was examined by 48S complex gel shift assay (Supplementary Fig. 2). To analyze Met-tRNA$_i^{Met}$ cleavages in hydroxyl radical cleavage reactions, 48S complexes were formed using 0.1 μM Met-[5′-^{32}P]tRNA$_i^{Met}$ instead of 1 μM unlabeled Met-tRNA$_i^{Met}$. Following hydroxyl radical cleavage reactions, the cleavage sites in tRNA$_i^{Met}$ were analyzed by 10% or 20% (w/v) denaturing PAGE. The tRNA ladders were prepared by digesting Met-[^{32}P]tRNA$_i^{Met}$ with RNase T1 (cleaves 3′ of G residue) or by incubating Met-[^{32}P]tRNA$_i^{Met}$ with 25 mM $NaHCO_3$ at 95 °C for 3 min. All cleavage assays were repeated two or more times to verify reproducibility of the results.

Toeprinting Assay. Toeprinting was performed as described[12]. Briefly, 5′-[^{32}P]-primer (5′-TTATTTGGATCTGGC-3′) was annealed to TP1 RNA (Sequence: 5′-GGAAUCUCUCUCUCUCUCUAUGCUCUCUCUCUCUCUCUCUCUCUCUC UCUCUCUCUCUCUCUCGCCAGATCCAAATAA-3') in 1X Recon buffer (30 mM HEPES [pH 7.6], 100 mM KOAc, 3 mM $Mg(OAc)_2$ and 2 mM DTT) by heating at 60 °C for 5 min and then transferring the tube directly to ice. 0.2 μM RNA–primer duplex was added to 10 μl binding reactions containing 1X Recon buffer, 5 mM EGTA, 0.8 U per μl RiboLock RNase inhibitor (Fermentas), 2 mM ATP-Mg^{2+}, 0.2 μM 40S ribosomal subunits, 1 μM eIF1, 1 μM eIF1A, 0.4 μM TC (preformed by mixing 1 mM GDPNP-Mg^{2+}, 0.4 μM Met-tRNA$_i^{Met}$, and 0.4 μM eIF2, and then incubating at 26 °C for 15 min), 0.4 μM eIF3, 1 μM eIF4A, 0.8 μM eIF4B, and 0.2 μM copurified eIF4E–eIF4G complex. An equivalent volume of Enzyme storage buffer (20 mM HEPES [pH 7.6], 100 mM KOAc pH 7.6, 10% (v/v) glycerol, 2 mM

DTT) was used in place of omitted factors. Complexes were assembled by incubating for 30 min at 26 °C, and then 2 μl of each reaction was diluted into 10 μl extension mix (10 mM Tris [pH 7.5], 10 mM $MgCl_2$, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol, 375 μM each dNTP, and 1 U per μl AMV Reverse Transcriptase [Northstar Bioproducts]) and extended for 30 min at 30 °C before resolving products on a sequencing gel (6% (w/v) polyacrylamide, 7 M Urea).

SUPPLEMENTARY NOTES

48S Complexes Used for Hydroxyl Radical Mapping are Functional

The 48S complexes used for hydroxyl radical mapping of eIF2 were assembled by mixing eIF2–GDPNP (slowly hydrolysable GTP analog)–Met-$tRNA_i^{Met}$ TC, eIF1, eIF1A, a model unstructured mRNA with a centrally located AUG codon, and 40S subunits. Using the unstructured model mRNA alleviated the requirement for cap recognition and unwinding of mRNA secondary structure, simplifying the initiation process and eliminating the requirement for additional translation initiation factors.

Two experiments were performed to test whether the 48S complexes were functional *in vitro*. In the first experiment, the position of eIF1 in the 48S complex was examined by hydroxyl radical mapping using Fe(II)-BABE derivatized eIF1. Yeast eIF1 has two native Cys residues, Cys64 and Cys89. The WT protein was reacted with Fe(II)-BABE and assembled into various ribosomal complexes, hydroxyl radicals were then generated by Fenton reactions and the cleavage

sites in 18S rRNA was analyzed by primer extension using ^{32}P-labeled primers. As shown in Supplementary Fig. 2**d**, Fe(II)-BABE modified eIF1 produced cleavages at nucleotides G1645, C1646 and G1757 to C1759 in 18S rRNA, corresponding to upper portion of helix h44. These cleavage sites are consistent with those obtained in similar studies with human eIF1[13]. The cleavage of helix h44 was detected in 40S–eIF1 binary complexes, and more prominently observed in complexes also containing eIF1A (Supplementary Fig. 2**d**, lane 2 versus 3). The cleavage sites on helix h44 are shown on the secondary structure of yeast 18S rRNA in Supplementary Fig. 2**e**. As Fe(II)-BABE could be linked to either or both Cys64 and Cys89 in eIF1, it is not possible to link the rRNA cleavage to either residue in eIF1; however, based on the crystal structure of the *Tetrahymena* 40S–eIF1 complex[14] and the hydroxyl radical probing studies with human eIF1[13], Cys64 is closer than Cys89 to helix h44. Interestingly, the cleavage of 18S rRNA at the top of helix h44 by Fe(II)-BABE modified eIF1 was not observed when eIF2 TC and mRNA(AUG) were included in the assay (Supplementary Fig. 2**d**, lane 4 versus 2). This lack of rRNA cleavage is consistent with the observation that eIF1 is released from 48S complexes upon AUG codon recognition, even in the absence of GTP hydrolysis by eIF2[15]. The binding of eIF1 near the top of helix h44 on the 40S subunit and the release of eIF1 upon AUG start codon selection indicate that the 48S complexes and the various reagents used in these studies are functional.

In the second experiment, the position of the 48S complex on the model mRNA was analyzed by primer extension assay (toeprinting). Whereas reactions

containing eIF1, eIF1A, mRNA and 40S subunits did not produce a toeprint on the mRNA, inclusion of eIF2 TC in the reaction produced a strong toeprint at 16 to 17 bases downstream of the AUG codon (Supplementary Fig. 1**f**, lanes 1 and 2). These results are consistent with previous reports in both yeast and mammalian systems that a 48S complex protects the mRNA to 16-17 bases downstream of the start codon[12,16]. Addition of eIF3 and-or eIF4F and eIF4B did not enhance the toeprint (Supplementary Fig. 1**f**, lanes 3-5), consistent with the notion that 48S complexes can stably assemble at the start codon of the unstructured model mRNA in the absence of the factors that promote mRNA binding. In addition to confirming that the reagents used in these studies support 48S complex formation, the toeprints at +16 and +17 are consistent with the notion that Met-tRNA$_i^{Met}$ is bound to the P-site of the 40S subunit and base-paired with the AUG start codon in the model mRNA.

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