eIF5A is an indispensable protein for eukaryotic cells

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File S2_supplementary Materials and Methods

Materials and methods

Yeast strains, plasmids, and growth conditions

All *Saccharomyces cerevisiae* strains and plasmids used herein are listed in Supplementary Tables S1 and S2 respectively. Cells were cultured in liquid YPD (2% glucose, 2% peptone, 1% yeast extract); synthetic complete media lacking uracil (SC-ura; 2% glucose, 0.67% Yeast Nitrogen Base [YNB], 0.193% Drop-Out Mix -URA [Kaiser, Formedium]); or rich-glycerol media (YPGly; 2% glycerol, 2% peptone, 1% yeast extract). Solid media were prepared by adding 2% agar.

Plasmid pRS416-TEF-*TIF51A*-*URA3* (PA317) and pRS416-TEF-*TIF51B*-*URA3* (PA319) were used for overexpression of Tif51A or Tif51B. Plasmid pRS416-TEF-*URA3* (PA298) was used as empty vector for negative control. Plasmids PA317 and PA319 were constructed from the plasmid PA298 by GAP-repair cloning in *S. cerevisiae* (Joska et al. 2014). The parental plasmid was linearized by digestion with *Eco*RI (Thermo Fisher Scientific). The *TIF51A* insert (including the coding sequence and 300 bp downstream of the STOP codon) and the *TIF51B* insert (including the coding sequence and 550 bp downstream of the STOP codon) were amplified by PCR using specific primers listed in Supplementary Table S3 ("GAP-repair cloning" subsection, File S1). PCR cassettes were co-transformed with the linearized plasmid in BY4741 yeast cells and cells with GAP-repaired plasmids were selected in SC medium lacking uracile. Plasmids were recovered from yeast cells and directly transformed in *E. coli* competent cells, then plasmids were extracted from bacteria and the correct cloning was confirmed by DNA sequencing. The plasmids were transformed in the corresponding strains following the lithium acetate-based method (Gietz et al. 1992) and transformants were selected in SC medium lacking uracile.

To generate a targeted deletion of the *TIF51B (ANB1)* gene, a PCR-based gene deletion strategy was employed to replace the full-length genomic *TIF51B* ORF with the selectable marker *HIS3*. The plasmid pFA6a-His3MX6 (Longtine et al. 1998) was used as template for the PCR reaction using primers listed in Supplementary Table S3 ("Gene deletion/tagging by PCR" subsection, File S1). The resulting cassette was integrated into the genome by homologous recombination in the corresponding strains, following the lithium acetate-based method (Gietz et al. 1992). Transformants were selected in SC medium lacking histidine. All the deletions were confirmed by conventional PCR using the genomic DNA of transformants.

A PCR-based genomic tagging technique was employed to tag the genomic full length *ROX1* ORF with myc at the C-terminal region. The plasmid pFA6a-13Myc-His3MX6 (Longtine et al. 1998) was used as a template for PCR reaction using primers listed in Supplementary Table S3 ("Gene deletion/tagging by PCR" subsection, File S1). The resulting cassette was integrated into the genome by homologous recombination in the corresponding strains, following the lithium acetate-based method (Gietz et al. 1992). Transformants were selected in SC medium lacking histidine. All the integrations were confirmed by conventional PCR using the genomic DNA of transformants.

Experimental assays were performed with exponentially growing cells for at least four generations until the required OD_{600} was reached at the corresponding temperature. For experiments involving temperature-sensitive strains, the cells were grown at 25°C (the permissive temperature) until the required OD_{600} was reached. Then, the cells were transferred to 37°C (the non-permissive temperature) for 4 or 6 h to completely deplete eIF5A. To study the protein stability, the media were supplemented with 100 µg/ml cycloheximide (CHX) (Sigma-Aldrich), and samples were collected over 3 h.

Isolation and characterization of spontaneous suppressor mutants

Spontaneous suppressor mutants of the temperature-sensitive strains *tif51A-1* and *tif51A-3* in the *TIF51B* and *tif51BΔ* backgrounds were isolated by selecting for the reversion of the

thermosensitive growth phenotype at 37°C. To achieve this, at least three independent cultures of each genetic background were grown at the permissive temperature of 25°C until exponential phase (OD_{600} approximately 0.5). The cells were then plated in YPD medium and the plates were incubated at 37°C for two days. Colonies exhibiting restored growth under restrictive conditions were selected as candidate suppressors. Individual isolates were restreaked onto YPD plates and grown at 37°C to confirm the stability of the phenotype. To determine the frequency of suppressor appearance, 400 µL of the undiluted culture containing approximately 10^7 cells was plated in duplicate onto YPD plates and incubated at 37°C. Concurrently, to determine the total number of viable cells, 200 µL of a $1:10^{-4}$ dilution were plated onto YPD plates and incubated at 25°C. The frequency of suppressor appearance was calculated as the ratio between the c.f.u. (colony-forming units) obtained at 37°C respect to the c.f.u. obtained at 25°C.

Genomic DNA extraction and Sanger sequencing

For genomic DNA extraction, 4 mL of an overnight culture was harvested by centrifugation and washed with sterile water. Pellets were resuspended in 500 µL of Buffer 1 (50 mM Tris-HCl pH 7.5, 20 mM EDTA, and 1% SDS) and transferred to screw-cap tubes containing 500 µL of 0.5 mm sterile glass beads. Cells were disrupted using a Precellys 24 tissue homogenizer (Bertin Technologies), followed by incubation at 70ºC for 10 min. After brief vortexing, 200 µL of 5 M potassium acetate and 150 µL of 5 M NaCl were added. Samples were then incubated on ice for 20 min and subsequently centrifuged at 13,000 rpm for 20 min at 4ºC. Supernatants were transferred to fresh tubes, and DNA was precipitated overnight at -20ºC with 96% ethanol. The resulting DNA pellets were collected by centrifugation at 13,000 rpm for 10 min at 4ºC, washed with 70% ethanol, dried in a SpeedVac Concentrator (Thermo Fisher Scientific) for 10 min and dissolved in TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA). DNA concentration and purity were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific). The *TIF51A* ORF was amplified by conventional PCR using the primers listed in Suplemmentary Table S3 ("Gene amplification for Sanger sequencing" subsection). The resulting PCR products were purified and sequenced by Sanger sequencing (Macrogen Spain).

Whole genome sequencing (WGS) and variant calling

For WGS, genomic DNA was extracted from overnight yeast cultures grown in YPD at 25°C using a modified zymolyase–SDS protocol adapted from (Querol et al. 1992). Briefly, 1.4 mL of culture was harvested by centrifugation (12,000 rpm, 3 min) at room temperature. Cell pellets were washed and resuspended in 500 µL of Solution 1 (0.9 M sorbitol, 0.1 M EDTA). To digest the cell wall, 30 µL of Zymolyase 20T (1 mg/mL; Carl Roth) was added, followed by incubation at 37ºC for 30 min with shaking at 800 rpm. After centrifugation, the resulting pellet was resuspended in 500 µL of Solution 2 (50 mM Tris-HCl pH 7.4, 20 mM EDTA). Cell lysis was performed by adding 13 µL of 10% SDS and incubating at 65ºC for 5 min.

To remove proteins, 200 µL of 3 M potassium acetate was added, and samples were incubated on ice for 15 min. After centrifugation at 14,000 rpm for 15 min at 4ºC, 700 µL of the supernatant was transferred to a fresh tube. DNA was precipitated with 700 µL of isopropanol for 10 min at room temperature. The DNA pellet was collected by centrifugation at 12,000 rpm for 10 min at 4ºC, washed twice with 500 µL of 70% ethanol, and dried in a SpeedVac Concentrator (Thermo Fisher Scientific) without heat for 15 min. The pellet was resuspended in 25 µL of nuclease-free water at 50ºC for 30 min. Remaining RNA was eliminated by adding 0.5 µL RNase A (10 mg/mL; Thermo Fisher Scientific) and incubating at 37ºC for 30 min. DNA concentration and quality were assessed using a Qubit Flex fluorometer (Thermo Fisher Scientific).

WGS was done at the FISABIO sequencing service (València). For this, DNA libraries were prepared following the Illumina DNA prep reference guide 1000000025416-10, with the Illumina DNA Prep kit (Illumina). Input DNA was diluted to 0.5 ng/µl to start the protocol. At the multiplexing step, Illumina DNA/RNA UD Indexes (Illumina) were used. Library size selection was performed

according to the Illumina Library Prep protocol with Sample Purification Beads (SPB) included in the library Prep kit. Library size was validated by using Fragment Analyzer 48-Capillary Array with the HS NGS Fragment Kit (1-6000bp) (Agilent).

The sample containing indexed libraries was loaded onto NextSeq™ 2000 P1 XLEAP-SBS™ Reagent Kit (300 Cycles) (Illumina) and onto the instrument, along with the flow cell. Automated cluster generation and paired-end sequencing with dual indexes reads was performed (2×150bp run).

Quality control of sequencing reads was performed using FastQC (Andrews 2023), and summary reports were generated with MultiQC (Ewels et al. 2016). Low-quality leading bases were removed by trimming the first 15 bp of each read using Trim Galore (Krueger 2025). In addition, an adapted version of SppIDer (Langdon et al. 2018) was used as a quality control step to assess genome-wide sequencing coverage and to confirm that all samples had no evidence of aneuploidy.

All analyses were performed using the *S. cerevisiae* S288C reference genome (assembly R64-1-1 (2011)), downloaded from the *Saccharomyces Genome Database* (SGD) (Cherry 1998). Trimmed reads were aligned to this reference genome using BWA-MEM (Li 2013), including read group information as required for downstream processing with GATK (McKenna et al. 2010). Alignments were converted to BAM format (Danecek et al. 2021), sorted, indexed, and duplicate reads were identified and marked using GATK MarkDuplicates.

Variant calling was performed following GATK best practices for germline short variant discovery (Auwera and O'Connor 2020). Initial variants were called using GATK HaplotypeCaller assuming haploid ploidy. Variant quality metrics were evaluated using a custom R-based workflow, and default GATK-recommended hard-filtering thresholds were applied to both SNPs and INDELs. High-confidence variants from the initial call set were used as known sites for base quality score recalibration. Recalibration was performed using GATK BaseRecalibrator and ApplyBQSR, and recalibration performance was assessed using AnalyzeCovariates. After recalibration, variants were re-called using GATK HaplotypeCaller with haploid ploidy and filtered using GATK-recommended parameters.

Functional effects of variants were predicted using SnpEff (Cingolani et al. 2012). As no pre-built database was available for the selected reference genome, a custom SnpEff database was constructed using the reference genome and its corresponding GFF annotation, the commands used for launching SnpEff are contained in *launch_snpeff.sh*. Since chromosome header names in the FASTA differed from those in the GFF annotation, a custom Python script (*change_head.py*) was used to rename the FASTA headers accordingly.

Annotated VCF files were parsed using a custom Python script (*parse_final.py*) to generate a wide-format table containing per-sample mutation presence and allelic states. Downstream filtering and identification of suppressor-specific variants based on genotype transitions between wild-type, eIF5A temperature-sensitive mutants, and suppressor strains were performed using custom R scripts (*parse_mutation.R*).

All custom scripts used for bioinformatic analyses are available at https://github.com/Alejandro-121/Scripts-asociated-to-Prieto-D-ez-et-al-2026.

CRISPR/Cas9 mediated genome editing

The CRISPR-Cas9 tool was used to generate point mutations in the *ROX1* gene and an insertion in the *MOT3* gene as previously described in (Shaw and Ellis 2018). Plasmid pWS158-Cas9-Ura3 (Addgene plasmid #90517) was a gift from Dr Tom Ellis (Imperial College, London, UK). The sgRNA target sequences were designed using the CHOPCHOP web tool (https://chopchop.cbu.uib.no). To construct the plasmids, pairs of complementary oligonucleotides containing the 20-bp protospacer and the appropriate overhangs were first

phosphorylated by incubating them at 37°C for 1 h with T4 Polynucleotide Kinase (New England BioLabs). Then, the oligonucleotides were annealed by heating them at 96ºC for 6 min and then gradual cooling to room temperature. The annealed sgRNA fragments were then cloned into the pWS158 backbone using a small-fragment Golden Gate assembly reaction with BsmBI-v2 (New England BioLabs) and T4 DNA ligase (Thermo Fisher Scientific). The assembly was performed in a thermocycler with 10 cycles of 42°C for 2 min and 16°C for 5 min, followed by a final digestion step at 60°C for 10 min and 80ºC for 10 min to linearize any remaining backbone. Repair templates (donor DNA) providing the desired mutations were synthesized via overlap extension PCR using Phusion DNA polymerase (Thermo Fisher Scientific) and the corresponding primers listed in Supplementary Table S3 ("Genome editing by CRISPR-Cas9" subsection, File S1). This strategy was used to introduce point mutations in *ROX1* [G158T (SNP1), G192T (SNP3), and T218C (SNP2)] and a 17-bp insertion (nucleotides 697–714) in *MOT3*, that causes a frameshift mutation. The corresponding strains were transformed using the lithium acetate-based method (Shaw and Ellis 2018), with approximately 100 ng of the assembled pWS158-sgRNA plasmid and >2 µg of the donor DNA repair template. Transformants were selected in SC-Ura. Subsequently, the loss of thermosensitivity was confirmed, as were the introduced mutations, by Sanger sequencing (Macrogen Spain).

Phenotypic assays

For the spot assays, cells were grown in YPD medium at 25ºC and harvested during the mid-exponential growth phase. 5-fold serial dilutions were prepared, starting at an OD_{600} of 0.3 and 5 µL of each dilution was spotted onto YPD, YPGly, or SC-Ura plates. Plates were then incubated at 25ºC, 30ºC, and 37ºC for 2 – 3 days.

For growth curve analysis in microtiter plates, cells were pre-cultured in deep 96-well plates with 500 µL of YPD, YPGly or SC-Ura media at 25ºC until saturation. After pre-culture, the OD_{600} was measured and adjusted to 1.0, and 20 µl of cell suspension were inoculated into 96-well plates (Thermo Fisher Scientific) at a final volume of 200 µL per well in YPD, YPGly or SC-Ura media (initial OD600 of 0.1). To monitor growth under different conditions, the plates were incubated in an SPECTROstar Nano (BMG LABTECH) plate reader at 25ºC, 30ºC or 37ºC. Absorbance at 600 nm was recorded every 30 min for 24 – 48 h under static conditions and the experiments were concluded once the strains reached stationary phase. Growth rates were estimated from the exponential growth phase by linear regression of the natural logarithm of OD_{600} values (ln OD_{600}) versus time. The exponential phase was identified automatically for each biological replicate using a custom R script (*specific_growth_rate.R*), which evaluated consecutive time intervals including a minimum of five data points, considering only OD_{600} values between 0.15 and 1.0 and requiring a minimum 1.5-fold increase in OD_{600} across the interval. Among the intervals fulfilling these criteria, the interval showing the best linear fit (highest adjusted R^2) and a positive growth rate was selected. The specific growth rate was calculated as the slope of the regression line. At least three biological replicates were analyzed for each strain.

All custom scripts used for bioinformatic analyses are available at https://github.com/Alejandro-121/Scripts-asociated-to-Prieto-D-ez-et-al-2026.

Western blotting

For protein analysis by western blotting, we followed the protocol described by (Zuzuarregui et al. 2015). Briefly, a cell culture volume corresponding to 10 OD_{600} units was harvested by centrifugation. For protein extraction, cell pellets were washed and resuspended in 200 µL of 0.2 M NaOH and incubated at room temperature for 5 min, and subsequently centrifuged at 12,000 rpm for 1 min. Samples were then resuspended in 100 µL of 2X SDS-PAGE loading buffer (24 mM Tris-HCl pH 6.8, 10% glycerol, 0.8% SDS, 5.76 mM β-mercaptoethanol, 0.04% bromophenol blue) and boiled at 95°C for 5 min. The resulting lysates were centrifuged at 3,000 rpm for 10 min at 4°C to remove cell debris and insoluble proteins, supernatants were then transferred into new

tubes and stored at -20°C. Total protein content in the extract was estimated by A_{280} using a Nanodrop spectrophotometer (Thermo Fisher Scientific) to ensure equal protein loading into the SDS-PAGE gels. The acrylamide percentage was adjusted according to the molecular weight of the protein of interest.

SDS-PAGE and immunoblotting were performed according to standard procedures (BioRad). Membranes were blocked with 5% skimmed milk in TBS-T (150 mM NaCl, 20 mM Tris-HCl pH 7.6, 0.1% Tween20) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies against eIF5A, hyp-eIF5A, or glyceraldehyde-6-phosphate dehydrogenase (G6pdh). Detailed information of the antibodies used in this study can be found in Supplementary Table S4. Bound antibodies were detected using the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescent signals were detected with an ECL Prime Western blotting detection kit (GE Healthcare) and quantified using Fiji (ImageJ) software (Schindelin et al. 2012). For quantitative analysis, the signal in each lane was first normalized to the mean signal across the entire blot to account for variation, followed by normalization against the corresponding G6PDH loading control. At least three biological replicates of each sample were analyzed for quantification purposes; however, cycloheximide-based assays were performed as a single replicate for qualitative assessment.

RT-qPCR analysis

To analyze the mRNA levels, total RNA was isolated from yeast cells following the acid phenol-chloroform method described in (Barba-Aliaga and Alepuz 2022). Briefly, a volume of an exponential phase culture corresponding to 10 OD_{600} units was harvested and flash frozen in liquid nitrogen. Cells were then resuspended in 500 µL of cold LETS buffer (0.1 M LiCl,10 mM EDTA pH 8.0, 10 mM Tris-HCl pH 7.4, 0.2% SDS) and transferred into a screw-cap tube already containing 500 µL of sterile glass beads and 500 µL of acid-phenol:chloroform (5:1, pH 4.5). Cells were disrupted using a Precellys 24 tissue homogenizer (Bertin Technologies) and centrifuged. The aqueous phase was transferred to a new tube for an additional extraction with 500 µL of acid-phenol:chloroform (5:1, pH 4.5), followed by a final extraction with 500 µL of chloroform:isoamyl alcohol (24:1). RNA from the upper aqueous phase was precipitated and finally dissolved in nuclease-free water. The concentration and purity of the RNA were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific).

Reverse transcription and quantitative PCR (qPCR) were performed as described by (Garre et al. 2013), with minor modifications. Briefly, 2.5 µg of the total RNA, previously treated with DNAse I (Roche), was reverse transcribed using oligo$(dT)_{18}$ primers and Maxima Reverse Transcriptase (Thermo Fisher Scientific). qPCR was performed using NZYSPeedy qPCR Green Master Mix (NZYtech), and quantification cycle (Cq) values were obtained using a CFX96 Touch™ Real-Time PCR Detection System (BioRad). Relative mRNA levels were normalized to the endogenous *ACT1* control. At least three biological replicates of each sample were analyzed. The specific primers used for gene amplification are listed in Supplementary Table S3 (“Gene expression detection by RT-qPCR and ChIP-qPCR” subsection, File S1).

Chromatin immunoprecipitation (ChIP)

For Rpb1 (RNA polymerase II catalytic subunit) and Rox1-13myc chromatin binding experiments, yeast cells were cultured in YPD at 25ºC until mid-exponential phase. For cross-linking, formaldehyde was added to 100 mL of culture to a final concentration of 1% and incubated for 15 min at 25°C. The reaction was quenched with 125 mM glycine for 5 min at room temperature with occasional inversion.

The chromatin immunoprecipitation (ChIP) experiments were performed as previously described in (Li et al. 2016) with minor modifications. Briefly, cells were harvested, washed, and resuspended in 300 µL of lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM EDTA, 1%

Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 1 mM benzamidine, and cOmplete Mini protease inhibitor cocktail [Roche]). Cells were lysed using glass beads and vortexed for 30 min at 4 °C. The volume was adjusted to 600 µL with lysis buffer, and chromatin was fragmented by sonication for 15 min (30 s ON/30 s OFF). After centrifugation at 12,000 rpm for 15 min, a 20 µL aliquot of the supernatant was collected as the whole-cell extract (INPUT). The remaining supernatant was incubated with orbital rotation for 2 h at 4 °C with Dynabeads Pan Mouse IgG (Invitrogen) previously conjugated to the corresponding antibody.

Beads were washed four times with PBS (150 mM NaCl, 40 mM Na_2HPO_4, 10 mM NaH_2PO_4) containing 0.02% Tween 20. Bound complexes were eluted twice with 40 µL of elution buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% SDS) by heating at 65°C for 8 min. Cross-linking was reversed by overnight incubation at 65°C with shaking. Samples were then treated with Proteinase K (Roche) for 90 min at 37 °C, and DNA was purified using the NZY Gelpure kit (NZYtech) following the manufacturer's instructions. To determine protein enrichment at specific DNA regions, qPCR was performed as described above using the primers listed in Supplementary Table S3 ("Gene expression detection by RT-qPCR and ChIP-qPCR" subsection). Data were normalized to the total INPUT DNA value from the corresponding whole-cell extract. Detailed information of the antibodies and Dynabeads used can be found in Supplementary Table S4. At least three biological replicates of each sample were analyzed.

Protein sequence alignments, structural modeling and protein stability prediction

Amino acid sequences for *S. cerevisiae* Tif51A (eIF5A1), Tif51B (eIF5A2), and their respective orthologs were retrieved from the Saccharomyces Genome Database (SGD) and UniProt. Multiple sequence alignments were performed using the Clustal Omega algorithm via the UniProt alignment service. The resulting alignments were imported into Jalview v2.11.5.1 (Waterhouse et al. 2009) for visualization, conservation analysis, and figure generation. To evaluate protein conservation, pairwise sequence alignments were performed with EMBOSS Needle (PSA) (https://www.ebi.ac.uk/jdispatcher/psa/emboss_needle; (Madeira et al. 2024)), and the percentage of sequence identity of eIF5A1 was calculated.

To assess the structural impact of the identified mutations, three-dimensional (3D) models of wild-type and mutant variants of Tif51A and Rox1 were predicted using AlphaFold2 via the ColabFold pipeline v1.5.5 (Mirdita et al. 2022). For each protein, the best-ranked model based on the predicted local distance difference test (pLDDT) score was selected for further analysis. Molecular graphics and analyses to compare conformational changes between the wild-type and mutated variants were performed with UCSF Chimera v1.18, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311 (Pettersen et al. 2004).

The effect of single-site mutations on protein stability was predicted using the I-Mutant2.0 server (https://folding.biofold.org/i-mutant/i-mutant2.0.html; (Capriotti et al. 2005)), based on the protein sequence. Stability changes were evaluated by calculating the free energy change ΔΔG at 25°C and pH 7.0.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software). Differences between two groups were analyzed using a two-tailed unpaired Student's t-test, whereas comparisons among multiple groups were performed using one-way analysis of variance (one-way ANOVA) followed by Bonferroni's multiple comparisons test. A minimum of three biological replicates was analyzed for each experiment.

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