Translation inhibitors cause abnormalities in ribosome profiling experiments

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ABSTRACT

Ribosome profiling and high-throughput sequencing provide unprecedented opportunities for the analysis of mRNA translation. Using this novel method, several studies have demonstrated the widespread role of short upstream reading frames in translational control as well as slower elongation at the beginning of open reading frames in response to stress. Based on the initial studies, the importance of adding or omitting translation inhibitors, such as cycloheximide, was noted as it markedly affected ribosome coverage profiles. For that reason, many recent studies omitted translation inhibitors in the culture medium. Here, we investigate the influence of ranging cycloheximide concentrations on ribosome profiles in *Saccharomyces cerevisiae* and demonstrate that increasing the drug concentration can overcome some of the artifacts. We subjected cells to various manipulations and show that neither oxidative stress nor heat shock nor amino acid starvation affect translation elongation. Instead, the observations in the initial studies are the result of cycloheximide-inflicted artifacts. Likewise, we find little support for short upstream reading frames to be involved in widespread protein synthesis regulation under stress conditions. Our study highlights the need for better standardization of ribosome profiling methods.
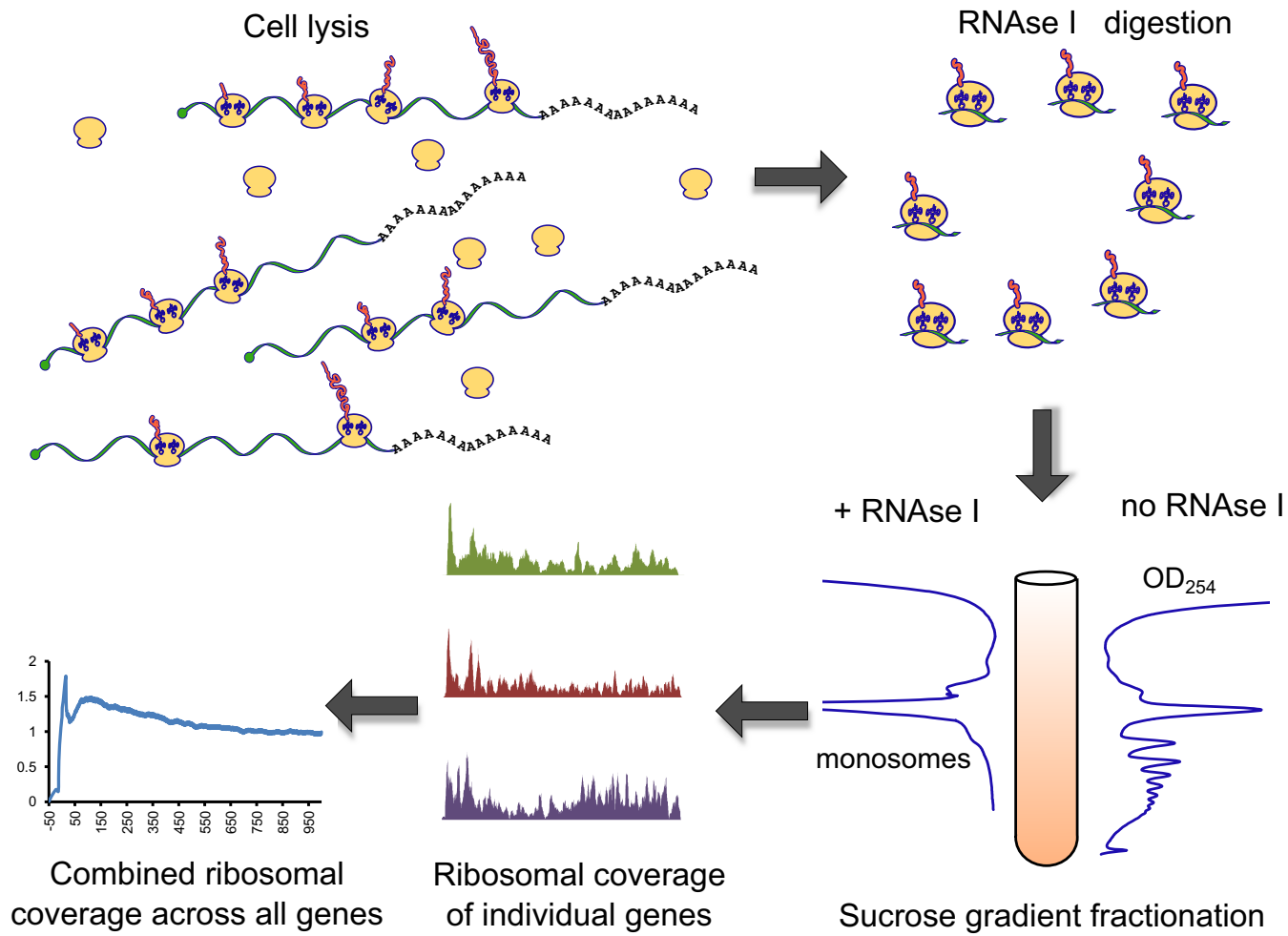
Figure 1. Ribosome profiling. Cell lysis releases a mixture of individual ribosomal subunits, assembled ribosomes in complex with mRNA and blank ribosomes with no RNA attached. Sucrose gradient fractionation allows separation and isolation of these components. Captured mRNA fragments are then sequenced on an Illumina platform.

MATERIALS AND METHODS

Extended material and methods can be found in Supplementary Information.

Yeast strains and growth conditions

Saccharomyces cerevisiae strain BY4741 was grown on YPD (Yeast extract, peptone, dextrose) agar plates for several days prior to experiments. Unless otherwise stated, the day before the experiment cells were transferred to a 50 ml flask of YPD medium and grown overnight at 30°C with shaking. A part of that culture was inoculated into 500 ml of fresh YPD at the initial $OD_{600} = 0.025$ and incubated at 30°C with shaking until the OD_{600} reached 0.5–0.6. If cultures were designated for cyloheximide treatment, the drug was added at the end of any additional stress-inducing incubation. Immediately after drug addition, cells were harvested by vacuum filtration on 65 um polyvinylidene difluoride (PVDF) filters (Millipore). It took exactly 5 min to collect the cells, which then were snap frozen in liquid nitrogen. If no drug treatment was needed, yeast cells were

beads. To prevent yeast thawing, pulverization was done in multiple 10 s cycles where vials were repeatedly submerged to liquid nitrogen after each cycle. Therefore, the content of vials was kept frozen during pulverization. Note that 1 ml of ice cold lysis buffer (20 mM Tris-HCl pH 8.0, 140 mM KCl, 5 mM $MgCl_2$, 1% Triton-X100, 100 μg/ml cycloheximide) was used to resuspend the pulverized cell powder. The lysates were clarified by centrifugation for 5 min. Note that 30 OD_{260} units of cell extract were treated with 600 units of RNAse I (Life Tech, Ambion) for 1 h at room temperature. The lysates were loaded on top of 10–50% sucrose gradient, prepared in the lysis buffer with no Triton.

collected in the same manner, but filtration was initiated 5 min before the stress had to finish.

Cycloheximide treatment

Concentrations of cycloheximide ranging from 1.56 to 10,000 μg/ml were used. We refer to 100 μg/ml as 'x1', because it was used to inhibit translation in all other studies cited in this report. Therefore, other concentrations were marked as x1/64, x1/16, x1/4, x8, x100. To achieve x8 concentration, we prepared the stock solution in dimethyl sulfoxide (DMSO). The highest possible concentration x100 was the most challenging. We first collected yeast cells by filtration, rapidly resuspended them in 5 ml of filtered YPD medium and added 5 ml of YPD with 20 mg/ml cycloheximide. This is the highest concentration possible considering drug solubility in water-based solvents. Stress conditions are listed in the Supplementary Information.

Cell lysis and ribosome isolation

Frozen cell paste pellets were pulverized in a Mini Bead Beater (BioSpec) using stainless steel vials and chromium

Ultracentrifugation in SW-41 Ti rotor for 3 h at 35 000 revolutions per minute and 4°C separated large ribosomal complexes from other cellular components. After fractionation in a sucrose gradient, the monosomal fraction was collected and footprints were isolated.

Sequencing library preparation

An oligonucleotide adapter was ligated to the 3′ end of footprints. It served as an anchor to a reverse transcription primer. Reverse transcription was followed by circular ligation and polymerase chain reaction amplification of li-

braries prior to sequencing on the Illumina HiSeq 2000 platform.

Footprint alignment

Bowtie software v. 0.12.7 ([6]) was used to align footprints to yeast genome (*S. cerevisiae* S288C genome, downloaded from SGD database with annotations). Custom Perl scripts were implemented to pre-process alignment files and plot ribosomal occupancy.

Ribosomal occupancy distribution plot

We selected all single exon genes longer than 1000 nucleotides expressed at reads per kilobase per million (rpkm) > 30. They were aligned by start codon and coverage at every nucleotide position of every gene was averaged. The plot covers 1000 nucleotides within reading frame plus 50 nucleotides upstream of the start codon. The average coverage density of the last 300 nucleotides was used to normalize ribosome occupancy so that each profile approached the value of 1.0. We used the entire footprint sequence to calculate coverage, therefore, the profile line appears smooth.

Alternatively, only 5′ or 3′ ends of footprints could be used, then the profile would be more irregular.

RESULTS AND DISCUSSION

Published studies on ribosomal profiling of both yeast and mammalian cells have used somewhat different sample preparation methods, which complicate direct comparison. Therefore, we reproduced some treatments and stresses using budding yeast as a model organism. *S. cerevisiae* cells were tested for amino acid starvation (as in ([1])), oxidative stress (as in ([2])) and heat shock (as in ([3])), with and without drug treatment in the culture medium. As expected, we observed a different distribution of ribosomal occupancy, wherein the broad peak downstream of the start codon decreased in the absence of the drug. Unexpectedly, there was no increase in response to stress (Figures [2]A–C and [3]). Apparently, none of the stress conditions tested targeted the translation elongation step.

To examine in more detail how the drug influences the ribosomal distribution, we performed a series of experiments with the cycloheximide concentrations ranging from 1.56 to
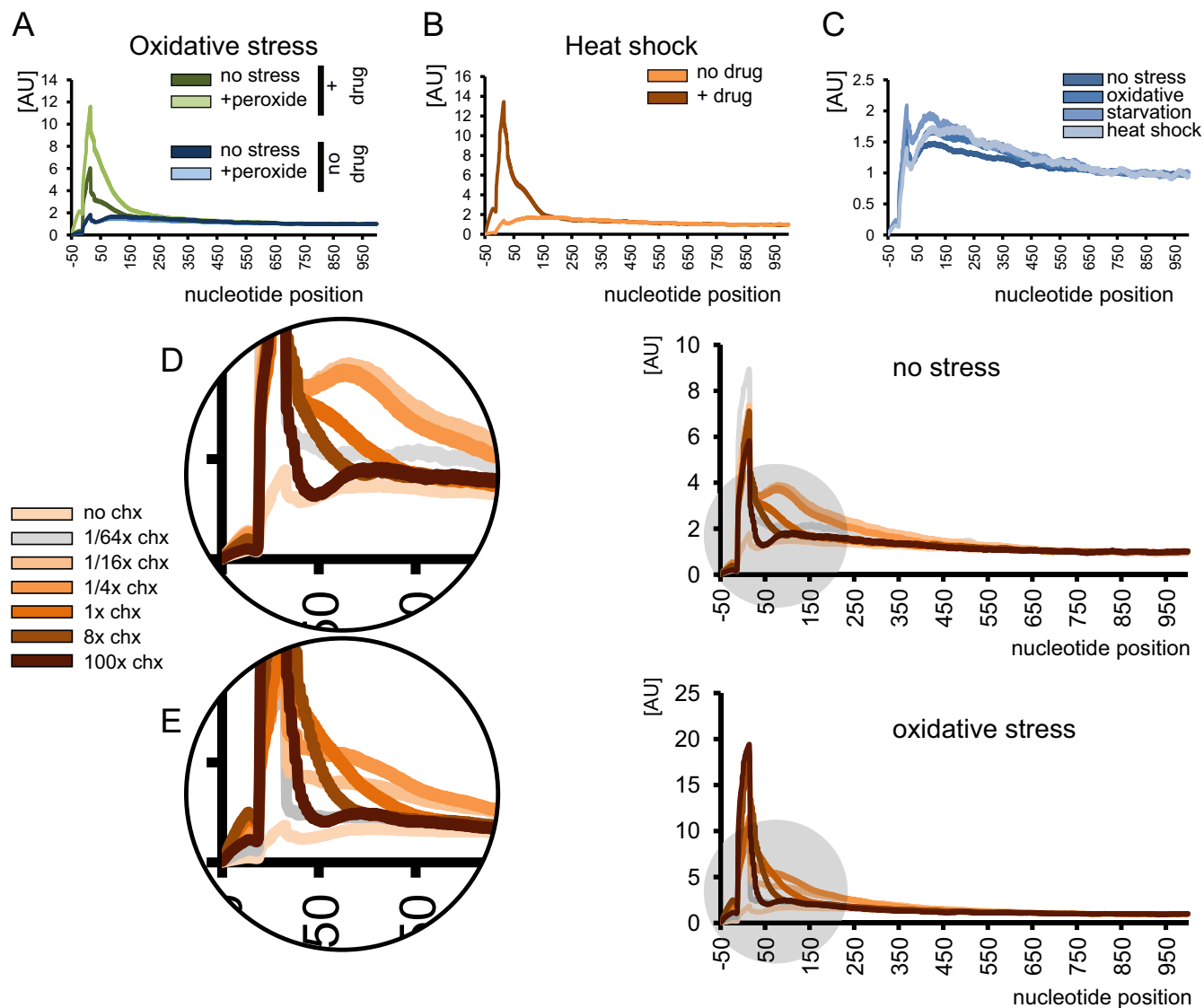
Figure 2. Ribosomal occupancy profiles and the effect of stress and drug treatment. (A) Control yeast cells and cells treated with hydrogen peroxide (0.2 mM) in the presence or absence of 100 μg/ml cycloheximide in the media. Nucleotide position count is relative to start codon. (B) Ribosome occupancy profiles of yeast cells undergoing heat shock (42°C, 20 min). The peak appears only when cycloheximide is added to the medium. (C) None of the three tested stress types lead to a significant increase of ribosomes at the 5′ proximities of reading frames in the absence of cycloheximide. Refer to Figure 3A and B for additional details on amino acid starvation. (D and E) Concentration of cycloheximide in the medium affects the shape of the profile, pointing to a passive diffusion model of cycloheximide entering live cells. Cells were grown in YPD medium in the absence of stress (D) or subjected to oxidative stress (0.2 mM hydrogen peroxide, 30 min) (E). Cycloheximide concentration does not immediately reach the threshold, under which all ribosomes are inhibited with 100% efficiency, instead increasing gradually. Therefore, following the treatment some ribosomes initiating translation continue protein synthesis until they encounter the drug, leading to a broad cumulative peak in the ribosomal occupancy profile.
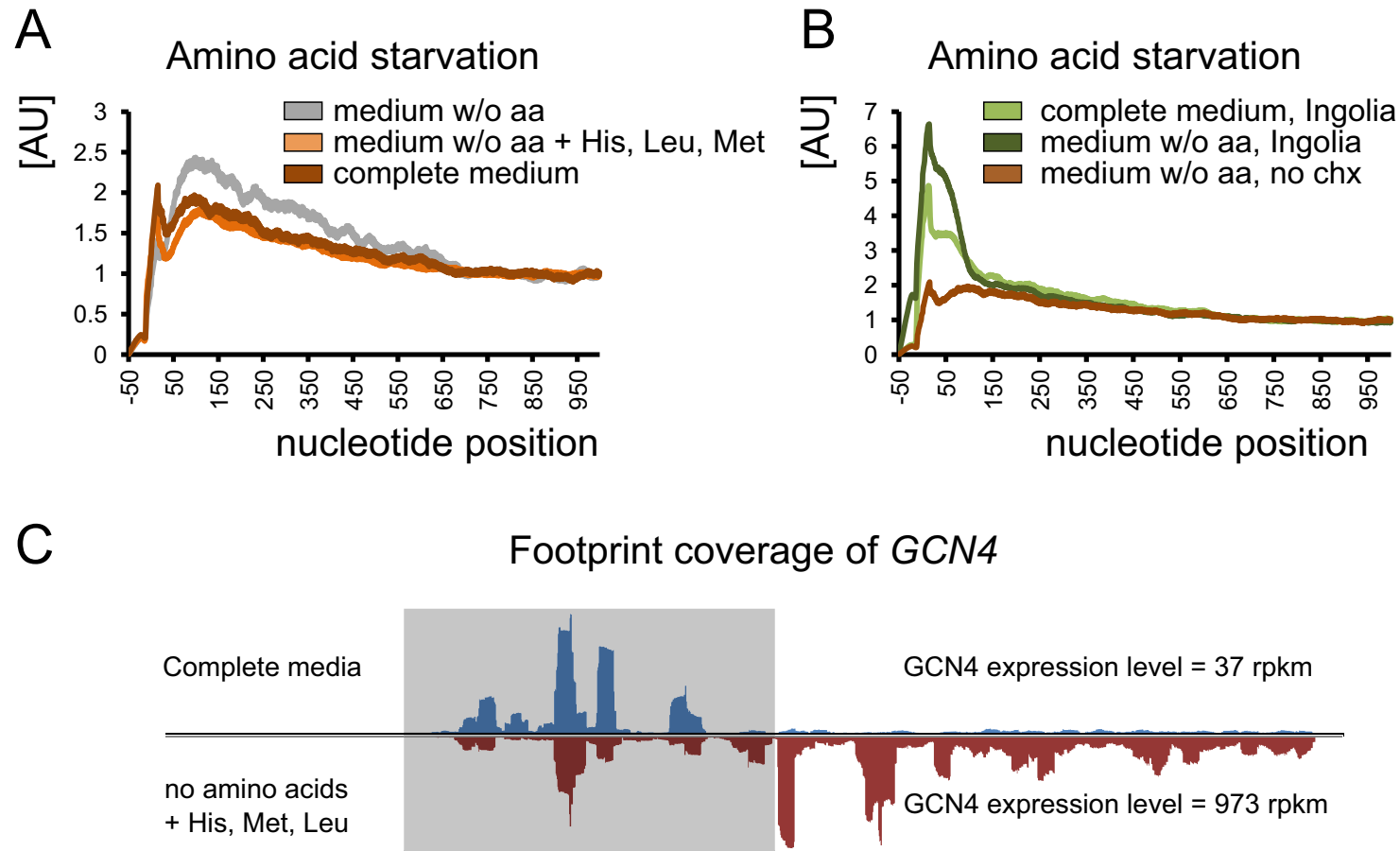
Figure 3. In-depth investigation of amino acid starvation and changes in the ribosomal profile. (A) Repeating the experiment as was done in (1) but without cycloheximide pre-treatment still leads to a slightly different ribosomal occupancy profiles (gray and dark brown lines on the graph). However, the yeast strain BY4741, used in that study is auxotroph in histidine, leucine and methionine, which are used as selective markers. Depletion of culture medium of all amino acids cannot be considered as starvation, because the lack of three essential amino acids will lead to cell death rather than to metabolism switching toward synthesis of its own amino acids. Therefore, we supplemented the medium without amino acids with normal levels of His, Met and Leu. As a result, the difference in ribosomal profiles between starved and non-starved conditions disappeared. Thus, amino acid starvation does not cause the accumulation of ribosomes at the beginning of ORFs or uORFs. The only scenario when this accumulation was observed is the absolute lack of the essential amino acids, leading to ribosome stalling at corresponding codons. This is a very extreme case, which has little in common with regulation *per se*. (B) Ribo-seq data published in (1) were processed with our analytical approaches (cycloheximide was present in the culture medium). Brown line is based on our experiment (same as in Figure 3A). (C) Footprint coverage of *GCN4* in response to amino acid stress derived from our experiments.
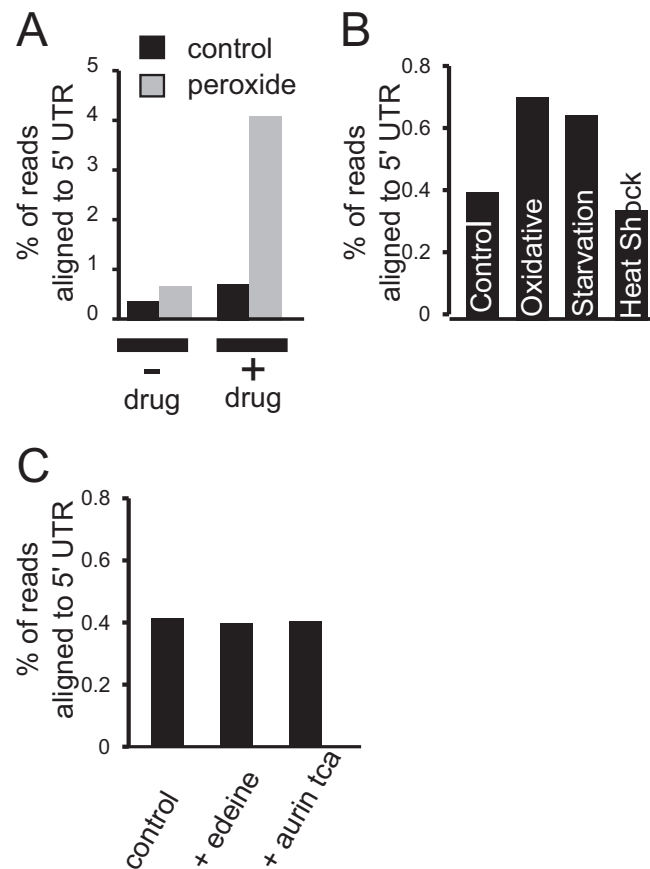
Figure 4. uORF and 5′ UTR coverage in ribosome profiling experiments. (A) There is a dramatic difference in cumulative 5′ UTR occupancy if cycloheximide treatment is omitted. (B) None of the three examined stresses significantly increase 5′ UTR ribosomal occupancy. Although oxidative stress and amino acid starvation do slightly increase 5′ UTR occupancy, the effect is minimal compared to what was previously found (1,2). (C) Addition of translation initiation inhibitors does not affect cumulative ribosomal occupancy at the 5′ UTR.
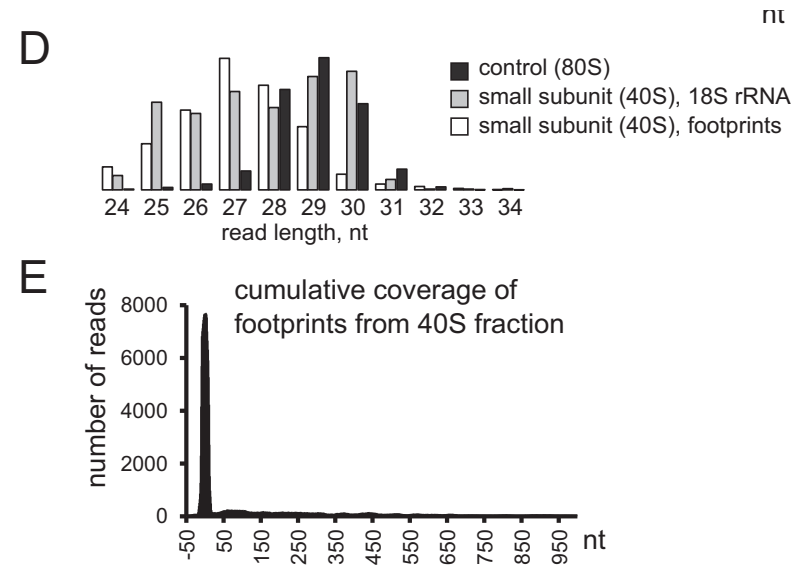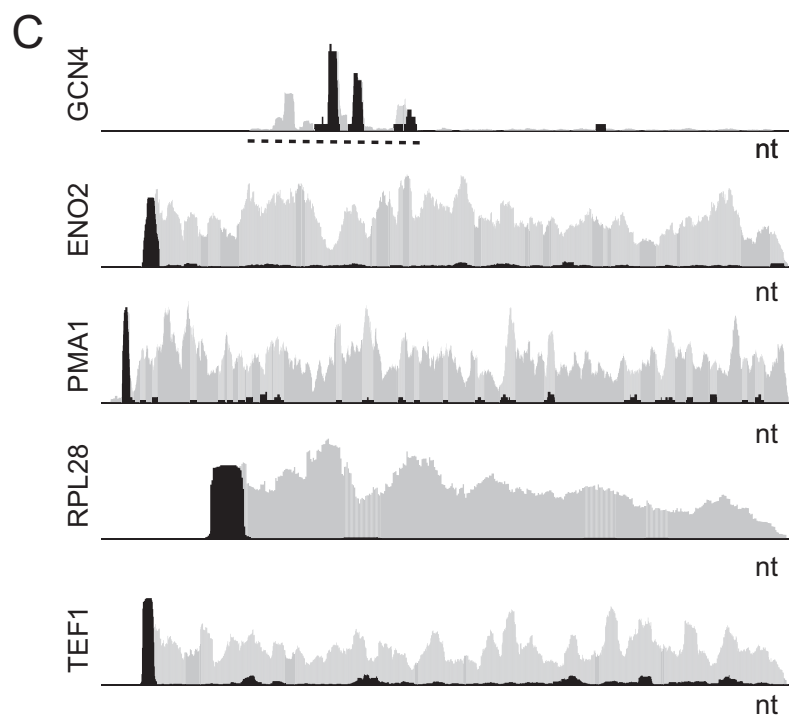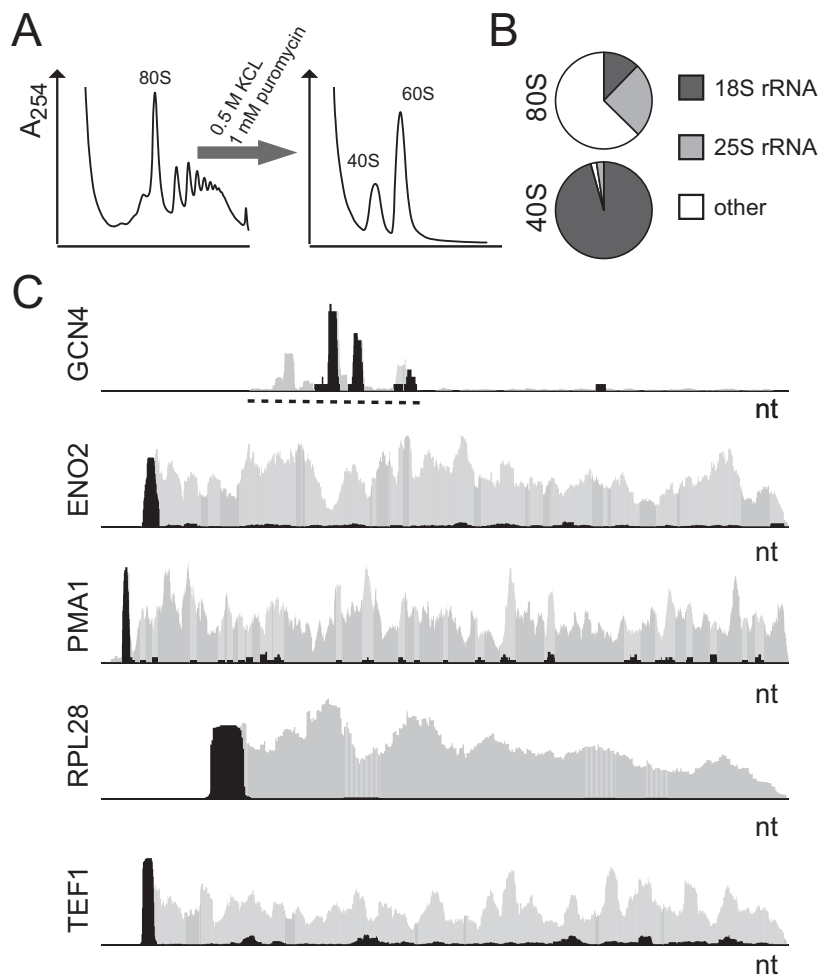
Figure 5. Ribo-seq of the small ribosomal subunit. (A) Dissociation of monosomes and polysomes into subunits. Fractions corresponding to the 40S small ribosomal subunit were collected for sequencing. (B) Shares of reads aligned to 18S and 25S rRNAs in Ribo-seq samples. The upper chart shows typical shares of reads in 80S fraction in control cells (YPD media, log growth phase, no stress, no cycloheximide pretreatment), and the lower shares of reads in the 40S fraction. Although not precisely quantitative, it gives an estimation of 40S fraction impurity. The 'other' category combines footprints and unaligned reads. (C) Representative footprint coverage profiles from normal Ribo-seq (gray) and 40S fraction (black). The dashed line marks the location of the *GCN4* uORFs. (D) Footprint length distribution in 80S and 40S fractions. Reads aligned to 18S rRNA are given as a size selection control for 40S fraction. Note: each distribution class has its own scale. (E) Cumulative coverage of footprints derived from 40S fraction. No normalization was applied prior to graph plotting. All genes regardless of their length were aligned by their start codon.
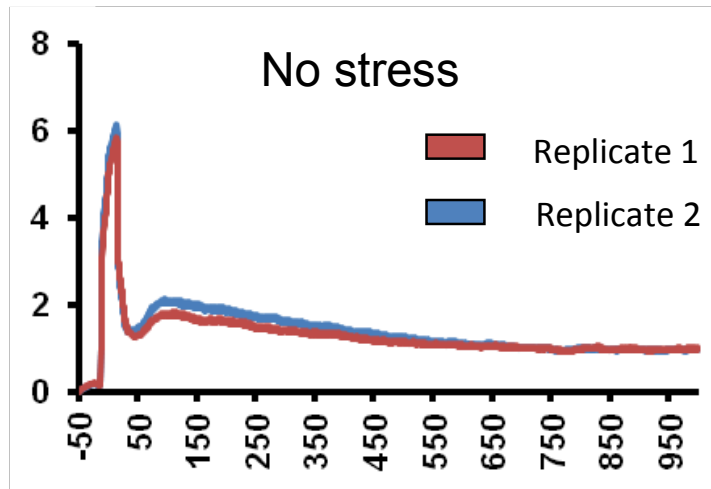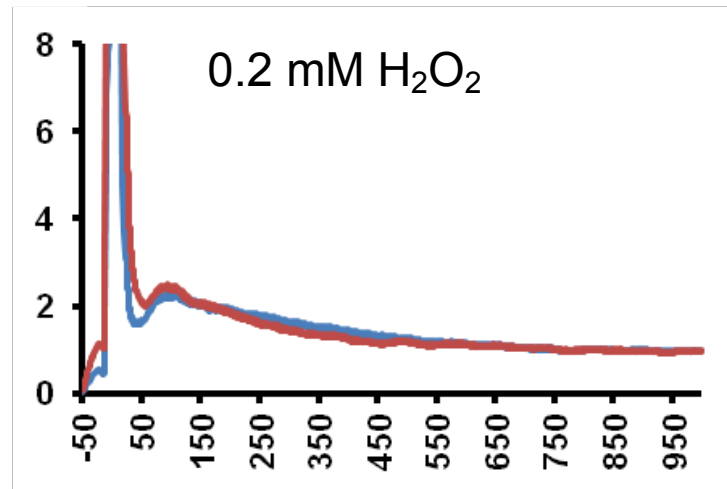
Supplementary Figure S1. Traces of ribosome occupancy for x100 concentration of cycloheximide were reproduced in two independent experiments. (A) No stress. (B) 0.2 mM hydrogen peroxide induced oxidative stress.