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## Selective profiling of ribosomes associated with yeast Upf proteins

ROBIN GANESAN<sup>1</sup>, JOHN LESZYK<sup>2</sup>, and ALLAN JACOBSON<sup>1,\*</sup>

<sup>1</sup>Department of Microbiology and Physiological Systems, University of Massachusetts Medical School, 368 Plantation Street, Worcester, MA 01655-0122

<sup>2</sup>Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 368 Plantation Street, Worcester, MA 01655-0122

### Abstract

Ribosomes associated with nonsense-mediated decay factors Upf1, Upf2, or Upf3 were purified by immunoprecipitation, and enrichment and stoichiometry of Upf factors and ribosomal proteins were analyzed by western blot and mass spectrometry. Using a small RNA library preparation protocol that eliminates in-gel RNA and cDNA size selection and incorporates four random nucleotides on each side of the ribosome-protected RNA fragment allowed recovery, detection, and analysis of all size classes of protected fragments from a sample simultaneously.

### Keywords

NMD; Selective ribosome profiling; Upf proteins

## 1. Introduction

Nonsense-mediated mRNA Decay (NMD) is a conserved translation-dependent mRNA decay pathway generally triggered when a premature termination codon (PTC) occupies the ribosomal A site [1–5]. NMD targets polysome-associated mRNAs [6–9] that account for as much as 15% of the complexity of an organism’s transcriptome [6, 10–18]. PTCs characteristic of NMD-targeted mRNAs can arise from mutations in genomic DNA, alternative pre-mRNA processing, or non-productive DNA rearrangements, but they are also inherent to a subset of normal transcripts, including those for which the ribosome fails to utilize or maintain the proper reading frame [19]. NMD substrates have substantial biological impact: not only does NMD ensure “junk” removal (e.g., byproducts of alternate splicing, cytoplasmic intron-containing transcripts, or pseudogene mRNAs [6, 20]), but it also effectively renders most nonsense alleles as null alleles [21, 22] and has been coopted to regulate the levels and locations of specific proteins [23, 24].

\*Corresponding author, Phone: (508) 856-2442, Fax: (508) 856-5920, allan.jacobson@umassmed.edu.

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*UPF1*, *UPF2*, and *UPF3* encode the key factors controlling NMD [1, 3, 4, 25–30] and their inactivation stabilizes nonsense-containing mRNAs while having no significant effects on most wild-type transcripts [6, 30, 31]. The Upf proteins are mostly cytoplasmic and interact with each other, the ribosome, and multiple mRNA decay and translation factors, but their exact roles in NMD, and their mechanism of association with a premature termination complex, remain to be clarified [1]. Upf1 is NMD's key regulator. Overexpression of *UPF1* compensates for the nonsense suppression phenotype of mutations in *UPF2* and *UPF3* (without an effect on NMD phenotypes), but not *vice versa* [32], and maximal *in vitro* activation of Upf1's helicase and ATPase activities requires both Upf2 and Upf3 [33]. The Upf proteins associate with ribosomes [34–38], but there are conflicting views as to whether this occurs stochastically, only to be activated at a PTC, or whether it is premature termination *per se* that leads to Upf recruitment and function [5]. Very little is known about when in the translation cycle Upf factors bind to ribosomes, whether NMD is triggered by these binding events, and which Upf factors, if any, confer degradation specificity to an NMD substrate.

To address this conundrum, we have established methods for enriching and profiling yeast ribosomes that are associated with specific Upf factors. Most ribosome profiling libraries are prepared using some version of the protocol developed by Ingolia et al. [39] and require in-gel size-selection of ribosome-protected RNA fragments, size selection and circularization of cDNA, and some method of PCR product size selection. In addition to being time-consuming and difficult to prepare libraries from small amounts of input RNA, these protocols are seldom designed to recover the full spectrum of potential ribosome-protected RNA fragment size classes. Further, unlike many small or micro-RNA library preparation protocols, most ribosome profiling library preparation methods do not include random nucleotide bar codes to eliminate PCR duplicates from downstream analysis.

We have developed a workflow (summarized in Fig. 1) which not only allows efficient recovery of Upf-associated ribosomes by immunoprecipitation, but also enables a snapshot of the translational status and stoichiometry of Upf factors and ribosomes. In addition, by incorporating a library preparation method which places four random nucleotides at each end of the ribosome protected fragment and eliminates in-gel RNA and cDNA size selection, our workflow has allowed us to eliminate PCR duplicates from our analyses, recover a broad range of ribosome-protected fragment size classes, and compare the read length distributions of all ribosome-protected fragment size classes present in a sample across the transcriptome.

## 2. Methods

### 2.1. Equipment and reagents

**2.1.1. Recommended equipment is listed in Table 1.**—Reagents and consumables necessary for performing this protocol are listed in Table 2.

**2.1.2. Buffers used in this protocol are:**

1. Footprinting buffer (FB): 20mM Tris, pH7.4, 150mM NaCl, 5mM MgCl<sub>2</sub>

2. Immunoprecipitation (IP) buffer: 150mM Tris, pH7.5, 150mM NaCl, 20mM MgCl<sub>2</sub>, 0.1% Igepal
3. 1.5X MES-NaOH buffer: 150 mM MES-NaOH, pH 5.5, 450 mM NaCl, 15 mM MgCl<sub>2</sub>, 15 mM β-mercaptoethanol
4. 1X PBS-T: 1X PBS, 0.1% Tween 20
5. Western stripping buffer: 2% SDS, 62.5mM Tris, pH6.8, 0.8% β-mercaptoethanol

## 2.2. High-copy expression of tagged Upf proteins

Yeast Upf proteins were tagged by N-terminal fusion of a FLAG epitope (amino acid sequence DYKDDDDK) [40] to the open reading frame of each gene. N-terminal tagging was chosen because previous experiments have demonstrated that these alleles are stably expressed and functional *in vivo* [29, 41]. FLAG-*UPF3* was expressed under the *TPI* promoter as this was determined to be necessary for high-level expression of this protein (data not shown).

**2.2.1. Yeast strains are described in Table 3.**—Plasmids [29, 41] are described in Table 4.

**2.2.2. Yeast strains were transformed with plasmids according to standard methods and grown on selective medium until a final shift to rich medium prior to harvest.**

## 2.3. Preparation of lysate and 80S ribosomes

**2.3.1. Growth and harvest of cells**—Cells are expanded in selective medium to maintain the plasmid bearing the FLAG-tagged *UPF* allele. Growth for ~2 doublings in rich medium prior to cell harvest ensures maximal occupancy of translating ribosomes on mRNA.

1. Inoculate 100ml selective medium from a single colony or cell patch grown on selective medium, shake at 30°C, 210 rpm, overnight. In the morning, dilute the culture to  $A_{600}=0.1$  in 100ml selective medium and shake at 30°C, 210 rpm, for 6–8 hours. The starting cell density for the next step may vary depending on the growth rate of the strain, but generally dilute culture to  $A_{600}=0.0015$ – $0.003$  into 2 liters prewarmed selective medium in a 6 liter flask, shake at 30°C, 120 rpm, 16 hours. In the morning, if the  $A_{600}$  has not exceeded 0.8, dilute the culture to  $A_{600}=0.15$ – $0.2$  into individual 2.8 liter Fernbach flasks containing 1 liter prewarmed YAPD medium (YEPD medium supplemented with adenine; [42]), 2 to 6 liters total, depending on the application. For immunoprecipitation experiments, a total of 6 liters of culture are grown per strain and cycloheximide condition; otherwise, 1 liter of culture is grown. Shake at 30°C, 210rpm, to  $A_{600}=0.6$  to 0.8.
2. Recover each liter of culture individually by vacuum filtration onto a 0.8μm cellulose acetate membrane filter, quickly collect cells with a spatula, flash

freeze by plunging into 50ml polyethylene centrifuge tubes filled with liquid nitrogen, and drip in 2.5ml footprinting buffer (FB) plus 1% TritonX-100, 0.5mM DTT, 1mM phenylmethylsulfonyl fluoride (PMSF), 1X protease inhibitors. When indicated, 100µg/ml final concentration cycloheximide is added to the FB and culture, with swirling to mix, immediately prior to vacuum filtration.

3. Pool frozen cells plus FB from 2 liters total of cell culture and lyse in a Cryomill (Retsch) in a 50ml stainless steel jar with a 20mm stainless steel ball; precool at 5Hz, 2 minutes, and grind at 10Hz, 15 minutes, precooling all tools and containers in liquid nitrogen and keeping all samples on dry ice. Pool frozen powder from up to 6 liters of culture from each culture condition into two new 50ml polyethylene centrifuge tubes and store at  $-80^{\circ}\text{C}$ .

**2.3.2. Purification of 80S ribosomes**—The following ribosome preparation protocol is adapted from Ingolia, *et al.* [39]:

1. Thaw pooled frozen cell powder by immersing the tube in  $30^{\circ}\text{C}$  water and stirring with a sterile pipette until just thawed, and then place on ice. Centrifuge twice for 5 minutes, 5,000 rpm (4,696 *g*), in a  $4^{\circ}\text{C}$  tabletop centrifuge. Place supernatant into chilled, balanced 10.4ml polycarbonate tubes and spin in an ultracentrifuge at 18,000 rpm (29,321 *g*), type 50Ti rotor,  $4^{\circ}\text{C}$ , 10 minutes. Carefully collect the supernatant with a Pasteur pipette, avoiding the lipid layer and pellet, into new chilled 10.4ml polycarbonate tubes; rebalance and spin again at 18,000 rpm (29,321 *g*), type 50Ti rotor,  $4^{\circ}\text{C}$ , 15 minutes. Carefully collect the supernatant as before into a chilled 50ml centrifuge tube on ice.
2. Measure lysate  $A_{260}$  in a spectrophotometer. Reserve 0.5ml lysate in 100–200µl aliquots on dry ice and store at  $-80^{\circ}\text{C}$  for later RNA extraction for use in making RNA-Seq libraries. Divide remaining lysate into 1ml aliquots in 1.7ml microcentrifuge tubes. Add 15U RNaseI per  $A_{260}$  unit of clarified lysate, and incubate for 1 hour at  $25^{\circ}\text{C}$  and 700rpm in a Thermomixer. Place digested lysate on ice and add Superase-In RNase Inhibitor to 0.8U per  $A_{260}$ .
3. Layer 3–4ml of digested lysate onto 6–7ml 1M sucrose cushion in FB plus 0.5mM DTT in a 10.4ml polycarbonate tube and centrifuge in a 50Ti rotor, 50,000 rpm (226,240 *g*), 105 minutes,  $4^{\circ}\text{C}$ . Dissolve ribosome pellet on ice in 0.5ml FB plus 0.5mM DTT, 1X protease inhibitors, 10U/ml Superase-In per tube, or 2ml total for ribosomes from 6 liters of culture. Transfer dissolved ribosomes to microcentrifuge tubes, spin in a microcentrifuge at 15,000 rpm (21,130 *g*), 1 minute, to pellet insoluble material and then transfer supernatant to new microcentrifuge tubes. Measure  $A_{260}$  of ribosomes in a spectrophotometer and freeze 100U aliquots for immunoprecipitation reactions. Additionally, freeze four aliquots of ribosomes equivalent to 20µl  $A_{260}=100$  for later RNA extraction if performing ribosome profiling on ribosomes which have not undergone immunopurification.

## 2.4. Immunopurification of Upf-associated ribosomes

### 2.4.1. Prepare affinity gel

1. All steps in this section are performed at 4°C.
2. Thaw anti-FLAG M2 Affinity Gel on ice; one 5 ml bottle is enough for two immunoprecipitation experiments of 6 bindings per condition. Aliquot 800µl 50% anti-FLAG M2 Affinity resin into each Amicon-Pro Affinity exchange device for a total of 6 devices per experiment. Spin in a refrigerated benchtop centrifuge at 1000g, 1 minute.
3. Resuspend each aliquot of affinity gel in 8ml cold IP buffer and spin at 1000g, 2 minutes; repeat.

### 2.4.2. Binding of ribosomes

1. Resuspend each washed aliquot of affinity gel in 8ml cold IP buffer plus 0.5mM DTT, 1X protease inhibitors, 100U/ml Suprase-In.
2. Save three 1µl aliquots of input ribosomes in 15µl IP buffer total from each condition on dry ice for later analysis by western blot and mass spectrometry; store aliquots at -80°C. Add 16 A<sub>260</sub> of ribosomes per aliquot of resuspended affinity gel (96 A<sub>260</sub> of ribosomes total per condition across 6 devices). Place any remaining ribosomes on dry ice and store at -80°C. Place a 3/8" Tough-Spot over the hole in the Amicon-Pro Affinity exchange device, rubbing firmly to secure. Close the cap tightly, and secure the cap with parafilm. Incubate for 90 minutes at 4°C with rocking at 12 rpm on a platform rocker.
3. After binding, remove parafilm, pierce the 3/8" Tough-Spot with a 20g hypodermic needle, and collect affinity gel by centrifugation at 4°C, 1000g, 2 min. Pool the flowthrough from each condition and save three aliquots of 15µl each as in step 2. Wash affinity gel 3 times with 8ml IP buffer plus 0.5mM DTT, 1X protease inhibitors, with centrifugation at 1000g for 2 minutes between each wash. Pool the final wash from each condition and save three 15µl aliquots as in step 2.
4. Place a new 3/8" Tough-Spot over the hole of the Amicon-Pro Affinity Exchange Device. Incubate each binding reaction with 2ml 0.15mg/ml 3X FLAG peptide in IP buffer plus 0.5mM DTT, 1X protease inhibitors, 100U/ml Suprase-In for 30min, 4°C, rocking as in step 2. Pierce the 3/8" Tough-Spot and collect eluate by centrifugation as in step 3.

### 2.4.3. Concentration of ribosomes

1. Spin 2ml eluate from each elution through an Amicon Ultra-2 100K Centrifugal Filter Unit at 4°C, 4000g, until the volume is ~200µl. Recover the concentrate by inverting the filter unit into the tubes provided by the manufacturer and spin at 1000g, 3 minutes. Pool the concentrate from each condition (6 tubes) into one Amicon Ultra-2 100K Centrifugal Filter Unit and spin at 4000g until the total volume is ~160µl; recover the concentrate as with the previous spin.

2. In thick wall polyallomer 7X20mm centrifuge tubes, layer 160 $\mu$ l concentrate on top of a 40 $\mu$ l 1M sucrose cushion in IP buffer plus 1X protease inhibitors, 10U/ml Superase-In, and spin in a tabletop ultracentrifuge at 66,000 rpm (194,173 *g*), 100 minutes, 4°C, in a TLA100 rotor. Carefully and completely remove the supernatant and resuspend the pellet in 60 $\mu$ l IP buffer plus 0.5mM DTT, 1X protease inhibitors, 20U/ml Superase-In. Determine A<sub>260</sub> with a spectrophotometer. Save three 1 $\mu$ l aliquots of the ribosome pellet in 15 $\mu$ l IP buffer total from each condition on dry ice for later analysis by western blot; save one 2 $\mu$ l aliquot of ribosomes in 15 $\mu$ l IP buffer total from each condition for mass spectrometry. Store remaining immunopurified sample as one aliquot for RNA preparation. Freeze ribosomes and aliquots on dry ice and store at -80°C.

## 2.5. Analysis of immunopurified ribosomes by western blot

### 2.5.1. Western blotting

1. Add 5 $\mu$ l 4X Laemmli Sample Buffer plus  $\beta$ -mercaptoethanol to each 15 $\mu$ l aliquot of input ribosomes, flowthrough, final wash, and ribosome pellet from the immunopurification steps. Incubate at 100°C for 5 minutes, cool briefly, and vortex for 2–3 minutes to completely disrupt the ribosomes. Pulse spin very briefly to collect the liquid.
2. Load the samples onto a 10-well, 30  $\mu$ l 4–20% precast polyacrylamide gel; include protein markers (4.6–300kDa) and run in 1X Tris/glycine/SDS buffer at 40mA per gel for 30 minutes. Transfer samples to a PVDF Membrane using a semi-dry electrophoretic transfer apparatus at 15V, 45 minutes.
3. If the FLAG-tagged protein of interest is larger than 55kD, cut each membrane with a razor blade at the 55kD protein size marker, block with 5% nonfat dry milk, 1X PBS-T, and probe the top membrane portion with 1:10,000 diluted anti-FLAG antibody in 3% BSA, 1X PBS-T followed by 1:5000 diluted ECL Rabbit IgG, HRP-linked whole Ab (from donkey) secondary antibody; probe the bottom membrane portion with 1:5000 diluted anti-Tcm1 antibody (gift of Jonathan Warner, [43]) followed by 1:5000 diluted Mouse IgG (H+L) secondary antibody. If the FLAG-tagged protein of interest is smaller than 55kD, it will be necessary to probe first with anti-FLAG antibody, followed by stripping in western stripping buffer at 50°C for 45 minutes with shaking and washing extensively with water, followed by probing with anti-Tcm1 antibody. Detect antibody signal with ECL homebrew reagents (The Collier Lab Protocol Book, <https://case.edu/med/collier/Collier%20Protocol%20Book.pdf>) using Hyperfilm ECL film.

## 2.6. Analysis of immunopurified ribosomes by mass spectrometry

### 2.6.1. Preparation of samples for mass spectrometry

1. Prepare input and pellet samples for mass spectrometry as for western blotting by incubating 1 $\mu$ l input ribosomes or 2 $\mu$ l pellet ribosomes in 15 $\mu$ l IP buffer total

with 5 $\mu$ l 4X Laemmli Sample Buffer plus  $\beta$ -mercaptoethanol at 100°C for 5 minutes followed by vortexing for 2–3 minutes.

2. Load samples onto a 10-well, 30  $\mu$ l 4–20% precast polyacrylamide gel and run at 40mA for 5 minutes. Do not exceed 5 minutes of run time.
3. Fix and stain gel with Colloidal Blue Staining Kit for 3 hours; decant stain and wash with distilled water just until sample bands are visible. Place gel onto plastic wrap and cut out the entire stained region of each sample with a razor blade, not more than 1cm x 1cm pieces. Place gel slice into a microcentrifuge tube and add 500 $\mu$ l water. Store at 4°C until the samples are sent to a mass spectrometry core facility, within a few days.
4. In the mass spectrometry facility, remove water and add 200 $\mu$ l of 250 mM ammonium bicarbonate. For reduction, add 20 $\mu$ l of a 45mM solution of 1, 4 dithiothreitol (DTT) and incubate at 50°C for 30 min. Cool samples to room temperature and then, for alkylation, add 20 $\mu$ l of a 100mM iodoacetamide solution and allow to react for 30 min. Wash gel slices 2 X with 1 ml water aliquots. Remove water and place 1ml of 50:50 (50 mM ammonium bicarbonate: acetonitrile) in each tube and incubate samples at room temperature for 1hr. Remove the solution and add 200  $\mu$ l of acetonitrile to each tube at which point the gels slices will turn opaque white. Remove acetonitrile and further dry gel slices in a Speed Vac. Rehydrate gel slices in 100 $\mu$ l of 4ng/ $\mu$ l of sequencing grade trypsin (Sigma) in 0.01% ProteaseMAX Surfactant (Promega): 50 mM ammonium bicarbonate. Add additional bicarbonate buffer to ensure complete submersion of the gel slices. Incubate samples at 37°C for 18 hrs. Remove the supernatant of each sample and place in a separate 1.5ml Eppendorf tube. Further extract gel slices with 200 $\mu$ l of 80:20 (acetonitrile: 1% formic acid). Combine the extracts with the supernatants of each sample, then dry the samples completely in a Speed Vac.

**2.6.2. LC/MS/MS Analysis**—Reconstitute tryptic peptide digests in 25 $\mu$ l 5% acetonitrile containing 0.1% (v/v) trifluoroacetic acid and separate on a NanoAcquity (Waters) UPLC. In brief, a 2.5 $\mu$ l injection is loaded in 5% acetonitrile containing 0.1% formic acid at 4.0  $\mu$ l/min for 4.0 min onto a 100  $\mu$ m I.D. fused-silica pre-column packed with 2 cm of 5 $\mu$ m (200Å) Magic C18AQ (Bruker-Michrom) and eluted using a gradient at 300nl/min onto a 75 $\mu$ m I.D. analytical column packed with 25cm of 3 $\mu$ m (100Å) Magic C18AQ particles to a gravity-pulled tip. The solvents are A, water (0.1% formic acid) and B, acetonitrile (0.1% formic acid). A linear gradient is developed from 5% solvent A to 35% solvent B in 60 minutes. Ions are introduced by positive electrospray ionization via liquid junction into a Q Exactive hybrid mass spectrometer (Thermo). Mass spectra are acquired over  $m/z$  300–1750 at 70,000 resolution ( $m/z$  200) and data-dependent acquisition selects the top 10 most abundant precursor ions for tandem mass spectrometry by HCD fragmentation using an isolation width of 1.6 Da, collision energy of 27, and a resolution of 17,500

**2.6.3. Data analysis**—Raw data files are peak processed with Mascot Distiller (version 2.5, Matrix Sciences, Ltd.) prior to database searching with Mascot Server (version 2.5) against the *S. cerevisiae* index of the SwissProt database. Search parameters include trypsin specificity with two missed cleavages. The variable modifications of oxidized methionine, pyroglutamic acid for N-terminal glutamine, N-terminal acetylation of the protein, and a fixed modification for carbamidomethyl cysteine are considered. The mass tolerances are 10 ppm for the precursor and 0.05Da for the fragments. Extracted ion chromatograms are generated for the precursor ions and quantitated using a variation of the average method described by Silva et al. [44] in Mascot Distiller. Search results and precursor intensity data are also loaded into the Scaffold Viewer (Proteome Software, Inc.) for peptide/ protein validation and label free quantitation.

## 2.7. Preparation of RNA and removal of ribosomal RNA

### 2.7.1. RNA preparation from lysate

1. Prepare RNA from the equivalent of 60µl  $A_{260} = 100$  undigested clarified lysate from step 3.2.2. with the miRNeasy mini kit as in 2.7.2, step 3. Quantitate on a spectrophotometer. Analyze RNA on a Fragment Analyzer with a High Sensitivity RNA Analysis Kit (15 nt) to check quality of RNA. (Fig. 2A, left panel)
2. Bring 30µg total RNA to 17µl in water. Add 2µl 10X Baseline-ZERO DNase Reaction Buffer, 1µl Baseline-ZERO DNase. Incubate at 37°C, 15–30 minutes in a thermocycler.
3. Add 2µl 10X Baseline-ZERO DNase Stop solution, incubate at 65°C, 5 min. Add 180µl water, extract 1X with Phenol:Chloroform:IAA, pH6.6. Reserve the aqueous layer and add 20µl 3M NaOAc, 2µl 10mg/ml glycogen, 600µl ethanol; place on dry ice 30 minutes; spin in a benchtop centrifuge at 12000 rpm (12,638 g), 4°C, 30 minutes; and wash the pellet twice with 70% ethanol. Decant ethanol, dry the pellet and dissolve in 30µl water. Measure the concentration on a spectrophotometer.

### 2.7.2. RNA preparation from ribosomes

1. Prepare RNA from the entire sample of immunoprecipitated ribosomes. If preparing a library from ribosomes which have not undergone immunoprecipitation, dilute the ribosomes in IP buffer to the same concentration as the immunoprecipitated ribosomes in 60µl IP buffer plus 0.5mM DTT, 1X protease inhibitors, 20U/ml Superase-In.
2. Add 2.5 U Turbo DNase to 60µl ribosomes; incubate on ice, 10 min.
3. Immediately extract RNA from ribosomes using the miRNeasy mini kit, following the manufacturer's protocol for the preparation of total RNA including small RNA, eluting into 30µl water in a 1.5ml flat cap microcentrifuge tube. Quantitate on a Qubit 3.0 Fluorometer with the RNA HS assay kit. For immunopurified ribosomal RNA, dilute 1:10 in water; for total ribosomes, dilute 1:50 prior to quantitation. Freeze purified RNA samples on dry ice and store at



–80°C until library preparation, up to 1 week. Analyze RNA on a Fragment Analyzer with a High Sensitivity RNA Analysis Kit (15 nt) to check quality of RNA (Fig. 2A, right panel).

### 2.7.3 Removal of ribosomal RNA

1. Ribosomal RNA is removed from RNA purified from both ribosomes and lysate using the Ribo-Zero Gold rRNA Removal Kit (Yeast).
2. For RNA from lysate, perform ribosomal RNA removal on one aliquot of 5µg RNA. Perform the rRNA removal using the manufacturer's protocol for 2.5–5µg input RNA. Transfer the supernatant to a fresh 1.5ml flat cap microcentrifuge tube and bring the volume up to 180µl with water.
3. For RNA from ribosomes, divide all the RNA from one sample and perform ribosomal RNA removal on two separate aliquots of ~14µl each. Perform the rRNA removal using the manufacturer's protocol for 1–2.5µg input RNA. Pool the supernatants from one sample for a combined total of 180µl and transfer to a 1.5ml flat cap microcentrifuge tube.
4. Add to 180µl RNA: 18µl 3M NaOAc, 2µl 1M MgCl<sub>2</sub> (to ribosome protected fragment (RPF) RNA only), 2µl 10mg/ml glycogen, 600µl cold 100% ethanol; place on dry ice, 30 minutes; spin in a benchtop centrifuge at 12,000 rpm (12,638 g), 4°C, 30 minutes; and wash the pellet twice with 80% ethanol. Carefully decant ethanol and dry the pellet. Dissolve RPF RNA in 23µl water. Dissolve RNA from lysate in 20µl Fragment/Prime/Finish mix from the TruSeq Stranded mRNA Sample Prep kit. Analyze RNA on a Fragment Analyzer using the High Sensitivity RNA Analysis Kit (15 nt) to verify removal of ribosomal RNA (Fig. 2B).

## 2.8. Preparation of RNA-Seq libraries

Libraries are prepared for RNA-Seq using the TruSeq Stranded mRNA Sample Prep kit following manufacturer's directions starting from the RNA fragmentation step (addition of Fragment/Prime/Finish Mix), without preceding steps to isolate polyA-containing mRNA.

## 2.9. Preparation of ribosome profiling libraries

**2.9.1. 3'-Dephosphorylation and 5'-phosphorylation of ribosome protected fragments**—The following protocol has been adapted from Guo, *et al.* [45].

1. Heat 20µl RPF RNA in a thermocycler at 80°C, 2 minutes. To 20µl RPF RNA, add 50µl 1.5X MES-NaOH buffer, 1µl SuperaseIn, and 1.2µl T4 polynucleotide kinase (T4 PNK). Incubate at 37°C, 2 hours; then 65°C, 20min. Add 72.2µl H<sub>2</sub>O.
2. Clean up with RNA Clean and Concentrator-5 kit but adjust RNA Binding Buffer (total volume as needed) by mixing an equal volume of buffer and ethanol. Add 290µl adjusted RNA Binding Buffer to each 145µl sample, mix. Proceed according to manufacturer's instructions, eluting into 16µl water.

3. Add to 15.6µl RNA: 2µl 10X T4PNK buffer, 2µl 10mM ATP, 0.4µl T4 PNK, 1µl SuperasIn. Incubate in a thermocycler 37°C, 1 hour, then 60°C, 10 minutes.
4. Clean up with RNA Clean and Concentrator-5, adjusting RNA Binding Buffer (total volume as needed) by mixing an equal volume of buffer and ethanol. Add 40µl adjusted RNA Binding Buffer to each 20µl sample, mix. Proceed according to manufacturer's instructions, eluting into 12µl water.

**2.9.2. Ribosome profiling libraries**—Ribosome profiling libraries are made using the NEXTflex Small RNA-Seq Kit v3 with the following modifications:

1. All steps can be performed in a 0.2ml 96-well PCR plate in a thermocycler.
2. Use 1:4 dilutions of both the NEXTflex 3' 4N Adenylated Adapter and NEXTflex 5' 4N Adapter.
3. Libraries will require 22–25 cycles of PCR amplification depending on the starting amount of RPF RNA. Check 1µl on a Fragment Analyzer after 22 cycles using the High Sensitivity NGS Fragment Analysis Kit, holding PCR reactions at –20°C during analysis. Thaw samples and perform 2–3 more PCR cycles if needed and check on a Fragment Analyzer again before gel-free size selection and cleanup. All samples for one study ideally should have the same number of PCR cycles.
4. Perform gel-free size selection and cleanup of the cDNA and final PCR products according to the manufacturer's supplemental protocol. For the PCR bead cleanup step, the protocol was altered by bringing the PCR reaction to 25µl with water, using 65µl beads, and eluting into 13.5µl Resuspension Buffer.

**2.9.3. Quantitate RNA-Seq and ribosome profiling libraries with a Qubit using the dsDNA HS Assay Kit.**—Check the quality of the libraries on a Fragment Analyzer using the High Sensitivity NGS Fragment Analysis Kit (Fig. 2C), prior to sequencing on a NextSeq500 using a NextSeq 500/550 High Output v2 kit (75 cycles) to generate single-end reads. Careful pipetting and dilution of samples and reagents for Qubit quantitation can eliminate the need for subsequent qPCR quantitation.

## 2.10. Important considerations

1. For efficient cell rupture, do not grind more than 15ml cell volume in the Cryomill at one time. Harvested cells from 2 liters of medium fulfill this requirement if the final  $A_{600}$  is less than 0.8.
2. Work quickly and efficiently when harvesting cells by vacuum filtration to ensure quality ribosome profiling libraries. Rapid transfer of cells to liquid nitrogen immediately after the growth medium is removed by filtration results in libraries with excellent three-nucleotide periodicity even in the absence of cycloheximide.
3. Pierce the caps of any centrifuge tubes used to hold liquid nitrogen prior to use to prevent rupture.

4. Cell pellets from 2 liters of each culture condition and ground cell powder can be pooled and stored at  $-80^{\circ}\text{C}$  for several weeks until needed. Be certain to prevent thawing or warming of the cell pellets or frozen cell powder until use.
5. When thawing the cell powder, only stir the tube in tepid water until most, but not all, of the frozen material is thawed; place the sample on ice and continue gently stirring until remaining ice is thawed. Keep cell lysate at  $4^{\circ}\text{C}$  until the RNaseI digestion step.
6. Do not exceed 0.5mM DTT in the IP buffer to avoid damaging the affinity gel.
7. Do not exceed 5 minutes of run time on a polyacrylamide gel when preparing samples for mass spectrometry, to minimize the amount of gel entering the mass spectrometry apparatus.
8. Do not use an old ( $>2$  months) previously opened miRNeasy kit for the RNA preparation steps, as we have found this to produce RNA of variable quality.
9. Small RNA fragments are susceptible to loss by adhesion to the sides of the microcentrifuge tube, or loss of pellet during ethanol precipitation steps. Always resuspend RNA pellets by pipetting, never vortex, and very carefully decant ethanol to avoid loss. Save all decanted ethanol until the final washed pellet is verified visually. After the final ethanol wash, pulse spin and carefully remove remaining ethanol with a round-tip gel loading pipette tip and dry before dissolving in buffer, being careful not to lose the pellet due to static charge. We have found that the microcentrifuge tubes used in this protocol are the least susceptible to RNA loss. Addition of  $\text{MgCl}_2$  to the RPF RNA ethanol precipitation step enhances recovery of small ( $<30\text{nt}$ ) RNA [46].
10. Four RNA-Seq or four ribosome profiling libraries can be pooled per sequencing run.
11. The first four and last four bases of each ribosome profiling library read following adapter trimming are random barcodes, thereby allowing removal of PCR duplicates from bioinformatic analysis if desired.

### 3. Results and Discussion

#### 3.1. Overexpressed N-terminally FLAG-tagged Upf1 proteins complement nonsense-mediated mRNA decay and growth phenotypes.

Preliminary co-immunoprecipitation experiments determined that expression of tagged Upf1 on a single-copy yeast plasmid and subsequent pulldown of purified ribosomes did not produce sufficient material or enrichment of Upf1 relative to ribosomal proteins to perform ribosome profiling analysis of Upf1-associated ribosomes; however, expression of FLAG-Upf1 under the *TDH3* [47] promoter on an episomal yeast plasmid and subsequent pulldown resulted in near stoichiometric relative amounts of Upf1 and ribosomal proteins as determined by mass spectrometry (data not shown). Therefore, FLAG-tagged Upf1 proteins expressed from yeast episomal plasmids were introduced into their respective yeast deletion

strains. In addition, Upf3 was placed under control of the *TPII* promoter for efficient expression.

Overexpression of Upf proteins has been demonstrated to rescue mutant NMD as well as nonsense suppression phenotypes in yeast and human cells [32, 48–53]. To ascertain whether the increased expression levels of these proteins were likely to yield biologically relevant results in downstream experiments, we measured the growth rate and NMD phenotype of strains bearing those plasmids. All three proteins were able to complement the NMD (Fig. 3) and growth phenotypes (Fig. 4) of their respective deletion strains. The growth curves of both FLAG-Upf1 and FLAG-Upf2 overexpression strains were indistinguishable from the wild-type strain (Fig 4, left panel), while the FLAG-Upf3 overexpression strain exhibited a growth curve intermediate between wild-type and *upf3* (Fig. 4, bottom row). That said, we recognize that overexpression of the three Upfs probably skews the equilibrium of ribosome association in favor of binding.

### 3.2. Immunopurification selects for and enriches Upf-associated ribosomes as determined by western blotting and mass spectrometry.

Ribosomes (80S) were purified from RNaseI-treated lysates prepared either in the presence or absence of cycloheximide and two biological replicates were generated for each combination of strain and cycloheximide condition. Yeast strains bearing FLAG-tagged Upf1, Upf2, or Upf3 were subjected to immunoprecipitation and elution from anti-FLAG M2 affinity gel and analyzed by western blot and mass spectrometry. Western blot analysis confirmed that FLAG-Upf-associated ribosomes were recovered from these preparations (Fig. 5A) and quantitation of mass spectrometry data by a variation of the average protocol [44] in Mascot Distiller was performed to determine the relative abundance of the Upf protein relative to all other proteins detected in the analysis (Supplementary Table 1) The ratio of the relative abundance of the Upf protein to the average relative abundance of the core ribosomal proteins (Upf:RP) present in the sample (Supplementary Table 2) was used to evaluate recovery and enrichment of Upf-associated ribosomes.

For all three proteins examined here, Upf:RP was greater than 1:1 after immunopurification (Fig. 5B, Pellet fraction). Enrichment of Upf protein in immunopurified ribosomes was evaluated by dividing the Upf:RP of immunopurified ribosomes by that of their corresponding input ribosomes (Fig. 5C). Inclusion of cycloheximide had no significant effect on enrichment of Upf proteins or Upf:RP after immunoprecipitation (Figs. 5C and 5B). In addition to ribosomal proteins and the Upf factors, spectra apparently from nascent peptides were represented in all samples. Because the Upf proteins are N-terminally tagged, it is likely that a proportion of the Upf spectra also represent pulldown of their nascent peptides.

### 3.3. Deep sequencing analyses

The ribosome profiling library preparation method used here eliminates gel size selection and has fewer precipitation steps than conventional methods. These changes should result in less sample loss and more efficient recovery of ribosome protected fragments compared to standard methods. Due to the limited number of ribosomes recovered after

immunopurification, efficient recovery of ribosome protected fragments was a particular concern. RNA-Seq and ribosome profiling libraries were generated from all samples, sequenced on a NextSeq500, and subjected to conventional bioinformatics analysis [19, 39, 54]. Inspection of read alignments from ribosome profiling libraries revealed that approximately 21% (Upf1), 6% (Upf2), and 9% (Upf3) of ribosomes were recovered subsequent to binding of the FLAG-tagged nascent peptide to the affinity gel (data not shown). While it is trivial to eliminate reads arising from these mRNAs from downstream analyses, it could be advantageous to use a C-terminal tag in future experiments, provided that such tagged proteins are biologically indistinguishable from their untagged cohort and that their associated ribosomes are recoverable by immunoprecipitation.

The incorporation of four random nucleotides on both sides of our ribosome protected fragments during library preparation allowed us to remove PCR duplicates from downstream analyses, thereby eliminating artificial read density “spikes” from the ribosome profiling data. Ribosome profiling libraries prepared with or without cycloheximide recovered a wide range of ribosome-protected RNA fragment sizes and allowed simultaneous observation of different fragment size classes within a given sample, fragment size class comparisons between total and immunopurified ribosome fractions, and read distribution across the transcriptome (manuscript in preparation).

In conclusion, high-level expression of epitope-tagged Upf proteins enabled purification and recovery of Upf factor-associated ribosomes, subsequent analysis of their protein content by mass spectrometry and, ultimately, identification of their bound mRNA fragments. The ribosome profiling library preparation method described here eliminates some unaddressed pitfalls of conventional methods and allows recovery and selective ribosome profiling analysis [55] of reads from purified, factor-bound ribosomes.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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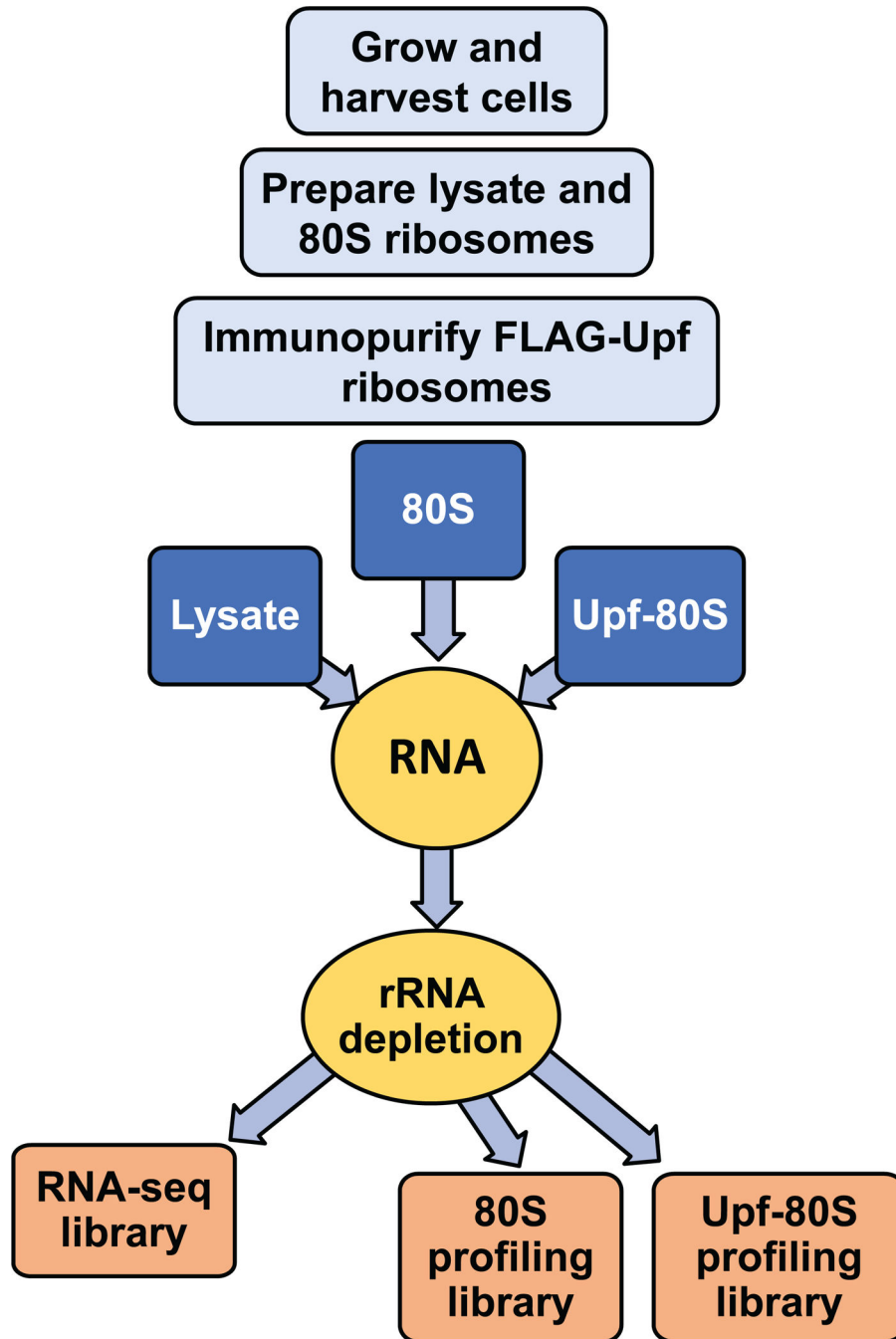
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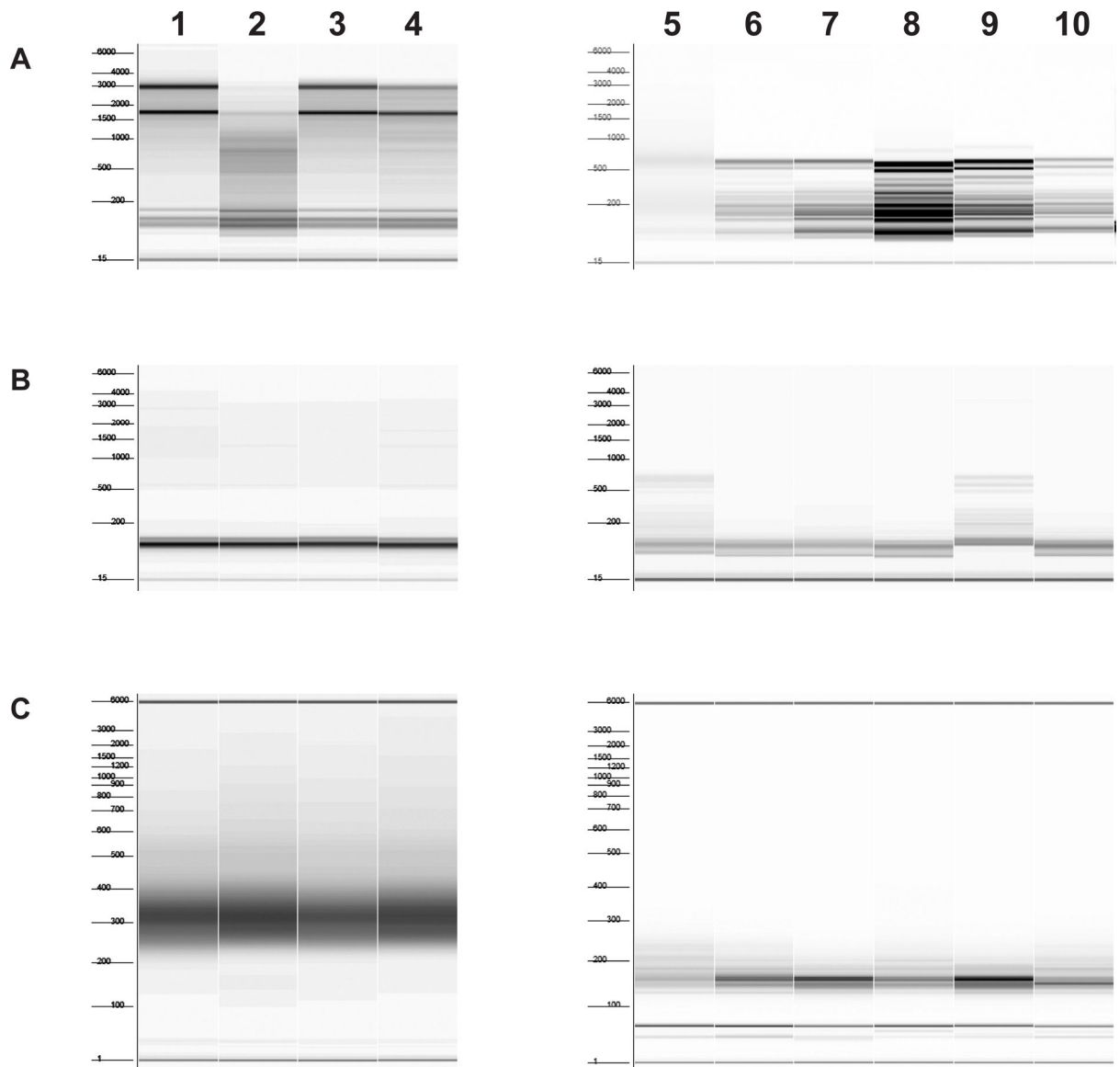


### Highlights

- Immunopurification enables detailed analysis of ribosomes associated with factors essential for nonsense-mediated mRNA decay (NMD).
- Stoichiometry and enrichment of NMD factors relative to ribosomal proteins pre-and post-purification can be estimated by mass spectrometry.
- Eliminating in-gel RNA and cDNA size selection and including random barcodes in the preparation of ribosome profiling libraries enables a translational snapshot from a wide range of ribosome-protected fragment sizes.

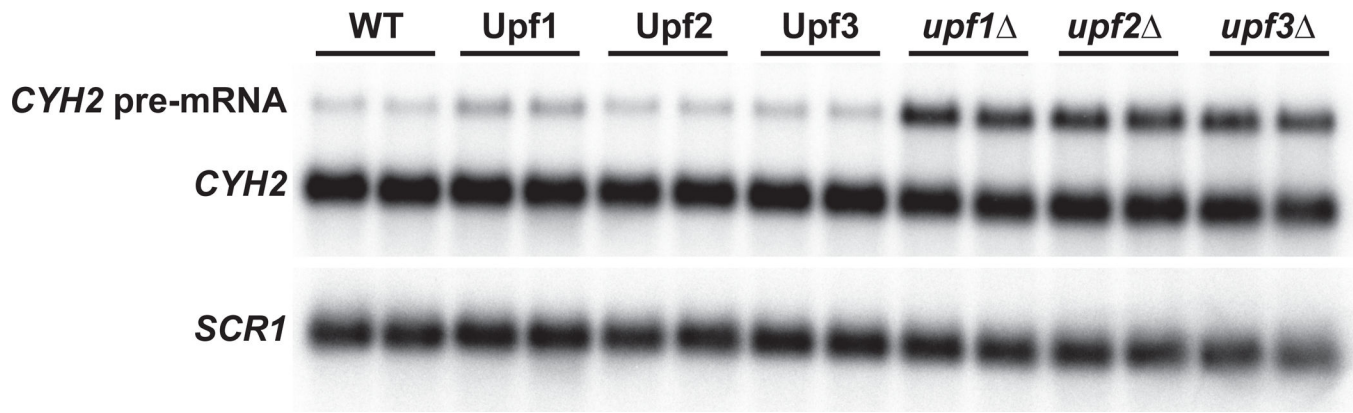


**Figure 1.**  
Diagram of protocol workflow.



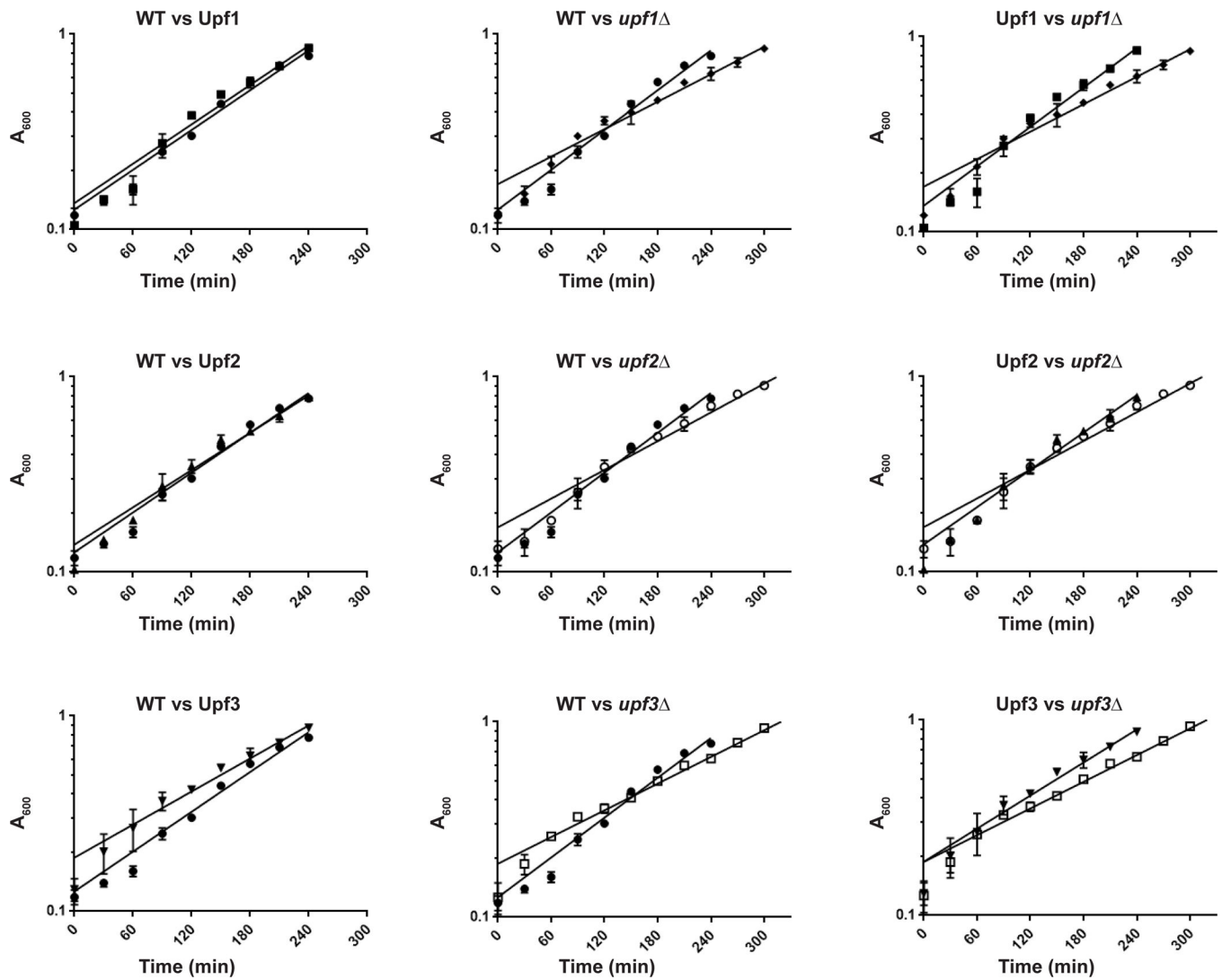
**Figure 2. Fragment analyzer examples.**

Starting material lanes 1–4, yeast lysate, untreated with RNase I; lanes 5–6, immunopurified ribosomes; lanes 7–10, total ribosomes. Numbers to the left of each figure indicate marker sizes in nt. **A.** RNA before removal of ribosomal RNA. Samples in lanes 2 and 5 are degraded and should be re-prepared; if the problem persists, it is necessary to make another preparation of lysate and ribosomes. Samples in lanes 5–10 have fragmented ribosomal RNA due to incubation of the lysate from which they were prepared with RNase I; this is expected. **B.** RNA after removal of ribosomal RNA. Samples in lanes 5 and 9 have incomplete removal of ribosomal RNA; these RNAs should be subjected to another round of ribosomal RNA removal. **C.** Finished libraries after PCR and cleanup. Sample in lane 5 is a library with a broad size range. This library should be prepared again if sequencing results indicate poor quality.



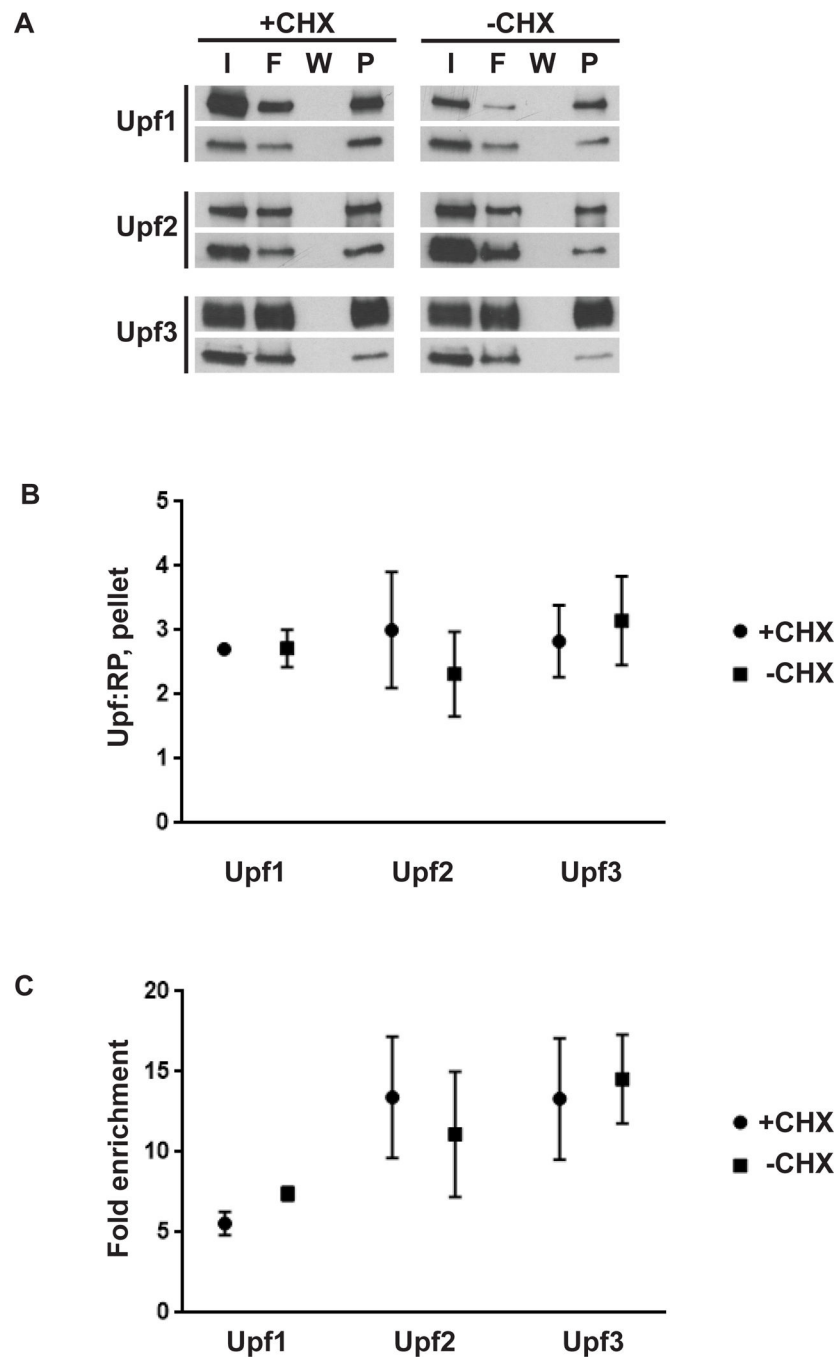
**Figure 3. Overexpressed N-terminally FLAG-tagged Upf proteins complement nonsense-mediated mRNA decay phenotypes.**

Northern blot of total RNA from yeast strains expressing: empty vector (WT); high-copy FLAG-tagged Upf1, Upf2, or Upf3 in the respective deletion strains; or bearing a deletion of the *UPF1*, *UPF2* or *UPF3* coding region (designated *upf1*, *upf2*, or *upf3*). NMD phenotype was determined by hybridization with a random-primed labeled probe for *CYH2*. *SCR1* was used as a loading control.



**Figure 4. Growth curve comparisons.**

Left panel, between WT and FLAG-tagged Upf strains; center panel, between WT and deletion strains; right panel, between FLAG-tagged Upf strains and corresponding deletion strains. WT, ●; FLAG-Upf1, ■; FLAG-Upf2, ▲; FLAG-Upf3, ▼; *upf1*, ◆; *upf2*, ○; *upf3*, □. Timepoints as mean  $A_{600}$  from cultures of two independent isolates. Error bars=range of values.



**Figure 5. Overexpressed N-terminally FLAG-tagged Upf proteins allow efficient recovery of Upf-associated ribosomes.**

**A.** Western blot of input (I), flowthrough (F), final wash (W), or pelleted ribosomes (P) from anti-FLAG immunoprecipitation reactions. Cultures were cycloheximide treated (+CHX) or untreated (-CHX) prior to cell collection. Western blots were probed with anti-FLAG (top panel per set) or anti-TCM1/RPL3 antibodies (bottom panel per set). **B.** Relative abundance of Upf protein per average relative abundance of core ribosomal proteins present in a sample (Upf:RP) in pellet as determined by mass spectrometry; mean and range of two biological

replicates per strain and condition. **C.** Fold enrichment of Upf protein in pellet vs input; mean and range of two biological replicates per strain and condition. FLAG-tagged proteins are indicated, all panels.

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**Table 1.****Equipment**

<b>Name</b>	<b>Manufacturer</b>
Shaking incubator	Thermo Scientific
2.8liter Fernbach flasks	Pyrex
1liter vacuum filtration device	Kontes
Cryomill	Retsch
Stainless steel jar	Retsch (01.462.0332)
20mm stainless steel ball	Retsch (05.368.0062)
Tabletop refrigerated centrifuge	Thermo Fisher
Ultracentrifuge	Beckman Coulter
Spectrophotometer	Beckman
Thermomixer	Eppendorf
Microcentrifuge	Eppendorf
Platform rocker	Fisher scientific
Tabletop ultracentrifuge	Beckman Coulter
Mini-PROTEAN Tetra Cell	BioRad
Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell	BioRad
Shaking water bath	VWR
Film processor	Kodak
Speed vac	Savant Instruments, Inc.
NanoAcquity UPLC	Waters
75 $\mu$ m I.D. analytical column	
Q Exactive hybrid mass spectrometer	Thermo
Mascot Distiller version 2.5	Matrix Sciences, Ltd.
Mascot Server version 2.5	Matrix Sciences, Ltd.
Scaffold Viewer	Proteome Software, Inc.)
Qubit 3.0 Fluorometer	Life Technologies
Thermocycler	Eppendorf
Fragment analyzer	Advanced Analytics



**Table 2.**

## Reagents and Consumables

Product	Manufacturer	Catalog number
1M Tris, pH7.5	Corning	46-030-CM
5M NaCl	Cellgro	46-032-CV
1M MgCl <sub>2</sub>	Ambion	AM9530G
RNase-free water	Ambion	AM9922
Triton X-100	Sigma	T8787
Dithiothreitol (DTT)	Sigma	D9779
Phenylmethylsulfonyl fluoride	Sigma	P7626
Igepal CA-630	Sigma	I8896
0.2M MES-NaOH, pH5.5	Boston Bioproducts	BB-109
10X PBS	Fisher Scientific	BP3994
Tween-20	Sigma	P1379
Sodium dodecyl sulfate (SDS)	Sigma	L3771
β-mercaptoethanol	Life Technologies	21985
0.8μm cellulose acetate membrane filter	Sterlitech	CA089025
Protease inhibitors	Pierce	88266
Cycloheximide	Calbiochem	239764
RNaseI	Invitrogen	AM2294
Superase-In	Ambion	AM2694
anti-FLAG M2 affinity gel	Sigma	A2220
Amicon-Pro affinity exchange device	Millipore	ACS500024
3/8" Tough-Spot	Diversified Biotech	SPRL-1100
20g BD PrecisionGlide hyperdermic needle	BD	305175
3X FLAG peptide	Sigma	F4799
Amicon Ultra-2 100K centrifugal filter unit	Millipore	UFC210024
Sucrose	Sigma	S0389
Thick wall polyallomer 7X20mm centrifuge tubes	Beckman Coulter	343621
4X Laemmli sample buffer	BioRad	161-0747
10-well, 30 μl 4-20% Mini-PROTEAN® TGX Precast Protein Gel	BioRad	4561093
ProSieve Quadcolor protein marker, 4.6- 300kDa	Lonza	00193837
10X Tris/glycine/SDS buffer	BioRad	161-0732
Immobilon-P PVDF membrane	Millipore	IPVH00010
Nonfat dry milk	Grocery store	
Bovine serum albumin	Sigma	A4503
anti-FLAG antibody	Sigma	F7425
ECL Rabbit IgG, HRP-linked whole Ab (from donkey)	GE Healthcare	NA934
Mouse IgG (H+L) secondary antibody	ThermoFisher Scientific	625620
Hyperfilm ECL	Sigma	GE28-9068-35
Colloidal Blue staining kit	Invitrogen	LC6025
Ammonium bicarbonate		

Product	Manufacturer	Catalog number
Dithiothreitol		
Iodoacetamide		
Acetonitrile		
Trypsin		
ProteaseMAX Surfactant	Promega	
Formic acid		
Trifluoroacetic acid		
5 µm (200Å) Magic C18AQ	Bruker-Michrom	
3 µm (100Å) Magic C18AQ	Bruker-Michrom	
Turbo DNase	ThermoFisher Scientific	AM2238
miRNeasy mini kit	Qiagen	217004
1.5ml flat cap microcentrifuge tube	Laboratory Products Sales	L259901
Qubit RNA HS assay kit	ThermoFisher Scientific	Q32852
High sensitivity RNA analysis kit (15 nt)	Advanced Analytical	DNF-472
Baseline-ZERO DNase	Epicentre	DB0715K
Phenol:Chloroform:IAA	Ambion	AM9732
3M sodium acetate (NaOAc)	Sigma	S7899
Glycogen	Roche	10901393001
Ethanol	Sigma	E7023
Ribo-Zero Gold rRNA removal kit (Yeast)	Illumina	MRZY1324
TruSeq Stranded mRNA sample prep kit	Illumina	RS-122–2101
NextSeq 500/550 High Output v2 kit (75 cycles)	Illumina	FC-404–2005
Qubit DNA HS assay kit	ThermoFisher Scientific	Q32851
High Sensitivity NGS fragment analysis kit	Advanced Analytical	DNF-474
T4 polynucleotide kinase	New England Biolabs	M0201
RNA Clean and Concentrator-5	Zymo Research	R1016
100mM ATP	Thermo Scientific	R0441
NEXTflex Small RNA-Seq Kit v3	BIOO Scientific	NOVA-5132–06
0.2ml 96-well PCR plate	USA Scientific	1402–9600
Qubit dsDNA HS assay kit	ThermoFisher Scientific	Q32851
<u>5X -TRP dropout mix (grams):</u>		
Uracil	2	Sigma U-0750
Histidine	4	“ H-8125
Arginine	4	“ A-5131
Methionine	4	“ M-9625
Tyrosine	4	“ T-3754
Isoleucine	4	“ I-2752
Lysine	4	“ L-5626
Adenine	2	“ A-8751
Phenylalanine	4	“ P-2126
Leucine	4	“ L-8000
Aspartic acid	4	“ A-6683

Product		Manufacturer	Catalog number
Valine	4	“	V-0500
Threonine	4	“	T-8625
Serine	4	“	S-4500
<u>-TRP liquid medium</u>			
Yeast nitrogen base		1.7 g	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		5.0 g	
Water		up to 850 ml	
Autoclave, then add (per liter):			
40 % glucose		50 ml	
5X-TRP (2g/100ml water)		100 ml	

**Table 3.**

## Yeast strains

<b>Name</b>	<b>Genotype</b>
HFY114	<i>MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 UPF2 UPF3</i>
HFY871	<i>MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 upf1::HIS3 UPF2 UPF3</i>
HFY115	<i>MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 upf2::HIS3 UPF3</i>
HFY861	<i>MATa ade2-1 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 can1-100 UPF1 UPF2 upf3::HIS3</i>

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**Table 4.**

## Plasmids

Name	Description
YEplac112	Yeast episomal plasmid ( <a href="https://www.addgene.org/vector-database/4892/">https://www.addgene.org/vector-database/4892/</a> )
pG1-FLAG-Upf1	Yeast episomal plasmid containing entire <i>UPF1</i> coding region as a 3.6kb BamHI fragment; FLAG-tag fused at the N-terminus; expressed under the <i>TDH3</i> promoter
YEplac112-FLAG-Upf2	YEplac112 containing entire <i>UPF2</i> coding region, and the endogenous promoter; FLAG-tag fused at the N-terminus; same as YEplac112-F2-NMD2* (*NMD2=UPF2)
YEplac195-TPI-FLAG-Upf3	Yeast episomal plasmid containing entire <i>UPF3</i> coding region as a 2.2 kb SalI-XbaI fragment; FLAG-tag fused at the N-terminus; expressed under the <i>TPI</i> promoter

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