## METHODS

**Plasmid and Yeast Strain Construction**. A *tif51a-td* integration plasmid (pC3286) was constructed by inserting an *Xho*I-flanked PCR (primers P1, 5'-CCGCTCGAGATGTCTGACGAAGAACATACCTTTGAA-3', and P2, 5'-CCGCTCGAGGTGAGTAGATGGAGACAAATCTTCCAAC-3') product containing the first 225 bp of the eIF5A open reading frame (ORF) into the *Xho*I site of p4437, a plasmid containing the copper-regulated *CUP1* promoter upstream of the ubiquitin coding region followed by an Arg codon (pCUP1-UBI-R) [16]. *TIF51A* (1.0 kb 5' flanking region, 0.5 kb ORF, and 0.5 kb 3' flanking region) was amplified from genomic DNA using primers P3 (5'-CTAGTCTAGAGCTGTCTCTACTAACAGATCTTGGCGTTTTTGAATCGTG-3') and P4 (5'-CTAGTCTAGAGAGAAAAATAACGACAACTGCAGGACTCGAACCTGCGCG-3'). The resultant PCR product was subcloned into *Xba*I digested single copy-number *LEU2* (YCplac111) and *URA3* (YCplac33) vectors or high copy-number *LEU2* vector YEplac181 [31] generating plasmids pC3287, pC3288, and pC3289, respectively. Plasmid pC3290 was generated by inserting a FLAG epitope tag at the C-terminus of the *TIF51A* ORF in pC3289 using primers P10 (5'-AGGAAGCTGCTAGAACCGATGATTATAAAGATGATGATGATAAATAAACCGGTT-3') and P11 (5'-ATCGGTTCTAGCAGCTTCCTTGAAGGAGATGG-3') and the GeneTailor Site-Directed Mutagenesis System (Invitrogen). Likewise, a K51R mutant derivative of pC3290 was constructed by site-directed mutagenesis using primers P14

(5'-CATGTCCACTTCTAAGACTGGTAGGCACGGTCACGC-3') and P15 (5'-ACCAGTCTTAGAAGTGGACATGTCGACAATCTTAC-3'), generating the plasmid pC3291.

To randomly mutate the *TIF51A* ORF, we first used site-directed mutagenesis and the primers P16 (5'-ACACACACAAATACCAACTCCTCGAGACAATGTCTGACG-3') and P17 (5'-GAGTTGGTATTTGTGTGTTTGGGAGTCTATG-3') to create an *Xho*I site immediately upstream of the *TIF51A* ORF and primers P18 (5'-CGATTAAACCGGTTAACATCACGGCCGGGGATATAAATG-3') and P19 (5'-TGATGTTAACCGGTTTAATCGGTTCTAGCAGCT-3') to create an *Eag*I site immediately downstream of the *TIF51A* ORF in pC3287 generating plasmid pC3292. The *TIF51A* ORF in pC3287 was amplified by error prone PCR using primers P12 (5'-GATCCGCTCGAGACAATGTCTGACGAAGAACATACCTTTG-3') and P13 (5'-GATCTCGGCCGTGATGTTAACCGGTTTAATCGGTTCTAGC-3'). The PCR products were digested with *Xho*I and *Eag*I and cloned into the *XhoI/EagI* site of pC3292 generating a *tif51a* mutant library. The plasmid pC3294 containing the *tif51a-S149P* mutant was generated by site-directed mutagenesis of the plasmid pC3292 using primers P20 (5'-CTATGGGTGAAGAAGCCGCCATCCCCTTCAAGGAAGCT-3') and P21 (5'-GATGGCGGCTTCTTCACCCATAGCGGAGATGAT-3').

Yeast strain J700 was derived from H2557 by linearizing plasmid pAJ2 [16] with *Pme*I and integrating it at the *UBR1* locus. Correct pAJ2 integration was verified by PCR amplification of the *UBR1* locus and also by demonstrating galactose-dependent over expression of myc-tagged *UBR1* in J700. Strain J700 was transformed with the single

copy-number *URA3* plasmid YCplac33 to generate strain J713. The *TIF51A* degron mutant (*tif51a-td*) strain J701 was generated by integrating pC3286 (linearized with *Sal*I) at the *TIF51A* locus in J700. The *tif51a-td* allele in J701 was confirmed by PCR analysis of genomic DNA and by Western analysis of WCEs from cells grown under permissive conditions (raffinose medium supplemented with copper where the *CUP1* promoter is induced and *UBR1* expression is low) versus non-permissive conditions (galactose medium lacking copper where the *CUP1* promoter is repressed and overexpression of *UBR1* triggers proteasomal degradation of the degron protein) using rabbit polyclonal antiserum against yeast eIF5A.

The *tif51b::KanMX4* module from the *tif51b* (YJR047c) knock-out strain in the yeast genome deletion collection was amplified by PCR using primers P5 (5'-GTTACCCTGAATCATATTCGACGATGTCGTCTCACACGGA-3') and P6 (5'-CACCCTCGTCGTGCAAAAAATATTTTGACTTCTACTCTTT-3') that hybridize ~600 bp upstream and downstream, respectively, of the *TIF51B* ORF. The PCR product was used to delete *TIF51B* in the haploid strains H2557, J701 and J713 generating strains J691, J702 and J714 respectively. The *KanMX4* marker in J691 was replaced by a *NAT* (nourseothricin-resistance) marker by transformation with *EcoRI* digested p4399[32] generating strain J692 (*tif51b::NAT*).

The haploid J692 strain was converted into a diploid strain (J693) by introducing a low copy-number *URA3* plasmid carrying the *HO* gene. Following isolation of a diploid transformant, cells lacking the *HO* plasmid were isolated on medium containing 5-fluoroorotic acid (5-FOA). Subsequently, the diploid strain J693 was transformed with the low copy-number *URA3, TIF51A* plasmid pC3288 generating strain J694. The

*tif51a::KanMX4* cassette from the heterozygous *tif51a*Δ strain from the yeast genome deletion collection (Open Biosystems) was amplified by PCR using primers P7 (5'-CGGGGTACCGTGTTATCCGAAGAGTCACTCACCAAAAAC-3') and P8 (5'-GCATACATGCATGCTCCTTTTCAAGATCATCACCGTCGTCATCAC-3') that hybridize ~1.5 kb upstream and 1 kb downstream, respectively, of the *TIF51A* ORF. This PCR product was used to replace the *TIF51A* gene in J694 with a *tif51a::KanMX* cassette creating strain J695. Strain J695 was sporulated, and subjected to tetrad analysis. Two of the four haploid spores carried the *tif51a::KanMX* allele in the chromosome and were unable to lose the *URA3, TIF51A* plasmid (unable to grow on medium containing 5-FOA). One of the two haploid strains, J696 (*tif51b::NAT tif51a::KANMX4* pC3288[*TIF51A,URA3*]), was transformed with the randomly mutated *tif51a* ORF library in pC3292 (low copy-number *LEU2*). Individual transformants were patched on SD plates supplemented with essential nutrients, and then replica-printed to two 5-FOA plates which were incubated at 25 ˚C and 37 ˚C, respectively. The *tif51a* mutant plasmids were isolated from cells that grew well at 25 ˚C, but failed to grow at 37 ˚C. The isolated plasmids were re-tested to confirm that the mutant phenotype was plasmid associated, and then sequenced to identify the mutation(s). One of the best temperature-sensitive mutants, *tif51a-D63V*, was selected for further analysis.

Strains J697, J698 and J699 were obtained by plasmid-shuffling following transformation of strain J696 with the *TIF51A* (pC3287), *tif51a-D63V* (pC3293), and *tif51a-S149P* (pC3294) plasmids, respectively. To purify Flag-tagged eIF5A (pC3290) and eIF5A$^{K51R}$ (pC3291), the indicated plasmids were introduced into strain J702 generating strains J703 and J704, respectively.

***In vivo* [$^{35}$S]Met incorporation**. Liquid cultures of yeast strains were grown under permissive conditions (SC-Met medium containing 2% raffinose and 100 μM $CuSO_4$) at 25 ˚C to an $A_{600}$ of ~1.0. Cells were washed at room temperature to remove $Cu^{2+}$, transferred to nonpermissive conditions (SC-Met medium containing 2% galactose and lacking $CuSO_4$), and incubated for 14 h at 25 ˚C to $A_{600}$ = 0.6-1.0. To start the labeling, 50 μM unlabeled methionine and 1 μCi/ml [$^{35}$S]Met was added to each culture. At 15-min intervals, the optical density of each culture was determined, 1 ml aliquots were removed, and [$^{35}$S]Met incorporation was monitored by trichloroacetic acid (TCA) precipitation.

**Polysome analysis.** Liquid cultures of cells were either treated or untreated with 50 μg/ml CHX for 5 min before harvesting, transferred to a 500-ml centrifuge bottle containing shaved ice, pelleted, and washed with 10 ml of Buffer B (20mM Tris pH 7.5, 50mM KCl, 10mM $MgCl_2$) supplemented with EDTA-free protease inhibitor tablet and 1mM DTT. For CHX-treated cells, the same concentration of CHX was present in all steps thereafter. Cell pellets were resuspended in an equal volume of Buffer B, and then an equal volume of glass beads was added, and cells were broken by vigorous mixing on a vortex. Following clarification, five $A_{260}$ units of WCEs were layered on 4.5-45% sucrose gradients prepared in 20 mM Tris pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT, and then centrifuged for 2.5 h at 39,000 rpm in a Beckman SW41 rotor. Gradients were fractionated while monitoring absorbance at $A_{254}$.

**Ribosome transit time measurement.** Assays were performed as described previously[21,33] with modifications as detailed below. Yeast cells grown at 25 ˚C in SC-Met medium were shifted to prewarmed (36 ˚C) medium, and after 2 h 0.1 μCi/ml [$^{35}$S]Met was added. At regular intervals, 10 ml aliquots were removed and mixed with 200 μg/ml CHX. Cells were resuspended in 0.2 ml cold Lysis Buffer (20 mM HEPES pH 7.4, 2 mM magnesium acetate, 0.1 M potassium acetate, 0.5 mM DTT, 0.1mg/ml CHX) and broken with glass beads by vigorous mixing on a vortex for 15 min. Cell lysates and two washes of the glass beads were combined and clarified by centrifugation. Total (nascent + completed) protein synthesis was determined by combining 0.5 ml lysate, 0.4 ml 60% sucrose, and 0.5 ml 50% TCA. Mixtures were incubated 10 min at 90 ˚C, 10 min on ice, and then collected on a GF/C filter that was pre-washed with 2 mM unlabeled Met. Following washes with 10% TCA and then acetone, filters were dried and counted by liquid scintillation. Completed protein synthesis was determined by layering 0.5 ml lysate on a 0.4 ml 60% sucrose cushion and by pelleting ribosomes by centrifugation at 55,000 rpm for 30 min in a Beckman TLA120.2 rotor. Radioactivity in the supernatant fraction (completed proteins) was determined as described above.

**Purification of eIF5A and eIF5A$^{K51R}$.** C-terminal FLAG-tagged proteins were overexpressed in yeast strains J703 and J704. Following growth to mid-log phase under permissive conditions, cells were washed, and then incubated under non-permissive conditions for 6 h to $A_{600}$ ~ 3.0. Cells were washed, resuspended in FLAG Binding Buffer (20 mM Na-phosphate pH-7, 500 mM NaCl, 0.1% Triton X-100, 1mM phenlmethylsulfonyl fluoride and EDTA-free complete protease inhibitor tablet) and

broken by high speed mixing with glass beads. WCEs were mixed overnight with anti-FLAG-M2 affinity gel (50% slurry, Sigma). Following washing, bound proteins were eluted with FLAG Binding Buffer containing 400 μg/ml FLAG-peptide, and dialyzed against 20 mM HEPES pH 7.5, 10% glycerol, 150 mM KCl and 2 mM DTT.

**Yeast *in vitro* translation assays.** *In vitro* assays of luciferase mRNA translation in crude extracts from yeast cells were performed as previously described[34] using capped and polyadenylated luciferase mRNA transcribed *in vitro* from plasmid T7 $LUC_{50}$ (50-mer poly (A) tail)[35].

**Reconstituted translation elongation assays.** 80S initiation complexes were assembled essentially as described[22] with the following modifications. A different reaction buffer (20 mM Tris-Cl pH 7.5, 100 mM potassium acetate pH7.6, 2.5 mM magnesium acetate, 0.25 mM spermidine, 2 mM DTT) was used and $tRNA_i^{Met}$ was labeled exclusively with [$^{35}$S]Met. Finally, the mRNA contained the ORF sequence AUG-UUC-UUC-UAA. After assembly, initiation complexes were layered onto reaction buffer containing 1.1 M sucrose and centrifuged for 1 hr at 100,000 rpm in a TLA-100.3 rotor. Complexes were resuspended in reaction buffer, flash-frozen, and stored at -80 ˚C. For each elongation reaction, Phe-$tRNA^{Phe}$ ternary complex was prepared. Each batch of ternary complex contained, as indicated in the figures, the following reagents: 45 pmol eEF1A, 21.6 pmol Phe-$tRNA^{Phe}$, 11.25 pmol eEF2, 11.25 pmol eEF3, 20 pmol eIF5A (or $eIF5A^{K51R}$), 2 mM GTP, 2 mM ATP, and 1x reaction buffer, and was incubated at 26 ˚C for 15 min. At the end of this incubation, the ternary complex was mixed with a single aliquot of thawed

initiation complex (~0.2 pmol), and incubated at 26 ˚C. Aliquots were removed at the indicated times and quenched in 0.5 N KOH. Reaction products were separated by electrophoresis on cellulose TLC plates (pyridine-acetate buffer, pH 2.8; 1400 V, ~40 min). [$^{35}$S]Met containing reaction products were detected by phosphorimaging and quantitated using ImageQuant 5.2 software (GE Healthcare Life Sciences).

**Reconstituted translation termination assays.** Initiation complexes were assembled as described above, except that the mRNA coding sequence was AUG-UUC-UAA. Simultaneously, Phe-tRNA$^{Phe}$ ternary complex was assembled. Equal volumes of Phe-tRNA$^{Phe}$ ternary complex and initiation complex were mixed and incubated at 26 ˚C for 15 min. The elongation complexes were then pelleted and stored as described above. Release factor complexes were assembled and incubated at 26 ˚C for 15 min, with the following components: 40 pmol eRF1, 40 pmol eRF3, 40 pmol eIF5A(or eIF5A$^{K51R}$), 1.3 mM GTP, and 1x reaction buffer. Single aliquots of elongation complex were mixed with release factor complex, and aliquots were withdrawn at the indicated times and quenched in 30% formic acid. Reaction products were separated, detected, and quantitated as described above.

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