

Genome-wide ribosome profiling reveals complex translational regulation in response to oxidative stress

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Information on unique and coordinated regulation of transcription and translation in response to stress is central to the understanding of cellular homeostasis. Here we used ribosome profiling coupled with next-generation sequencing to examine the interplay between transcription and translation under conditions of hydrogen peroxide treatment in *Saccharomyces cerevisiae*. Hydrogen peroxide treatment led to a massive and rapid increase in ribosome occupancy of short upstream ORFs, including those with non-AUG translational starts, and of the N-terminal regions of ORFs that preceded the transcriptional response. In addition, this treatment induced the synthesis of N-terminally extended proteins and elevated stop codon read-through and frameshift events. It also increased ribosome occupancy at the beginning of ORFs and potentially the duration of the elongation step. We identified proteins whose synthesis was regulated rapidly by hydrogen peroxide posttranscriptionally; however, for the majority of genes increased protein synthesis followed transcriptional regulation. These data define the landscape of genome-wide regulation of translation in response to hydrogen peroxide and suggest that potentiation (co-regulation of the transcript level and translation) is a feature of oxidative stress.

Gene expression may be controlled at multiple levels. Globally, it is regulated by histones and satellite proteins. Locally, promoters, enhancers, and other regulatory elements are used to guide transcription. Numerous studies have yielded datasets involving the networks of transcription factors and described the associated mechanisms of transcriptional regulation. Developments in microarray technology have facilitated such studies and made them affordable for individual laboratories. Accordingly, a vast number of studies has emerged that describe transcriptional responses to various treatments, stimuli, knockouts, and other interventions. Conversely, the investigation of the regulation of gene expression at the level of translation lagged behind because of the lack of accessible high-throughput methods.

It often is assumed that changes in mRNA abundance are proportional to changes in protein synthesis in the cell, but numerous exceptions are known. One powerful approach to assess changes in protein abundance directly is the use of whole-proteome mass spectrometry, but this method is inferior to mRNA profiling in its throughput and can detect only a fraction of protein products in the cell (1). Other high-throughput approaches, such as fluorescent protein reporter libraries, are available (2–4). However, they are designed for the quantification of individual proteins rather than for addressing the details of translation. Indirect approaches, such as comparative microarray profiling of mRNAs within monosomes and polysomes, are popular as well (5–8). These methods enable estimation of the mRNA transcripts that are being translated. Recent advances in next-generation sequencing have enhanced data acquisition, improved sensitivity, and made this method superior to microarrays in its throughput (9). Importantly, it allowed mRNA abundance and protein translation to be examined in the same sample with high accuracy (with subcodon resolution) (10, 11). This experimental strategy involves deep sequencing of mRNA fragments (footprints) buried inside the actively translating ribosomes. Protein translation can be inferred from footprint abundance. Coupled with regular mRNA-sequencing (mRNA-seq)

analyses, these data give information on the actual mRNA sequences that are being translated, identity of the reading frames used, and ribosomal density at each position within these mRNAs. Hereafter, we refer to this method as “ribosome profiling” or Ribo-seq. Another promising application of Ribo-seq is measuring translational regulation by monitoring translation efficiency (TE), which is the amount of footprint normalized to underlying mRNA abundance.

In the current study, we applied Ribo-seq to investigate the fine details of *Saccharomyces cerevisiae* response to oxidative stress caused by hydrogen peroxide treatment. A key advantage of this method is the much higher sensitivity than obtained with microarrays. With this method we were able to detect changes in transcription and its regulation within 5 min of treatment. Oxidative stress is one of the best-studied regulators of transcription (12), but little is known about how this stress changes protein abundance and posttranscriptional regulation. Previous studies pointed to a weak correlation between transcriptional and translational gene responses, i.e., elevated mRNA transcripts in stressed cells did not match the set of proteins that changed abundance. Microarray analyses revealed that only 15% of genes involved in translational response showed the corresponding changes at the mRNA levels (6). Our study focused on using Ribo-seq to examine precisely translation and its regulation by oxidative stress.

Results

Ribo-Seq. An overview of the Ribo-seq method that we used to examine the regulation of translation by oxidative stress is given in Fig. 1A. Each translating ribosome protects ~28 nucleotides on the translated mRNA, and the unprotected regions are removed by subjecting mRNAs to RNase I digestion. The protected mRNA pieces (footprints) are extracted and analyzed by deep sequencing. Because their length is known, the exact codons that occupy the A and P sites of the ribosome can be determined. This information is used to identify frameshifts, read-through events, and altered codon use. Additionally, quantification of footprints provides an opportunity to estimate changes in translation for every mRNA species.

A key factor that decreases throughput of this method is that only 5% of total yeast RNA consists of mRNA in rapidly growing yeast cells (13). Previously, contamination was eliminated during footprint preparation by ultrafiltration, which is not very efficient; i.e., the fraction of ribosomal RNA fragments in sequencing libraries approached 80%, with an average value of about 60%, as observed in previous studies (10) and our own pilot experiments. To improve the throughput of the method, we examined the content of contaminating rRNA fragments. In our footprint samples a particular fragment of the 28S ribosomal subunit was responsible for 90% of contamination. An additional

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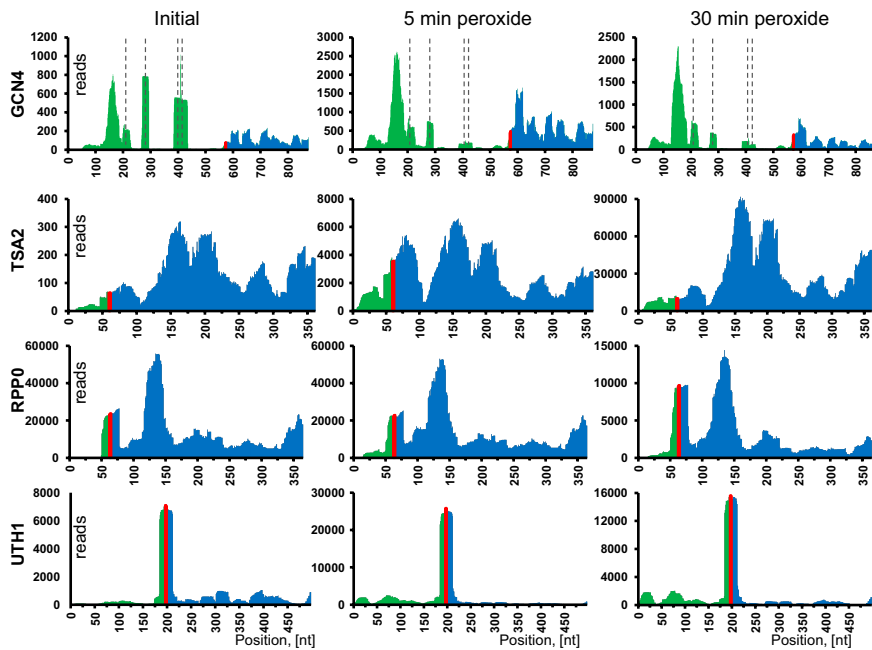


Fig. 2. Examples of 5'-UTR translation during oxidative stress. Ribosome footprint coverage for four different mRNAs discussed in the text illustrates various patterns of translation. Panels show the footprint coverage of certain mRNAs with no in-frame stop codons upstream of annotated genes. For each mRNA, translation following 5- and 30-min hydrogen peroxide treatment is given. Untreated yeast cells served as a control. The entire 5' UTR and 300 nt of the gene sequence were used to generate the coverage density map. The 5'-UTR part of the mRNA is shown in green, the AUG start codon in red, and the annotated gene in blue. Dashed lines in GCN4 graphs indicate positions of known uORFs.

5- and 30-min samples. We also developed a simple method for frameshift search and validation that is technically similar to the search for N-terminal extensions. A short region downstream of the stop codon for each annotated gene was examined for the presence of ribosomal footprints with coverage comparable to the gene itself. A handful of candidates were confirmed manually. For validation, the 5' ends of footprints aligned to the regions upstream or downstream of the known frameshift were quantified and assigned to the matching reading frame. The frame with the highest count would correspond to the actual ORF. This approach is shown in Fig. 3 and Fig. S2 for two known frameshifts in *S. cerevisiae*, antizyme and protein ABP140, respectively (19). Further analysis of genes for read-through of annotated stop codons yielded four additional genes with +1 frameshifts (i.e., ribosome slipping one nucleotide towards 3'

end) (Table S4). An example is shown in Fig. 3B. All these frameshifts were detected under conditions of oxidative stress.

Correlation Between Transcriptional and Translational Responses to Oxidative Stress. In *S. cerevisiae*, ~1,700 genes are regulated by hydrogen peroxide at the level of transcription, including ~900 genes of the environmental stress response cluster, which encompasses genes regulated in response to various stresses such as heat shock, starvation, and oxidative stress (12). Next-generation sequencing technologies can improve the sensitivity and dynamic range of gene-expression analysis significantly. We found that after 5-min treatment with hydrogen peroxide transcriptional changes were observed for 116 genes, of which 10 were down-regulated and 106 were up-regulated. The 30-min treatment yielded transcriptional changes in

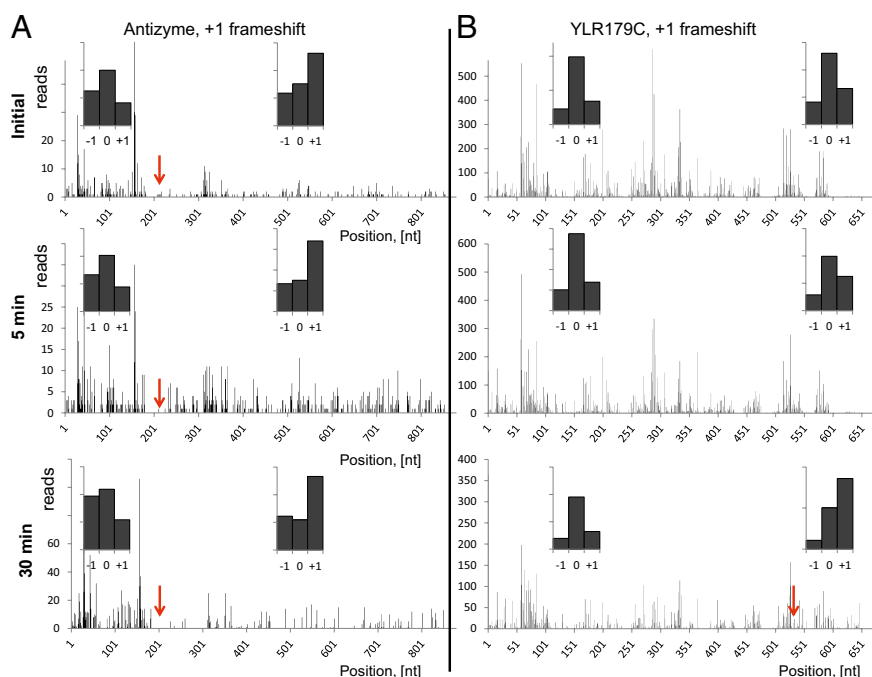


Fig. 3. Ribo-seq allows identification of frameshifts (red arrows). (A) Validation of the known frameshift in the antizyme gene. (B) Oxidative stress leads to a frameshift in the product of the YLR179C gene. We observed a change of frame, leading to translation of a longer protein in the 30-min peroxide treatment sample. The 5' ends of footprints were mapped to the genomic sequence of YLR179C. (Insets) Histograms show the count of footprints, matching one of three possible frames either to the left or to the right of the frameshift. The "0" frame is the one with the annotated start codon. The highest count of footprints matched the "0" frame before the frameshift and the "+1" frame after the frameshift.

1,497 genes (529 down-regulated and 968 up-regulated) with the threshold of 2.6-fold (see [Datasets S1](#) and [S2](#) and [Fig. S34](#) for comparison of mRNA-seq with microarrays from ref. 12).

One of our major goals was to examine genome-wide translational changes and posttranscriptional regulation of translation in response to oxidative stress. Sequencing of ribosomal footprints enabled direct and absolute quantification of mRNAs undergoing translation. It should be noted that Ribo-seq does not provide protein concentrations but instead estimates the relative translation for a given protein. Using this method, we showed that protein synthesis cannot be inferred securely from mRNA abundance. There were genes whose translation did not correlate with mRNA abundance ([Fig. S4E](#)). In addition, a significant fraction of genes showed essentially no translation, although their mRNAs were present. We detected translational response for 97 genes after the 5-min hydrogen peroxide treatment. Only four genes showed decreased protein synthesis at this time point. After 30 min, relative protein synthesis was decreased in 593 genes and increased in 766 ([Dataset S2](#)). Some proteins increased expression between 5 and 30 min, some reached a plateau at 5 min, and others declined during the longer treatment time.

Interestingly, the values of translation change in response to hydrogen peroxide did not match those for mRNA transcripts exactly, even if we only consider coregulated genes ([Fig. 4A](#), black dots), although in most cases the changes in values are in the same direction. For instance, the footprint density of a representative protein increased 10-fold, but its mRNA expression increased only twofold. These data suggest a specific posttranscriptional control of protein expression. Indeed, by comparing changes in TE with changes in mRNA transcripts, we observed multiple proteins in which translational regulation was greater than transcriptional regulation ([Fig. 4C and D](#)). The TE is the ratio of Ribo-seq read counts to mRNA-seq read counts, and it describes the

propensity of mRNA to undergo translation. The higher the TE, the better is the mRNA translated. Posttranscriptional regulation can be simply permissive, allowing an mRNA transcript to be translated under stress conditions. However, based on our analysis, posttranscriptional regulation usually makes an addition to transcription changes, modulating protein synthesis (see [Fig. S3B](#) for the TE error rate). Because we observed an immediate increase in uORF footprint density in response to hydrogen peroxide treatment, we further examined a possible effect on the TE of downstream genes. In our reference database, 3,830 genes had annotated 5' UTRs with an unambiguous sequence longer than 23 nt. Among them, nearly 1,800 were covered by ribosomal footprints in at least one of the samples, and 1,217 had increased footprint density after the 30-min peroxide treatment. We analyzed the potential coregulation of translation and increased ribosomal density at 5' UTRs in these 1,800 genes and found that, on a genome-wide scale, ORF translation and ribosomal density at uORFs were mostly independent under oxidative stress conditions ([Fig. 4B](#)).

Oxidative Stress Regulates Translation Elongation. We found that the density of elongating ribosomes on the mRNAs was consistently higher within the first 100–150 nt from the start codon. This observation may be explained by codon use and the corresponding tRNA copy number (20). Hydrogen peroxide treatment caused a significant increase in ribosome occupancy and, therefore, in the density of footprint coverage within the beginning of the ORF ([Fig. 5A](#)), and this effect was similar for the 5- and 30-min treatment samples. Treatment affected transcripts regardless of their length or expression level [similar to the previous observations (10)]. The data suggest that oxidative stress influenced elongation, forcing ribosomes to spend more time at the beginning of their ORFs. Together with the increased utilization of the 5' UTRs it explains the contradiction with previous experimental observations (6). The fact that ribosome density increased so rapidly upon addition of hydrogen peroxide implies a direct effect of the oxidant, which targets ribosomes and elongation factors.

Ribo-Seq Enables Codon Occupancy Quantification in Vivo. Because Ribo-seq can track translation at a single-nucleotide resolution, we examined the experimental relative frequency of translated codons and compared the experimental observations with the predicted values. Assuming that all codons are translated at the same rate, one would expect the distribution of codons trapped at the ribosomal A site to be identical to the frequency distribution of codons across mRNAs (normalized to expression levels). However, our experimental data showed that some codons were more enriched ([Fig. 5B](#), bars above the baseline), meaning that they are met more frequently in ribosomes and are translated less efficiently. Codons such as CAC or GGT fit into the relative synonymous codon use (RSCU) table, which is used for calculations of the codon adaptation index (21) that rely partially on tRNA copy numbers in the yeast genome (20). The number of experimental replicates does not allow us to compare a particular codon directly in untreated and peroxide-treated yeast. Nevertheless, by analyzing the whole distribution ([Fig. 5B](#)), we observed that the difference between predicted and experimental codon occupancy was less in stressed than in unstressed yeast. In other words, untreated, logarithmically grown yeast cells have more selective pressure on translation machinery (e.g., the availability of charged tRNA). Oxidative stress causes a rapid decrease in translation but, perhaps, less of a decrease in the pool of tRNAs and in the amount of mRNA, thus relaxing the competition of ribosomes for tRNAs. Therefore, the observed codon occupancy tends to be similar to the codon distribution of genes. Increasing the number of experimental replicates can make this method sensitive enough to detect changes in individual codon translation upon stress or any other change in condition.

Discussion

Our data define the landscape of translational control of oxidative stress in yeast. We made several interesting observations. First, we

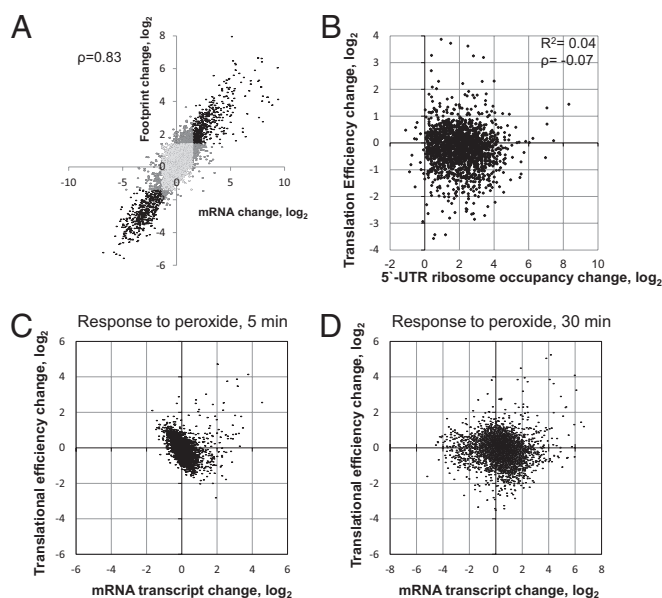


Fig. 4. Interplay between translation and transcription. (A) Correlation between changes in footprint and transcript abundances in response to hydrogen peroxide. Light gray dots represent genes whose footprint count and mRNA count were not affected by peroxide treatment; dark gray dots represent genes with only the footprint or mRNA affected; and black dots represent coaffected genes. Changes in transcript and in footprint abundance between the initial and the 30-min peroxide samples are plotted on the axes (for further details see [SI Materials and Methods](#)). (B) Increased ribosomal occupancy at the 5' UTR does not affect the TE of a downstream gene. (C) Relationship between change in TE and change in mRNA transcript change after 5-min incubation with peroxide. (D) Relationship between change in TE and change in mRNA transcript after 30-min incubation with peroxide.

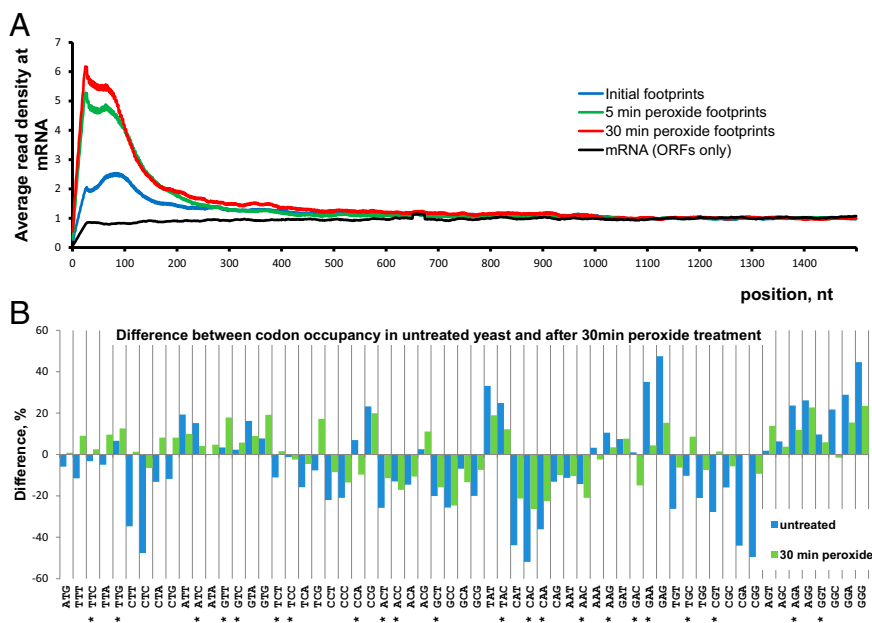


Fig. 5. Global features of translation examined by Ribo-seq. (A) Density of footprint coverage along the mRNA. Profiles of read coverage were calculated for each mRNA longer than 1,500 nt and rpk_m >10. The profiles were normalized based on the average density in the region from 1,000–1,500 nt. Densities for each nucleotide position were averaged across all mRNAs. An average between the two experimental replicates is shown. (B) Ribosomal occupancy of individual codons measured *in vivo*. Percentage of difference is calculated between the predicted codon distribution across mRNAs and the experimental codon appearance at the ribosomal A site. For details of normalization and prediction, see *SI Materials and Methods*. Codon values greater than zero are encountered more often at the A site of ribosome. Asterisks mark codons with the highest RSCU values (21). Blue bars represent untreated control; green bars represent samples treated for 30 min with 0.2 mM hydrogen peroxide.

found widespread translation of uORFs under conditions of oxidative stress. A dramatic increase in uORF ribosome occupancy occurred only 5 min after the addition of hydrogen peroxide and greatly exceeded the overall changes in protein translation. Comparisons between our study and the previously identified uORFs under conditions of starvation revealed a more extensive use of the 5' UTRs under oxidative stress. Two times as many genes showed increased ribosome occupancy at their 5' UTRs under oxidative stress than under starvation (Fig. S3C). The greater fraction of ribosomes bound to the 5'-UTR regions may be caused by two opposite events. First, translation of these regions may be up-regulated, thus producing short, cryptic peptides. On the other hand, ribosomes may move to the 5'-UTR regions slowly, accumulating footprints without affecting polypeptide yields. We think the second explanation is more likely. It agrees with the elevated density in the first 30–50 codons within the mRNA and with the reported increase in elongation time under conditions of oxidative stress (6). It also is consistent with the complex relationships between gene translation and 5' UTR translation. Mass-spectrometry analyses would show the real yield of uORF-produced peptides and would be useful for the development of future Ribo-seq applications. We did not detect up- or down-regulation of known translation initiating factors at the 5-min time point, so the observed effects on the 5' UTR likely were caused by posttranslational modifications of initiation factors or ribosomal proteins. Phosphorylation of eIF2, a component of the ternary initiation complex, is known to inhibit translation initiation and, consequently, protein expression (22). In some cases, this factor was shown to induce translation of proteins, such as ATF4 or GCN4, through the intricate system of translation and reinitiation events at the uORFs (14, 23, 24). An additional reason for increased ribosome occupancy at the 5' UTRs may involve initiation at non-AUG codons. The majority of our detected uORFs had no AUG start codons. eIF1 and eIF5 are the factors that control the recognition of start codons during translation initiation in eukaryotes (25, 26). We suggest that hydrogen peroxide impairs the fidelity of these factors, which normally restrict initiation to AUG codons, thereby facilitating non-AUG initiation of translation as the ribosome scans the mRNA. Our observations imply a mechanism that slows down the ribosome at uORFs and the beginnings of ORFs. It can be achieved by impairing the exchange of elongation factors, incomplete dissociation of initiation factors, or binding additional stress-activated proteins. In addition, hydrogen peroxide may damage tRNAs (27),

amino acids (28), and aminoacyl-tRNA synthetases (29). The exact molecular mechanism requires further studies.

Translatome and transcriptome in yeast are regulated conjointly in response to various stresses, such as amino acid depletion, osmotic shock, and sorbitol treatment (7). Thus the genes up-regulated at the level of transcription also yield more protein product as well, a process that is termed “potentiation.” However, in the response to hydrogen peroxide only ~15% of transcriptionally regulated genes were believed to be linked by potentiation (6). Our data indicate that the overlap is greater and that oxidative stress is not unique in this respect (Fig. 4A). We compared our results directly with the published reports on the translation response to oxidative stress (6). All proteins with high scores from that study were present in our list, and the two studies also had several down-regulated proteins in common. However, about 70% of peroxide-regulated proteins from that study did not overlap with our hits, perhaps because the greatly increased ribosomal density at the 5' UTRs and at the beginnings of regular ORFs, which does not reflect the actual increase of translation, compromises the microarray-based approaches. In this regard, Ribo-seq has an advantage over microarrays. Overall, our study offers a more detailed view of the translational response to oxidative stress and leads to reevaluation of many translational targets of peroxide. We also observed a significant difference between mRNA abundance and its translation (Fig. S4E). Some mRNAs were not translated at all. Several genes had remarkably permissive posttranscriptional regulation upon hydrogen peroxide treatment. For example, *Srx1*, coding for sulfiredoxin, is present in unstressed yeast cells as a moderately transcribed gene with no detectable ribosomal occupancy. Its translation increases immediately after the addition of peroxide, increasing the TE by orders of magnitude. *Srx1* reduces cysteine-sulfenic acid, formed upon reaction with hydrogen peroxide in the active sites of peroxiredoxins. Among them, *Tsa1* is one of the major proteins contributing to stress resistance (30). An opposite example is PAB1, a polyA-binding protein mediating interactions between the 5' cap structure and the 3' mRNA poly (A) tail and facilitating translation. Treatment with hydrogen peroxide greatly decreased the TE of PAB1, but its transcript abundance remained unchanged.

Importantly, the degree of translational response to hydrogen peroxide did not match the transcriptional response precisely. There are multiple cases of posttranscriptional regulation in addition to the general transcriptional response. For example, 5-min

incubation with the oxidant increased the TE of 32 genes and decreased the TE of 13. A longer incubation up-regulated 62 genes and down-regulated 122 (Dataset S1). This finding highlights our incomplete understanding of molecular mechanisms controlling gene expression. Increasing numbers of high-throughput studies involving *S. cerevisiae* and mammalian cells that address an interplay between translation and transcription suggest that these processes do not correlate perfectly with each other in either single-cell or culture-wide conditions (3, 31, 32).

Ribo-seq offers an improved experimental alternative to the codon adaptation index (21). It is able to detect differences between the TEs of synonymous codons. Ribo-seq may become a valuable tool for addressing the effects of deliberate starvation and amino acid depletion on codon-specific translation. Overall, our study defined the genome-wide regulation of translation by oxidative stress.

Materials and Methods

Additional details can be found in *SI Materials and Methods*. Primers used in library preparation are listed in Table S5.

Yeast Strains and Growth Conditions. One milliliter of BY4741 strain (MATa his3 leu2 met15 ura3) from a frozen stock (OD₆₀₀ of 0.6 in 15% glycerol) was added to 50 mL of yeast extract-peptone-glucose (YPD) medium, and the cells were grown for 16 h at 30 °C. A 1-mL aliquot of that culture was added to 400 mL of fresh YPD and grown to an OD₆₀₀ of 0.5. This culture then was used for treatments and sample collection.

Preparation of Lysates. The initial protocol was based on a previously described procedure (10, 11). Before the addition of peroxide, a 50-mL aliquot of culture was taken rapidly and pelleted by centrifugation for 1 min at 3,400 × g at 4 °C; then the pellet was frozen immediately in liquid nitrogen. This aliquot was used for mRNA isolation, and the rest of culture was used for footprints. The peroxide concentration used in this study was 0.2 mM with incubation times of 5 and 30 min.

Ribosome Fractionation and RNA Extraction. A 50-U aliquot of cell extract (OD₂₆₀) was used for footprints extraction. It was treated with 1,000 U of *Escherichia coli* RNase I (Ambion) and was incubated for 1 h at room

temperature with gentle shaking to digest the mRNA. After fractionation in sucrose gradient, the monosomal fraction was collected, and footprints were isolated.

Library Construction for Footprint Sequencing. Libraries were prepared with the strand information preserved to minimize ambiguously aligned reads. A protocol that included polyadenylation of RNA fragments and subsequent DNA circularization was used. The resulting libraries were sequenced on the Illumina GLx2 or HiSeq2000 platforms.

Bioinformatics Analyses. In-house Perl scripts were used to prepare reference databases. Alignment of sequencing reads was performed by Bowtie software v.0.12.7 (33), allowing two mismatches per read. Because every read bears a polyA tail at the end, we omitted all "A" from the 3' ends of sequences before aligning. A detailed description is given in *SI Materials and Methods*.

Codon Translation Analysis. In an ideal situation, ribosomal footprints should be 28 nt in length. However, RNase I used to degrade unprotected mRNA segments occasionally left extra nucleotides or cut off extra nucleotides. By plotting a distribution of the footprint length, we found that RNase creates footprints that are mostly 27–29 nt in length (Fig. S4C). These footprints can be aligned to the reference ORFs, and the position of a footprint's 5' end relative to the reading frame can be obtained. If the 5' end of a footprint matched the exact border of a codon, we considered it "ideal." If the 5' end of a footprint matched the position of a codon ± 1 nt, we deleted or added the first nucleotide, respectively. Thus, we minimized the error in determining the ribosome position and defined which codon was located in the A site.

Differential Gene Translation Analysis. All experimental samples were collected in duplicates. Based on correlation between the replicates, we set up a reads per kilobase per million mapped reads (rpkm) threshold of 10 for the genes whose translation and transcription could be determined reproducibly (Fig. S4 A and B). The gene was considered regulated if its rpkm value changed more than 2.6-fold (1.4 in log₂ scale). This threshold eliminated most false-positive hits (Fig. S4D).

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