Altered proteome in translation initiation fidelity defective eIF5G31R mutant causes oxidative stress and DNA damage

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The recognition of the AUG start codon and selection of an open reading frame (ORF) is fundamental to protein biosynthesis. Defect in the fidelity of start codon selection adversely affect proteome and have a pleiotropic effect on cellular function. Using proteomic techniques, we identified differential protein abundance in the translation initiation fidelity defective eIF5G31R mutant that initiates translation using UUG codon in addition to the AUG start codon. Consistently, the eIF5G31R mutant altered proteome involved in protein catabolism, nucleotide biosynthesis, lipid biosynthesis, carbohydrate metabolism, oxidation-reduction pathway, autophagy and re-programs the cellular pathways. The utilization of the upstream UUG codons by the eIF5G31R mutation caused downregulation of uridylate kinase expression, sensitivity to hydroxyurea, and DNA damage. The eIF5G31R mutant cells showed lower glutathione levels, high ROS activity, and sensitivity to H2O2.

The fidelity of start codon selection is critical for the synthesis of a normal proteome. Defect in the genes involved in the general translation initiation that alters the fidelity of start codon selection could have a pleiotropic effect on cellular function as they change the cellular proteome. However, no studies have been conducted to understand the change in cellular proteome and its effects on cellular physiology caused by the defect in eukaryotic translation initiation factor fidelity. In eukaryotic translation initiation, the AUG start codon is predominantly selected to establish an open reading frame (ORF) to decode the genetic code while scanning the mRNA from 5′ to 3′ direction by the translation initiation machinery. The Saccharomyces cerevisiae translation initiation factors critical for the start codon selection consist of eIF2-GTP-Met-tRNAi Ternary complex (TC), eIF1, eIF1A, eIF5, eIF3, and 40S ribosomal subunit together constitute 43S pre-initiation complex (PIC) that is involved in the selection of AUG start codon on the mRNA1,2. The mRNA binds to the eIF4F complex through its 5′ m7Gppp cap and it is recruited laterally to the PIC by placing the mRNA into the mRNA entry channel results in the formation of 48S initiation complex3,4. The factor eIF1A binds to the A-site of the ribosome and promotes mRNA scanning by its C-terminal tail (CTT)5. The factor eIF1 binds near P-site to monitors anti-codon:codon interaction and prevents non-AUG codon selection1,2. During the scanning for AUG start codon, the N-terminal GTase activating protein (GAP) domain of eIF5 interacts with the G-domain of eIF2y subunit to get GTP hydrolysis to form GDP and P. Once the AUG start codon enters the P-site of the 40S ribosome, the anti-codon:codon interaction causes major rearrangement in the scanning complex. The eIF1A-CTT interacts with the N-terminal domain of eIF5, whereas the eIF1 moves out of the P-site of the ribosome, causing the Met-tRNAi to move from POUT to PIN conformation resulting in the selection of AUG codon and release of Pi from the eIF2-GDP-Pi complex3,6,7.

In yeast, the mitochondrial version of glycyl-tRNA synthetase and alanine-tRNA synthetase is translated using UUG and ACG start codons, respectively8,9. However, the molecular mechanisms involved in the non-AUG codon selection in these cases are unknown. The mRNA features such as cis-acting long structured 5′ untranslated region (UTR) or the short unstructured 5′ UTR and the trans-acting mRNA recruitment and assisting factors such as eIF4B, eIF4G, PABP, or Ded1p plays an important role in the effective translation of a selective mRNAs10-14. However, defects in the assembly of the 48S initiation complex could adversely affect start codon selection and translation efficacy of global mRNA during the scanning process that can alter general proteome. Mutations isolated in some of the initiation factors such as eIF5G31R, eIF2βS264Y and eIF2γN135D cause...
relax stringency in the recognition of AUG start codon (Suppressor of initiation codon:  Sui− phenotype) proposed to be caused due to the premature P release, intrinsic GTPase reaction and altered tRNAi binding defect, respectively15–17. The  Sui− mutants can utilize the in-frame or the out-of-frame UUG, CUG, or GUG codons as a translation start site at the 5′ UTR of the mRNA16. The dominant-negative  eIF5G31R hyper GAP mutant is the strongest  Sui− mutation identified that preferentially recognizes UUG as a start codon and shows severe slow growth, whereas in the recessive conditions the  eIF5G31R mutation is lethal18. This preferential recognition of UUG start from the 5′ regulatory region of the  GCN4 transcript causes repression of  GCN4 expression (general control non-derepressed:  Gcn− phenotype)19. We hypothesized that the aberrant translation initiation at the upstream UUG (upUUG) codon by the  Sui− mutant might change the cellular proteome that adversely affects cellular physiology and severely cause growth defects. To gain insights in the underpinning cause of growth defect by the translation initiation fidelity defective  eIF5G31R mutation, we used gel-based proteomics approach 2D-PAGE followed by MALDI-TOF/MS and gel-free labeling approach iTRAQ (isobaric tag relative absolute quantification) along with nLC-MALDI-TOF/MS technique to quantitate differential protein abundance. Our results suggest a change in the proteome of the  eIF5G31R mutant that has major implications in DNA damage and oxidative stress.

Materials and methods

Yeast strains used in this study.

The Saccharomyces cerevisiae yeast strains used in this study were: YP823;  Mat α, ura3-52, leu2-3,112, trp1Δ63, GCN2 + 19. YP824;  Mat α, ura3-52, leu2-3,112, trp1Δ63, GCN2 +, his4Δ::KanMx6 20. Ctt1-loxP-Ura3-loxP-Ctt1 cassette was PCR amplified using oligonucleotides oPA1534 and oPA1535 and B4033 vector as a template. This PCR product was transformed into yeast strain YP823 to delete chromosomal  CTT1 gene to give rise to yeast strain YP939;  MATα leu2-3, -112, ura3-52, trpΔ63, GCN2 +, Gal2 +, ctt1∆::Ura3. The  ctt1∆ in the YP939 strain was confirmed by PCR using oligonucleotides oPA1569 and oPA584.

Plasmids used in this study are listed in Table 1 and oligonucleotides are listed in the Supplementary Table S1.

Cloning. The 2.2 kb  eIF5G31R encoding gene was digested from vector A70350 using EcoRI-SalI restriction endonuclease (RE) and cloned into pYCplac22 to generate pYCplac22-eIF5G31R (A838) vector. The pYcplac33-Ura6-lacZ reporter construct (A1074) was generated as follows—first, the 534 bp of  URA6 promoter and the N-terminal 12 amino acids region was PCR amplified using oligonucleotides oPA1534 and oPA1535 and B4033 vector as a template. This PCR product was transformed into yeast strain YP823 to delete chromosomal  CTT1 gene to give rise to yeast strain YP939;  MATα leu2-3, -112, ura3-52, trpΔ63, GCN2 +, Gal2 +, ctt1∆::Ura3. The  ctt1∆ in the YP939 strain was confirmed by PCR using oligonucleotides oPA1569 and oPA584.

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| Sr. no. | Plasmid number | Plasmid name | Type | References |
|---------|----------------|--------------|------|------------|
| 1       | A823           | pYCplac22    | Single copy (s.c) | 21 |
| 2       | A838           | pYCplac22-eIF5G31R | s.c | This study |
| 3       | B4033          | pUG72        | s.c | 22 |
| 4       | A308           | pYCplac111   | s.c | 21 |
| 5       | A309           | pYCplac33    | s.c | 21 |
| 6       | B1378          | pRS425       | High copy (h.c) | 22 |
| 7       | A1074          | pYCplac33-Ura6-lacZ | s.c | This study |
| 8       | A1140          | pYCplac33-upUUGless Ura6-LacZ | s.c | This study |
| 9       | A1141          | pYCplac33-Ura6-3xHA | s.c | This study |
| 10      | A1142          | pYCplac33-upUUGlessUra6-3xHA | s.c | This study |
| 11      | A1144          | pYCplac33-CCT1-3xHA | s.c | This study |
| 12      | A1147          | pRS425-Ura6   | h.c | This study |
| 13      | A1149          | pRS425-CCT1   | h.c | This study |
| 14      | A1170          | pRS425SOD1-SOD2 | h.c | This study |
| 15      | A1311          | pYCplac111-YAP1_LacZ | s.c | This study |
| 16      | A1312          | pYCplac111-YAP1-upTTGless_LacZ   | s.c | This study |
| 17      | A1340          | pYCplac33-upUUG1in-frame_lacZ | s.c | This study |
| 18      | A1341          | pYCplac33-upUUG1in-frame_lacZ | s.c | This study |
| 19      | A1342          | pYCplac33-upUUG3in-frame_lacZ | s.c | This study |
| 20      | A1343          | pYCplac33-upUUG3in-frame_lacZ | s.c | This study |
| 21      | A1346          | pRS425-YAP1   | h.c | This study |

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oPA1011 followed by a second round of PCR amplification using oPA1005/oPA1467 and cloned into HindIII-Sall site of vector A1074 to generate pYcplac33-upUUG3in-frame_lacZ (A1340) vector. The vector A1340 was modified to insert AUG codon at −95 position and cloned at HindIII-Sall site of vector A1074 to generate pYcplac33-upUG3in-frame_lacZ (A1341) vector. The upUUG codon at the −22 position was put in-frame with the URA6-lacZ reporter by fusion PCR using oligonucleotides oPA1005, oPA1012, oPA1013, and oPA1470 and cloned at HindIII-Sall site of vector A1074 to generate pYcplac33-upUG3in-frame_lacZ (A1342) vector. The vector A1342 was modified to insert AUG codon at −25 position by fusion PCR using oligonucleotides oPA1005 and oPA1471 using A1342 as a template and cloned at HindIII-Sall to generate pYcplac33-upUG3in-frame_lacZ (A1343) vector. A 1636 bp of URA6 gene containing promoter and terminator region from genomic DNA was PCR amplified using oligonucleotide oPA1005/oPA1089 and cloned in pRS425 (B1378) vector at HindIII-Sall site to generate pRS425-Ura6 (A1147) vector.

C-terminal 3xHA tag was introduced into the CTT1 gene as follows—first, the 520 bp CTT1 3’UTR region was PCR amplified using oligonucleotides oPA1036/oPA1037 and cloned at BamHI-KpnI sites of pYcplac33 (A309) vector to generate intermediate vector. Using oligonucleotide oPA1034/oPA1040, a C-terminal 3xHA tag was introduced into the promote-ORF region of the CTT1 gene by PCR amplification and cloned into the intermediate vector at the BamHI-HindIII site to generate pYcplac33-CTT1-3xHA (A1144) vector. The CTT1 overexpression construct pRS425-CTT1 (A1149) was generated by amplifying the 2716 bp CTT1 genomic DNA region using oligonucleotide oPA1108/oPA1109 and cloned into high copy pRS425 vector (B1378) at NotI-BamHI site.

The 1478 bp SOD1 genomic DNA region was PCR amplified using oligonucleotides oPA1173/oPA1174 and the 1722 bp SOD2 genomic DNA region was PCR amplified using oligonucleotides oPA1175/oPA1176, digested with NotI-BamHI-HindIII restriction endonuclease and cloned at the NotI-HindIII site of the high copy pRS425 vector to generate pRS425SOD1-SOD2 (A1170) vector.

The pYcplac111-YAP1_LacZ reporter construct (A1311) was generated as follows—first, the 544 bp YAP1 promoter and the N-terminal 8 amino acids region were PCR amplified using oligonucleotide combination oPA1607/oPA1609 and cloned into vector pYcplac111 (A308) vector at SpeI-BamHI site. The LacZ region and YAP1 3’UTR region were PCR amplified using oligonucleotides combination oPA1559/oPA1560 and oPA1608/oPA1610 respectively and cloned at BamHI-Sall and Sall-HindIII sites. The six upstream UUG codons from the YAP1 5’UTR were removed using multiple PCR as follows—First, using A1311 vector template and oligonucleotides combination oPA1607/oPA1640, a PCR product was generated that removed upUUG codons from −63, −45, and −14 position. This PCR product was used as a template to amplify and remove upUUG codon from the −96 and −91 position using oligonucleotides combination oPA1609, oPA1638, oPA1639, and oPA1607. Using the last PCR product as a template, the upUUG codon at the −128 position was removed by PCR amplification using oligonucleotides combination oPA1609, oPA1636, oPA1607, and oPA1637. This PCR product was cloned at the SpeI-BamHI site of the A1311 vector to generate pYcplac111-YAP1-upTTGless_LacZ (A1312) vector. The YAP1 overexpression construct pRS425-YAP1 (A1346) was generated by amplifying 2792 bp YAP1 genomic DNA region using oligonucleotide oPA1607/oPA1608 and cloned into pRS425 vector at Spel-HindIII site.

2D-PAGE followed by MALDI-TOP/MS proteomics approach. Three biological replicates of wild type (WT) and eIF5G31R mutant cells were grown till OD600~1.0. The cells were suspended in 2D lysis buffer (7 M Urea, 2 M Thiourea, 65 mM DTT, 2% CHAPS, and a Complete Protease Inhibitor table) and mechanically lysed by vortexing using acid-washed glass beads (200 µm) at 4 °C. The supernatant containing total cell extract was separated at 13,000×g for 30 min at 4 °C and the total proteins were quantitated by Bradford assay. The total proteins (400 µg) were resolved on 18 cm IPG strip pH 3–11 using Ettan IPGphor 3 isoelectric focusing system (GE) under the following conditions: 12 h (h) at 50 V; 30 min at 250 V; 1 h at 1000 V; 1 h at 2500 V; 1 h at 5000 V; 1 h at 8000 V and held at 8000 V until total Vh reached 50,000 Vh. After IEF, the IPG strips were equilibrated for 30 min in a reduction buffer containing 1% DTT in an equilibration buffer (75 mM Tris—Cl pH 8.8, 6 M urea, 30% v/v glycerol, 2% SDS, 0.002% bromophenol blue), and subsequently alkylated for 30 min in an alklylation buffer containing 2.5% Ioda-acetamide in equilibration buffer buffer. After equilibration, the strips were placed on 12% polyacrylamide gels and immobilized by overlaying 0.5% agarose on the strips. The second-dimensional separation was carried out in the Bio-Rad Protein II xi Cell and electrophoresed at 80 V for 24 h. The gel was stained with Coomassie brilliant blue (CBB), scanned with GS-800 Calibrated Densitometer (Bio-Rad Laboratories), and analyzed using GE Image Master 2D Platinum v7.0 software. The altered spots were excised, followed by in-gel digestion using trypsin (1 µg/µl trypsin in 1:10 enzyme to protein ratio; AB Sciex #4352157) at 37 °C for 16 h. iTRAQ 4-plex reagents (AB Sciex iTRAQ 4-plex kit #4352135) were used to label the resultant peptide digest according to the manufacturer’s instructions. The different isobaric tags labeled peptides were pooled together and subjected to the strong cation exchange chromatogram using the ICAT column (AB Sciex ICAT).
NanoLC-MALDI TOF/MS. All the fractions were reconstituted in 0.1% triluoracetic acid and chromatogram on reverse phase C18 column (Eksigent, 3 µm, 120 Å pores, 0.075 x 150 mm) using Eksport nanoLC Ultra 2D plus system with 5–90% acetonitrile gradient (2 µl/min flow in 68 min). Eluant from each fraction was deposited with α-CHCA matrix at 15 s/spot and spotted onto the MALDI plate with the help of Eksport MALDI spotter (AB Sciex). The spotted LC-MALDI plates were subjected MALDI TOF/TOF (AB Sciex). Reelectro mode mass calibrations were performed externally with a calibration mixture (Cal mix TOF/TOF, AB Sciex #4333604), whereas MS/MS mode mass calibration was executed using the fragments of the Glu-fibrinopeptide B precursor ion. MS and MS/MS data were acquired for all the fractions using defined LC-MALDI parameters. Abundant peptides with a precursor mass of 3568 Da with a mass window ±100 Da were sent for MS/MS with a 0.8 ppm error. Data Dependent Acquisition (DDA) was activated with automatic Collision Induced Dissociation (CID) and automatic MS/MS acquisition. The MS and MS/MS data of the peptides were used for protein identification and peak areas of the iTRAQ reporter ions suggest the relative abundance of the proteins in the respective samples. The schematics of the iTRAQ and nanoLC-MALDI-TOF/MS approach are outlined in Supplementary Fig. S1a.

Data processing and analysis. Quantification and identification of protein were performed on ProteinPilot 4.0.8085 Software (AB Sciex) integrated with Mascot search engine version:2.3. The Paragon algorithm in the ProteinPilot software was used for the peptide identification. User-defined search parameters were as follows: (i) Sample type, iTRAQ 4-plex (Peptide Labeled); (ii) Cysteine alkylation, MMTs; (iii) Digestion, Tryptsin; (iv) Instrument, MALDI TOF/TOF AB Sciex 5800, (v) Mass tolerance 100 ppm for precursor ion and 0.8 ppm for daughter ions, (v) Database, S. cerevisiae database from Uniprot, SC strain S288C-20160606.fasta and the corresponding reverse sequence (decoy: for false discovery rate (FDR = 2.0 × decoy hit/total hit) estimation), (vi) Keratin excluded. The peptides were automatically selected by the Pro Group algorithm to calculate the reporter peak area, error factor (EF), and P-value for quantification. Post quantification dataset was auto bias-corrected to eliminate any variation due to unequal mixing while pooling different labeled peptides. The False Discovery Rate (FDR) was estimated using the Proteomics System Performance Evaluation Pipeline (PSPEP) algorithm integrated into the ProteinPilot, and the Global FDR of 5% was used for further analysis. Quantified proteins from both sets were first normalized against the pooled internal control used in both experimental sets. Post normalization, the dataset was filtered based on unused score cut-off 1.3 or more for further analysis from both sets with at least one unique peptide identified. Proteins present in both sets were further tested for statistical significance by performing Student’s t-test, P-value ≤ 0.05. A cut-off ≥1.5-fold and ≤0.66-fold were used for differential protein abundance estimation for up-regulation and down-regulation, respectively. The summary of iTRAQ data analysis is outlined in Supplementary Fig. S1b. In addition, the mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE24 partner repository with the data set identifier PXD029460.

Western blotting. Yeast cells were grown till OD600 ~ 0.8. The cell pellet was resuspended in PBS and mechanically lysed as described above. The supernatant containing total cell extract was separated by centrifugation at 13,000xg for 20 min at 4 °C. Total proteins were quantified by Bradford assay. Total proteins were separated on SDS-PAGE followed by electro-blotting to a PVDF membrane. The blots were probed using an anti-HA antibody (Sigma #H9658, 1:10,000 dilution) and horseradish peroxidase (HRP) conjugated mouse secondary antibody (Sigma, #A9044). The blot was developed using SuperSignal (Thermo Scientific #34075) and quantitated using EvolutionCapt Solo S 17.00 software.

Real-time PCR. Yeast cells were grown till OD600 ~ 0.8 in 30 ml culture. The cell pellet was resuspended in 400 µl TRIzol (Ambion #15596026) and mixed with a 1:1 chloroform-isooamyl alcohol mixture. The supernatant containing total RNA was separated by centrifugation at 13,000xg for 2 min at 4 °C. The total RNA was precipitated by isopropanol and after 75% ethanol wash, the pellet was suspended in distilled water and treated with DNase-I at 37 °C for 30 min. The reaction was terminated by heat inactivation at 70 °C for 10 min. The total RNA (0.5 µg) was subjected to cDNA synthesis using SuperScript III enzyme (Invitrogen #18080044) and oligonucleotide oPA1024, oPA1102, oPA1114, and oPA1642 for ACT1, URA6, CTT1, and YAP1 genes, respectively, and the reaction was carried at 50 °C for 60 min. The reaction was stopped by heat inactivation at 70 °C for 15 min. The cDNA was diluted fivefold and a 3 µl of cDNA was subjected to real-time quantitative PCR using Power SYBR Green PCR Master Mix (Applied Biosystem #1601516) and BioRAD CFX384 Touch Real-Time PCR Detection System. The oligonucleotides combination oPA1023/oPA650, oPA1101/oPA1102, oPA1113/oPA1114, and oPA1641/oPA1642 were used for PCR amplification of ACT1, URA6, CTT1, and YAP1 cDNA, respectively.

ROS assay. Yeast cells were grown till OD600 ~ 0.8, cells were washed with PBS, followed by incubation in PBS containing 100 µM H2DCFDA dye (Sigma # D6883) and 5 µM MitoSOX Red (Invitrogen #M36008) for 30 min in the dark at 37 °C followed by washing with PBS. Slides were mounted and observed under Leica SP8 confocal microscope using a 63x objective lens with the FITC (Em λ328) filter to measure H2DCFDA fluorescence and TRITC (Em λ580) filters to measure MitoSOX Red fluorescence. The images were quantitated using NIH ImageJ software (1.52p).
β-Galactosidase reporter assay. Yeast cells were transformed with appropriate reporter plasmids. Five colonies from each transformant were grown overnight at 30 °C with shaking at 220 rpm in synthetic dextrose (SD) media with appropriate nutrient supplements and grown till OD_{600} ~ 0.8. Cells were harvested by centrifugation at 13,000×g for 20 min at 4 °C and resuspended in LaCZ buffer (60 mM Na_{2}HPO_{4}, 40 mM NaH_{2}PO_{4}, 10 mM KCl, and 1 mM MgSO_{4}, pH 7.0) and cell lysis was performed as described above. Clarified cell extract (~30 μg) was mixed with LaCZ buffer, followed by addition of 180 μl of ONPG (4 mg/ml in LaCZ buffer). After 30 min of incubation, absorbance was measured at 420 nm (Molecular Devices Spectra Max). Protein estimation was done using Bradford assay and the β-galactosidase activity (nmol of O-nitrophenol-β-d-galactopyranoside cleaved per min per mg) was calculated after normalization with total protein.

Estimation of cellular glutathione. Yeast cells were grown till OD_{600} ~ 0.8. The cells were washed with PBS and treated with 30 μM monochlorobimane (mCBM) dye for 30 min at 30 °C. Fluorescence adduct formation was imaged at Ex λ_{350} and Em λ_{460} using a Leica SP8 confocal microscope at ×63 magnification. The images were quantitated using NIH ImageJ software (1.52p).

Tunel assay. Tunel assay was performed using DeadEnd Fluorometric Tunel System (Promega #G3250). Yeast cells OD_{600} ~ 0.8 (100 μl) was spun down at 4000×g for 2 min, followed by fixation in 3.7% formaldehyde for 30 min at room temperature. Cells were washed with PBS and treated with 10 U of lyticase in PBS at 37 °C for 60 min. The cells were washed with PBS and permeabilized using 0.1% triton-X-100 in 0.1% sodium citrate for 5 min on ice. These cells were equilibrated with rTdT and nucleotide mix in 45 μl of equilibration buffer followed by incubation at 37 °C for 1 h. The cells were washed three times with PBS, followed by DAPI staining for 15 min in the dark. The cells were washed with PBS and imaged using Leica SP8 confocal microscope using DIC, DAPI (Em λ_{415}) filter and FITC (Em λ_{520}) filter. The images were quantitated using NIH ImageJ software (1.52p).

Results

Translation fidelity defective eIF5G31R Sui− mutants alter cellular proteome. Historically suppressor of initiation codon (Sui′) mutations were identified by performing genetic screening to check the growth of Saccharomyces cerevisiae his4-303 mutant (AUG start codon of HIS4 gene was mutated to AUU codon) on histidine minus auxotrophic media. The Sui′ mutant could utilize the third in-frame UUG codon as an initiation codon and grow on histidine minus medium [5,16]. Our bioinformatic analysis suggests ~44.6% of Saccharomyces cerevisiae CDS contain at least one UUG codon in the 5′ untranslated region (UTR) within 50 bp of 1 AUG start codon (Supplementary Sheet 1). If Sui′ mutants can utilize these upstream UUG codons (upUUG) as translation initiation codon while scanning the mRNA from 5′ to 3′ directions, then that would lead to the formation of either short upORF (ORF with UUG start codon), upORF overlapped out-of-frame with the main ORF or upORF in-frame with main ORF (N-terminal extension) (Fig. 1a). Translation from these out-of-frame upORFs may preclude the translation of main ORF, resulting in the altered proteome in Sui′ mutant cells. The eIF5G31R mutation is one of the strongest Sui′ mutants that preferentially recognize UUG as a start codon, unlike other Sui′ mutants that can also utilize CUG or GUG as a start codon [16,18]. The eIF5G31R mutation in the recombinant region of URA6 is repressed by 0.55-fold less abundant (P-value, 0.0069) uridylate kinase protein (Fig. 1d). To validate this analysis showed ~0.55-fold less abundant (P-value, 0.0069) uridylate kinase protein (Fig. 1d). To validate this data, we modified the URA6 gene by adding a C-terminal 3xHA tag and checked the plasmid-borne expression of this modified gene in the WT and eIF5G31R mutant yeast strain by Western blot using Anti-HA tag antibody. Consistent with the iTRAQ data analysis, the Western blot showed ~0.23-fold less abundance of uridylate kinase.
protein (Fig. 2a, left). To check if the lower uridylate kinase protein levels were not due to the lower transcription of its mRNA, we performed RT-qPCR using URA6 ORF specific oligonucleotides. To our surprise, we observed ~3.5-fold higher URA6 mRNA expression levels in the eIF5G31R mutant, suggesting that the lower levels of uridylate kinase protein indicate translation defect (Fig. 2a, right). It may be possible that the presence of the four upUUG codons at the ~160 nucleotides (nt) long 5′UTR has generated an out-of-frame upORFs that prematurely terminate translation before reaching the URA6 main ORF in the eIF5G31R mutant. To test this, we mutated all four upUUG codons (at −95, −92, −22, and −13 positions) and checked the uridylate kinase protein levels by Western blot. The removal of all upUUG codons caused an overall reduction in uridylate kinase protein levels in the WT cells. However, the relative expression of uridylate kinase protein increased (ratio 0.66)
in the eIF5G31R mutant after the removal of upUUG codons from the mRNA as compared to the WT (ratio 0.23) mRNA (Fig. 2a). These results suggest that the eIF5G31R mutant utilizes upUUG codons to translate the out-of-frame upORFs and the ribosomes dissociate before reaching the main ORF causing lower uridylate kinase protein expression.

The utilization of upUUG codons at the 5′ UTR region by the eIF5G31R mutant creates two upORFs. The upUUG1 (−95) and upUUG2 (−92) codons are in the same reading frame and constitute upORF1 that terminates before the main ORF of URA6 gene. The upUUG3 (−22) and upUUG4 (−13) codons are in the same reading frame and constitute the second upORF2 that partially overlaps out of frame with the main URA6 ORF (Fig. 2b). To probe which upUUG codons are utilized by the eIF5G31R mutant, we made the URA6-LacZ reporter construct (A1074). This construct was modified to remove all upUUG1-4 codons (A1140), removal of upUUG3-4 and keeping upUUG1 intact in-frame with URA6-LacZ (A1340), removal of upUUG3-4 and changing upUUG1 into AUG codon in-frame with URA6-LacZ (A1341), removal of upUUG1, 2, and 4 and keeping upUUG3 intact in-frame with URA6-LacZ (A1342), and removal of upUUG1, 2 and 4 and changing upUUG3 to AUG codon in frame with URA6-LacZ (A1343). These constructs were transformed into the WT or eIF5G31R mutant yeast cells to check the resultant β-galactosidase activity. Consistent with the results obtained with Western blot,

Table 2. List of altered protein spots identified by gel-based 2D-PAGE/MALDI-TOF/MS approach.

| Sr. no. | Accession # (UniProt database) | Spot ID | Gene | Fold change (eIF5G31R/WT) | P-value | Actual mol. wt (kDa) | On gel approx. mol. wt (kDa) |
|---------|--------------------------------|---------|------|---------------------------|---------|---------------------|-----------------------------|
| 1       | P00925                         | 270     | ENO2 | 0.3448                    | 0.0456  | 47                  | 47                          |
| 2       | 76                             | ENO2    | 2.0886 | 0.0191                |         | 47                  | 24                          |
| 3       | 269                             | ENO2    | 0.5060 | 0.0009                |         | 47                  | 47                          |
| 4       | P14540                         | 231     | FBP1 | 0.6040                    | 0.0401  | 38                  | 38                          |
| 5       | P38013                         | 36      | AHP1 | 0.6047                    | 0.0370  | 19                  | 19                          |
| 6       | P06169                         | 9       | PDC1 | 1.6507                    | 0.0026  | 61                  | 13                          |
| 7       | 173                             | PDC1    | 2.6654 | 0.0299                |         | 61                  | 34                          |
| 8       | P14540                         | 188     | FBP1 | 1.7153                    | 0.0067  | 38                  | 34                          |

Table 3. List of altered proteins identified by gel-free labeled iTRAQ along with nLC-MALDI-TOF/MS approach.

| Sr. no. | Accession # (UniProt database) | Gene | Fold change (eIF5G31R/WT) | P-value |
|---------|--------------------------------|------|---------------------------|---------|
| 1       | P21147                         | OLE1 | 0.5477                    | 0.0124  |
| 2       | P15700                         | URA6 | 0.5546                    | 0.0069  |
| 3       | P32451                         | BIO2 | 0.6493                    | 0.0081  |
| 4       | P08456                         | CHO1 | 0.6500                    | 0.0246  |
| 5       | P32916                         | SRP101| 0.6570                    | 0.0304  |
| 6       | P38779                         | CIC1 | 0.6613                    | 0.0200  |
| 7       | P32891                         | DLD1 | 0.6661                    | 0.0050  |
| 8       | P50861                         | RIB4 | 1.5247                    | 0.0261  |
| 9       | P31382                         | PMT2 | 1.5368                    | 0.0344  |
| 10      | P07807                         | DFR1 | 1.5977                    | 0.0424  |
| 11      | Q07825                         | FRA1 | 1.6199                    | 0.0396  |
| 12      | P40943                         | RGI1 | 1.6674                    | 0.0044  |
| 13      | Q01454                         | CTF4 | 1.7217                    | 0.0309  |
| 14      | P46992                         | TOH1 | 1.7233                    | 0.0489  |
| 15      | P32528                         | DUR12| 1.7275                    | 0.0011  |
| 16      | Q02725                         | VTC3 | 1.7513                    | 0.0068  |
| 17      | P47075                         | VTC4 | 1.8045                    | 0.0040  |
| 18      | P40159                         | YNL208W| 1.8591                    | 0.0191  |
| 19      | Q04432                         | HSP31| 1.8778                    | 0.0026  |
| 20      | P22943                         | HSP12| 1.8912                    | 0.0252  |
| 21      | P43585                         | VTC2 | 1.9087                    | 0.0453  |
| 22      | P12962                         | CAF30| 1.9685                    | 0.0059  |
| 23      | Q21223                         | DCS2 | 2.2230                    | 0.0340  |
| 24      | P38736                         | GOS1 | 2.3314                    | 0.0344  |
| 25      | P06115                         | CTT1 | 2.4573                    | 0.0475  |
| 26      | P54785                         | MOT3 | 3.3204                    | 0.0459  |
removal of all the upUUG codons increased the expression of URA6-LacZ reporter in the eIF5G11R mutant. Moreover, whereas the translation from the −95 position (upUUG1 or AUG) codon severely down-regulated reporter expression, the translation from the −22 position (upUUG3 or AUG) showed better reporter expression (Fig. 2b). These results suggest that eIF5G11R mutant initiated translation from the second upORF2 that partially overlaps out-of-frame with the main URA6 ORF by utilizing the upUUG3 codon.

If the uridylate kinase protein levels are downregulated in the eIF5G11R mutant strain, it should also affect the catalysis of UMP to UDP. The UDP is further converted into UTP and CTP. Since the UTP and CTP are the precursors for DNA and RNA biosynthesis, we propose that the eIF5G11R mutant affects nucleic acid biosynthesis. Our iTRAQ data analysis showed a significant abundance of proteins previously reported to be associated with DNA replication stress (TDH2, VTC4, PRB1, HSP31, HSP12, TOH1, DUR1,2 & RGI1). Our iTRAQ data analysis showed a significant abundance of proteins previously reported to be associated with DNA replication stress (TDH2, VTC4, PRB1, HSP31, HSP12, TOH1, DUR1,2 & RGI1). Yeast cells under DNA replication stress show sensitivity to hydroxyurea (HU). To check if the eIF5G11R mutation is sensitive to DNA replication stress, we checked the growth on 25 mM HU. The eIF5G11R mutation was sensitive to HU and the sensitivity was partially rescued by a high copy overexpression of URA6 gene (Fig. 2c). The DNA replication stress also induces DNA damage that can be tested by a TUNEL assay. Consistently, the eIF5G11R mutant cells showed a twofold increase in the TUNEL positive cells (Fig. 2d).

eIF5G11R mutation causes oxidative stress. The catalase-T enzyme (CTT1) showed higher abundance in the eIF5G11R mutant as per our iTRAQ analysis. The higher abundance of the catalase-T enzyme indicates oxidative stress in the eIF5G11R mutant cells. To test this, we first checked the levels of catalase-T by adding a C-terminal 3xHA tag and analyzed the plasmid-borne expression of this modified gene in the WT and eIF5G11R mutant yeast strain by Western blot using anti-HA tag antibody. Consistent with the iTRAQ data analysis, the Western blot showed 1.3-fold upregulation of catalase-T protein (Fig. 3a, left). We also checked the transcription level of CTT1 mRNA by RT-qPCR using CTT1 ORF specific oligonucleotides. There was ~ threefold higher expression of CTT1 mRNA in the eIF5G11R mutant cells (Fig. 3a, right). The discrepancy between the CTT1 mRNA expression and protein expression could be because of the presence of the four upUUG codons (~105, −76, −66 and −40) in the 112 nucleotides long 5′ UTR of CTT1 mRNA. The upUUG codons at the −105 and −66 position are in-frame with the main AUG start codon, whereas the upUUG codon at −76 and −40 are out-of-frame with the main AUG start codon. Since the upUUG codon at the −105 position is very close to the 5′ cap of the mRNA and could be skipped by the eIF5G11R mutant containing 485 scanning ribosome. However, the eIF5G11R mutant could initiate translation from the out-of-frame ~76 or ~40 upUUG codon causing premature termination, whereas initiation from the in-frame upUUG codon at −66 position could result in translation of the catalase-T protein. Taken together, this results in ~1.3-fold increase in the catalase-T protein expression as the CTT1 mRNA levels are high. These results suggest eIF5G11R mutant cells must be under oxidative stress and in response to this stress, the expression of the CTT1 transcript could have been upregulated. The yeast cells under oxidation stress should show sensitivity to H2O2. Consistently, the eIF5G11R mutant showed strong sensitivity to H2O2 treatment (Fig. 3b). The sensitivity to oxidative stress was partially rescued in the eIF5G11R mutant cells when the CTT1 gene was deleted (Fig. 3d). The oxidative stress in the eIF5G11R mutant may have been triggered by the accumulation of reactive oxygen species (ROS) such as hydrogen peroxide (H2O2), the hydroxyl radicals (OH·), and the superoxide anion (O2·−). To check if the eIF5G11R mutant has higher ROS levels, we treated the eIF5G11R mutant cells with H2DCFDA and MitoSOX red dye to
check cytosolic and mitochondrial ROS levels, respectively. The 1.48-fold high fluorescence for H$_2$DCFDA and 2.06-fold high fluorescence for MitoSOX red suggests high ROS levels in the eIF5G31R mutant cells (Fig. 3e,f).

**Lower levels of glutathione and YAP1 gene expression exacerbate the oxidative stress in the eIF5G31R mutant.** Our data showed higher levels of ROS in the eIF5G31R mutant cells. Catalase, superoxide dismutase, glutathione peroxidase, and thioredoxin system neutralize the intracellular ROS$^{47–49}$. Our iTRAQ data analysis showed 20% downregulation of GSH1 protein levels in the eIF5G31R mutant (Supplementary Sheet 2), suggesting that there may be inadequate levels of glutathione to detoxify the cellular ROS. We check the intracellular glutathione levels by monochlorobimane dye, which reacts with glutathione in the presence of glutathione-S-transferase to form an adduct that shows fluorescence at 380 nm$^{50,51}$. Microscopic image analysis showed a 30% reduction of

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**URA6-LacZ expression**

\[
\begin{align*}
1 & \text{A1074} \\
2 & \text{A1140} \\
3 & \text{A1340} \\
4 & \text{A1341} \\
5 & \text{A1342} \\
6 & \text{A1343} \\
\end{align*}
\]

\[
\begin{align*}
\text{AUG} & \text{URA6-LacZ} \\
\text{UGU} & \text{URA6-LacZ} \\
\text{ACC} & \text{URA6-LacZ} \\
\text{-X} & \text{URA6-LacZ} \\
\text{X} & \text{URA6-LacZ} \\
\text{X} & \text{URA6-LacZ} \\
\end{align*}
\]

**β-Galactosidase activity (U/mg)**

\[
\begin{align*}
1.0 & \text{m} \\
1.5 & \text{m} \\
2.0 & \text{m} \\
2.5 & \text{m} \\
3.0 & \text{m} \\
3.5 & \text{m} \\
4.0 & \text{m} \\
4.5 & \text{m} \\
\end{align*}
\]

---

**TUNEL Assay**

\[
\begin{align*}
\text{WT} & \text{eIF5G31R} \\
\text{DAPI} & \text{DAPI} \\
\text{FITC} & \text{FITC} \\
\text{Fluorescence intensity} & \text{WT} \text{eIF5G31R} \\
\text{Fluorescence intensity} & \text{WT} \text{eIF5G31R} \\
\text{n = 300} & \text{p < 0.0001} \\
1.08 \pm 0.52 & 27.83 \pm 6.78 \\
\end{align*}
\]
glutathione level in the eIF5G31R mutant cells (Fig. 4a). The growth defect associated with the eIF5G31R mutant was partially rescued in the presence of the 5 mM glutathione (Fig. 4b). However, the high copy overexpression of GSH1 and GSH2 genes did not rescue the slow growth of the eIF5G31R mutant (data not shown). It could be possible that other components of the redox system critical for ROS detoxification are not fully expressed in the eIF5G31R mutant cells.

YAP1 is a AP-1 family transcription factor that regulates ~28 genes associated with ROS detoxification, including GSH1, GSH2, GLR1, SOD1, SOD2, GPX2, TSA1, TPR1, AHP1, and TRX2. These genes are involved in glutathione biosynthesis, superoxide dismutase activity, glutathione peroxidase function, thioredoxin, and thioredoxin peroxidases action. The YAP1 protein was not identified in our iTRAQ or 2D-PAGE data analysis. However, analysis of the YAP1 gene transcript suggests the presence of six out-of-frame upUUG codons at the YAP1′ UTR that may affect its translation. We made YAP1-LacZ reporter constructs with native 5′ UTR region containing the six upUUG codons (A1311) and the modified construct devoid of the six upUUG codons (A1312) and these constructs were transformed in the WT or eIF5G31R mutant cells. The eIF5G31R mutant showed 2.4-fold less YAP1 reporter expression from the native constructs. However, upon removing six upUUG codons, the reporter expression in the eIF5G31R mutant was similar to the WT (Fig. 4c). This result suggests that the YAP1 protein expression was downregulated in the eIF5G31R mutant, which may have affected the transcription of the target genes involved in the redox pathway. Consistently, we observed down-regulation of the AHP1 mRNA (Supplementary Fig. S6) and AHP1 protein levels that protect against oxidative damage (Table 2). However, overexpression of the YAP1 gene from a high copy plasmid should increase the YAP1 protein levels and show a detoxifying effect on ROS. Consistently, the YAP1 overexpression showed partial growth rescue of the eIF5G31R mutant in the presence of H2O2 (Fig. 4d).

Discussion
Previously, we reported that the eIF5G31R mutation recognizes UUG codon but does not initiate translation from CUUG or GUG codon16. In this study, we decipher the underpinning effect of the eIF5G31R mutation on UUG codon recognition that alters cellular proteome in dominant conditions. Our iTRAQ data suggests that seven proteins abundance was downregulated, while nineteen proteins abundance was upregulated. The most striking of them was the 0.55-fold downregulation of the uridylyl kinase protein (URA6), which takes part in the de-novo pyrimidine biosynthesis pathway. This downregulation of uridylyl kinase was not due to the lower levels of URA6 transcript; rather, our analysis suggests that the utilization of the upstream UUG codons could generate out-of-frame upORFs that impedes translation initiation from the URA6 main ORF. Removal of all the four upUUG codons from the URA6 transcript caused an overall reduction in the uridylyl kinase translation in the WT cells. We suspect mutating the upUUG codons may have changed the secondary structure of the mRNA resulting in the lower translation of the URA6 transcript. However, the relative expression of uridylyl kinase increased in the eIF5G31R mutant cells after removing the four upUUG codons (Fig. 2a). The eIF5G31R mutant cells could have unsuccessfully tried to mitigate the low abundance of uridylyl kinase by upregulating the URA6 mRNA expression to relieve the lower nucleotide availability stress. The upregulation of DNA replication stress markers (TDH2, VTC4, PRB1, HSP31, HSP12, TOH1, DUR1,2 & RGI1) observed in our iTRAQ data is consistent with the sensitivity of the eIF5G31R mutant to hydroxyurea because of the low uridylyl kinase expression and the attendant DNA damage (Fig. 2c,d).

The upregulations of catalase-T in the eIF5G31R mutant proteome suggest oxidative stress. Consistently, the eIF5G31R mutant showed sensitivity to H2O2, which was partially rescued by the high copy overexpression of either CTT1 or SOD1/SOD2 genes (Fig. 3b,c). The sensitivity to H2O2 is consistent with the high cytosolic and mitochondrial ROS levels in the eIF5G31R mutant (Fig. 3e,f). Compounding this oxidative stress, glutathione levels were 30% lower in the eIF5G31R mutant cells (Fig. 4a), and supplementing 5 mM glutathione could partially rescue its growth defect (Fig. 4b). However, we could not identify a major source of ROS generation in...
the eIF5G31R mutant through our proteome analysis. Previous report from Salmon and co-workers, suggest that the DNA damage alone can elicit an increase in the intracellular ROS levels. Thus the DNA damage observed in the eIF5G31R mutant (Fig. 2d) could trigger an intracellular ROS generation, and the components involved in ROS detoxification were not optimum due to the downregulation of regulatory genes, thus tipping the balance towards oxidative stress. YAP1 is an AP-1 family transcription factor that regulates ~28 genes associated with ROS detoxification and the YAP1-LacZ reporter expression was observed to be downregulated in the eIF5G31R mutant because of the upUUG codon utilization. Consistently, the overexpression of the YAP1 gene partially rescued the sensitivity to oxidative stress (Fig. 4c,d).

Our results indicate that the defect in translation initiation fidelity in the eIF5G31R mutant reprograms the cellular pathways to cause DNA damage and oxidative stress. Moreover, the upregulation of proteins involved
Figure 4. Lower level of glutathione and YAP1 gene expression exacerbates oxidative stress in eIF5G31R mutant cells. (a) Glutathione quantification. Yeast strain YP823 carrying EV pYCplac22 (A823) or pYCplac22-eIF5G31R (A838) constructs were grown in SD plus leucine, uracil medium till OD<sub>600</sub> ~ 0.8 at 30 °C. 200 μl cells suspension were stained with 30 μM of monochlorobimane (mCBM) followed by fluorescence microscopic imaging at Em λ460. The values are average (n = 300) along with the standard error of the mean (SEM). (b) Growth analysis in the presence of glutathione. Yeast strains as per (a) were spotted on either SD plus leucine, uracil plus 2 mM H₂O₂ or supplemented with 5 mM glutathione plates and incubated at 30 °C for 3 days. (c) Quantification of YAP1-LacZ expression. Yeast strain YP823 carrying EV pYCplac22 (A823) or pYCplac22-eIF5G31R (A838) constructs were transformed with pYCplac111-YAP1_LacZ (A1311) or pYCplac111-YAP1-upTTGless_LacZ (A1312) construct, were grown till OD<sub>600</sub> ~ 0.8 at 30 °C and the β-galactosidase activity was measured as per Fig. 2b. The error represents an average deviation. (d) Growth analysis in the presence of H₂O₂. Yeast strain YP823 carrying EV pYCplac22 (A823) or pYCplac22-eIF5G31R (A838) constructs were transformed with either high copy EV pRS425 (B1378) or (h.c) pRS425-YAP1 construct (A1346), grown overnight, serially diluted, and spotted on SD, uracil plus 2 mM H₂O₂ as per Fig. 3c. Statistical differences were determined by the two-tailed Student’s t-test. ns non-significant.
in the protein degradation and micro-autophagy pathways suggests an enhanced turnover of proteins in the eIF5G31R mutant cells. Consistently, we observed enhanced degraded products of FBPI, PDC1 and ENO2 proteins in 2D-PAGE MALDI-TOF/MS analysis (Table 2). We did not observe significant changes in the 40S, 60S, and 80S ribosomal (monosome) peaks in the eIF5G31R mutant (Supplementary Fig. S2), suggesting that the mutation may not have affected the general assembly of PIC during the translation initiation process and only affected the 5′ to 3′ mRNA scanning where the eIF5G31R mutation preferentially selected UUG as initiation codon. In support of our finding, the cryo-EM data suggest that the N-terminal domain of eIF5 displaces eIF1 from the P-site of the 48S complex during AUG codon recognition. It is suggested that the eIF5G31R mutation may prematurely displace eIF1 while scanning the mRNA from 5′ to 3′ direction and stabilizing the base pairing of Met-tRNAi anticodon with the UUG codon. Furthermore, the 35S-Methionine labelling experiment suggested a less than 10% reduction in the protein biosynthesis in the eIF5G31R mutant cells (data not shown). This subtle decrease in the protein translation may be an effect of oxidative stress in the eIF5G31R mutant, as oxidative stress increases ribosomal transition time on mRNA and shows an inhibitory effect on translation elongation. Consistently, we observed lower polysomes in the eIF5G31R mutant (Supplementary Fig. S2).

Earlier in-vitro biochemical data suggest that the eIF5G31R mutation disfavors the AUG base pairing while favors UUG base pairing in the ‘Closed/PIN’ conformation with the Met-tRNAi, suggesting a possible mechanism of UUG codon recognition. However, it is not clear whether the eIF5G31R mutant also disfavors AUG codon recognition in vivo. Our data can resolve this issue; as upon removal of the upUUG codons from the URA6-LacZ or YAPI-LacZ reporter constructs, the levels of reporter expression initiated from the AUG codon in the eIF5G31R mutant and WT cells were essentially identical (Figs. 2b, 4c), suggesting that the eIF5G31R mutant can recognize AUG start codon in vivo. Thus the dominant slow-growth phenotype and the recessive lethality could be because of the additional UUG codon recognition and the attendant proteome change in the eIF5G31R mutant. The eIF2γN135D mutation confers a Sui− phenotype, a slow-growth phenotype, and Met-tRNAi binding defects that are consistent with its Gcd− phenotype (de-repression of GCN4 expression). However, the slow-growth phenotype of the eIF2γN135D mutations is suppressed by the overexpression of Met-tRNAi, but not by the eIF1 overexpression, which only suppresses the Sui− phenotype. Taken together, it appears that the slow growth phenotype of the eIF2γN135D mutation is because of the TC formation defects. However, the dominant slow growth phenotype in the eIF5G31R mutant is primarily because of the UUG codon recognition (Sui− phenotype) and the attendant proteome change that causes oxidative stress and DNA damage, as the eIF5G31R mutation does not have TC formation defect and show Gcd− phenotype. However, we do not imply that oxidative stress and DNA damage are the only defects caused by the upUUG codon recognition in the eIF5G31R mutant cells. It could be possible that the mRNA transcript having multiple out of frame upUUG codons (examples, URA6 or YAPI) may be disproportionately affected compared to the mRNA transcript having a single out of frame upUUG codon. Moreover, not all upUUG codons can be utilized efficiently to initiate translation by the Sui− mutants as the sequence context surrounding the upUUG codon may also play an important role. Consistently, the eIF5G31R mutation showed ~2.6-fold decrease in UUG codon recognition efficiency when the UUG codon is in a poor sequence context (Supplementary Fig. S7). Probably genome-wide ribosomal profiling of the Sui− mutants will give more clarity on global upUUG codon utilization in different sequence contexts and identification of affected proteins involved in various cellular pathways.

The significance of studying defects in the translation initiation process is that it uncovers the fundamentals of the information decoding process and sheds light on the diseases associated with this process. Diseases such as Alzheimer’s, amyotrophic lateral sclerosis, Vanishing White Matter (VWM) are linked with the defects associated with integrated stress response (ISR) that lower TC levels. Mutations isolated in the human eIF2γ subunit are associated with X-linked intellectual disability characterized by mental deficiency, epilepsy, hypogenitalism, microcephaly, and obesity (MEHMO syndrome). Interestingly, the yeast model of MEHMO syndrome mutant that uncovered DNA damage and oxidative stress due to altered proteome could serve as an impetus to investigate further the proteome change and the attendant cellular defects caused by the eIF2γ mutants in the MEHMO syndrome patients.

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References
1. Hinnebusch, A. G. Molecular mechanism of scanning and start codon selection in eukaryotes. Microbiol. Mol. Biol. Rev. 75, 434–467 (2011).
2. Hinnebusch, A. G. The scanning mechanism of eukaryotic translation Initiation. Annu. Rev. Biochem. 83, 779–812 (2014).
3. Llácer, J. I. et al. Conformational differences between open and closed states of the eukaryotic translation initiation complex. Mol. Cell 59, 399–412 (2015).
4. Llácer, J. I. et al. Translational initiation factor eIF5 replaces eIF1 on the 40S ribosomal subunit to promote start-codon recogni-
5. Fekete, C. A. Molecular mechanism of scanning and start codon selection in eukaryotes. Microbiol. Mol. Biol. Rev. 75, 434–467 (2011).
6. Fekete, C. A. The scanning mechanism of eukaryotic translation Initiation. Annu. Rev. Biochem. 83, 779–812 (2014).
7. Llácer, J. I. et al. Conformational differences between open and closed states of the eukaryotic translation initiation complex. Mol. Cell 59, 399–412 (2015).
8. Llácer, J. I. et al. Translational initiation factor eIF5 replaces eIF1 on the 40S ribosomal subunit to promote start-codon recogni-
9. Fekete, C. A. et al. The eIF1A C-terminal domain promotes initiation complex assembly, scanning and AUG selection in vivo. EMBO J. 24, 3588–3601 (2005).
10. Alme, P. V. & Dever, T. E. Direct binding of translation initiation factor eIF2gamma-G domain to its GTPase-activating and GDP-
11. GTP exchange factors eIF5 and eIF2B epsilon. J. Biol. Chem. 281, 12636–12644 (2006).
12. Maag, D., Fekete, C. A., Gryczynski, Z. & Lorsch, J. R. A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. Mol. Cell 17, 263–275 (2003).
8. Chang, K.-J. & Wang, C.-C. Translation initiation from a naturally occurring non-AUG codon in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**, 13778–13785 (2004).
9. Chen, S.-J., Lin, G., Chang, K.-J., Yeh, L.-S.S. & Wang, C.-C.C. Translational efficiency of a non-AUG initiation codon is significantly affected by its sequence context in yeast. *J. Biol. Chem.* **283**, 3173–3180 (2008).
10. Iwasaki, S., Floor, S. N. & Ingolia, N. T. Rocaglates convert DEAD-box protein eIF4A into a sequence-selective translational repressor. *Nature* **534**, 558–561 (2016).
11. Guenther, U. P. et al. The helicase Ded1p controls use of near-cognate translation initiation codons in 5′UTRs. *Nature* **559**, 130–134 (2018).
12. Costello, J. et al. Global mRNA selection mechanisms for translation initiation. *Genome Biol.* **16**, 1–21 (2015).
13. Mitchell, S. F. et al. The 5′-7-methylguanosine cap on eukaryotic mRNAs serves both to stimulate canonical translation initiation and to block an alternative pathway. *Mol. Cell* **39**, 950–962 (2010).
14. Gupta, N., Lorsch, J. R. & Hinnebusch, A. G. Yeast Ded1p promotes 48S translation preinitiation complex assembly in an mRNA-specific and eIF4F-dependent manner. *Elife* **7**, 1–25 (2018).
15. Castillo-Valverius, R., Yoon, H. & Donahue, T. F. Genetic characterization of the *Saccharomyces cerevisiae* translational initiation suppressors *sui1, sui2* and *SUI3* and their effects on HIS4 expression. *Genetics* **124**, 483–495 (1990).
16. Huang, H. K., Yoon, H., Hannig, E. M. & Donahue, T. F. GTP hydrolysis controls stringent selection of the AUG start codon during translation initiation in *Saccharomyces cerevisiae*. *Genes Dev.* **11**, 2396–2413 (1997).
17. Alone, P. V., Cao, C. & Dever, T. E. Translation initiation factor 2gamma mutant alters start codon selection independent of Met-suppressors. *Mol. Cell. Biol.* **28**, 6877–6888 (2008).
18. Antony, A. C., Ram, A. K., Dutta, K. & Alone, P. V. Ribosomal mutation in helix 32 of 18S rRNA alters fidelity of eukaryotic translation start site selection. *FEBS Lett.* **593**, 852–867 (2019).
19. Gupta, N., Lorsch, J. R. & Hinnebusch, A. G. Ded1p promotes 48S translation preinitiation complex assembly in an mRNA-specific and eIF4F-dependent manner. *Elife* **7**, 1–25 (2018).
20. Guenther, U. P. et al. The helicase Ded1p controls use of near-cognate translation initiation codons in 5′UTRs. *Nature* **559**, 130–134 (2018).
21. Costello, J. et al. Global mRNA selection mechanisms for translation initiation. *Genome Biol.* **16**, 1–21 (2015).
22. Gueldener, U., Heinisch, J., Koehler, G. J., Voss, A. & Hegemann, J. H. A second set of loxP marker cassettes for Cre-mediated gene targeting. *EMBO J.* **17**, 2173–2181 (1998).
23. Skoneczny, A., Miciałkiewicz, A. & Skoneczny, M. The PRIDE database and related tools and resources in 2019: Improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450 (2019).
24. Perez-Riverol, Y. et al. The PRIDE database and related tools and resources in 2019: Improving support for quantification data. *Nucleic Acids Res.* **47**, D442–D450 (2019).
25. Tkach, J. M. et al. Dissecting DNA damage response pathways by analysing protein localization and abundance changes during DNA replication stress. *Nat. Cell Biol.* **14**, 966–976 (2012).
26. Uttenweiler, A., Schwarz, H., Neumann, H. & Mayer, A. The vacuolar transporter chaperone (VTC) complex is required for mitophagy. *Mol. Cell Biol.* **18**, 166–175 (2007).
27. Jager, S., Strayle, J., Heinemeyer, W. & Wolf, D. H. Cic1, an adaptor protein specifically linking the 26S proteasome to its substrate, is associated with eIF4G for binding to eIF4E. *Saccharomyces cerevisiae* Genetics **534**, 558–561 (2016).
28. Kumánovics, A. & Melero, A. Protein O-mannosyltransferases participate in ER protein quality control. *Biochim. Biophys. Acta BBA Bioenergy** **1830**, 1596–1605 (2013).
29. Altmann, M., Schmitz, N., Berset, C. & Trachsel, H. A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. *EMBO J.* **16**, 1114–1121 (1997).
30. Saito, K., Kuroda, M. & Ishino, Y. Suppression of the nonsense mutant phenotype by the yeast suppressor gene *DFR1*. *Saccharomyces cerevisiae* Genetics **124**, 143–153 (1991).
31. Aihara, M., Schutt, H., Neumann, H. & Mayer, A. The vacuolar transporter chaperone (VTC) complex is required for mitophagy. *Mol. Cell Biol.* **18**, 166–175 (2007).
32. Malys, N. & McCarthy, J. G. E. Dcs2, a novel stress-induced modulator of m7G pppX pyrophosphatase activity that localizes to P bodies. *J. Mol. Biol.* **363**, 370–382 (2006).
33. Skoneczna, A., Miciałkiewicz, A. & Skoneczny, M. *Saccharomyces cerevisiae* Hsp31p, a stress response protein conferring protection against reactive oxygen species. *Free Radic. Biol. Med.* **42**, 1409–1420 (2007).
34. Jang, Z. R., Abigad, L. T., Huang, S. H., Cai, B. & Jong, A. Y. A. Y. Molecular characterization of *Saccharomyces cerevisiae* UR46 gene. DNA sequence, mutagenesis analysis, and cell cycle regulation relevant to its suppression mechanism to cdc8 mutation. *J. Biol. Chem.* **266**, 18287–18293 (1991).
35. Patzer, L. & Stucki, K. Characterization and isolation of the yeast iron regulon in response to iron limitation. *FEBS Lett.* **205**, 74–81 (1986).
36. Jang, Z. R., Abigad, L. T., Huang, S. H., Cai, B. & Jong, A. Y. A. Y. Molecular characterization of *Saccharomyces cerevisiae* UR46 gene. DNA sequence, mutagenesis analysis, and cell cycle regulation relevant to its suppression mechanism to cdc8 mutation. *J. Biol. Chem.* **266**, 18287–18293 (1991).
37. Kurutas, E. B. The importance of antioxidants which play the role in cellular response against oxidative/nitrosative stress: Current status. *Nutr. J.* **15**, 71 (2016).
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Author contributions

P.V.A., A.K.R., and A.R.S. analyzed the data and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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