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Recommended Citation
Zinskie, Jessica A; Ghosh, Arnab; Trainor, Brandon M; Shedlovskiy, Daniel; Pestov, Dimitri G; and Shcherbik, Natalia, "Iron-dependent Cleavage of Ribosomal RNA During Oxidative Stress in the Yeast Saccharomyces cerevisiae" (2018). School of Osteopathic Medicine Faculty Scholarship. 115.
https://rdw.rowan.edu/som_facpub/115

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Iron-dependent cleavage of ribosomal RNA during oxidative stress in the yeast Saccharomyces cerevisiae

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Received for publication, May 25, 2018, and in revised form, July 16, 2018 Published, Papers in Press, July 18, 2018, DOI 10.1074/jbc.RA118.004174

Stress-induced strand breaks in RNA have been observed in many organisms, but the mechanisms by which they originate are not well-understood. Here we show that a chemical rather than an enzymatic mechanism initiates RNA cleavages during oxidative stress in yeast (Saccharomyces cerevisiae). We used cells lacking the mitochondrial glutaredoxin Grx5 to demonstrate that oxidant-induced cleavage formation in 25S rRNA correlates with intracellular iron levels. Sequestering free iron by chemical or genetic means decreased the extent of rRNA degradation and relieved the hypersensitivity of grx5Δ cells to the oxidants. Importantly, subjecting purified ribosomes to an in vitro iron/ascorbate reaction precisely recapitulated the 25S rRNA cleavage pattern observed in cells, indicating that redox activity of the ribosome-bound iron is responsible for the strand breaks in the RNA. In summary, our findings provide evidence that oxidative stress–associated rRNA cleavages can occur through RNA strand scission by redox-active, ribosome-bound iron that potentially promotes Fenton reaction–induced hydroxyl radical production, implicating intracellular iron as a key determinant of the effects of oxidative stress on ribosomes. We propose that iron binding to specific ribosome elements primes rRNA for cleavages that may play a role in redox-sensitive tuning of the ribosome function in stressed cells.

Reactive oxygen species (ROS) are omnipresent stressors for all biological systems. Low levels of ROS play roles in many biochemical processes and act as signaling molecules, but at supraphysiological levels, ROS can damage cellular components, including lipids, proteins, and nucleic acids. Through oxidation of rRNA and proteins, ROS can influence the functionality of ribosomes, the complex ribonucleoprotein machines responsible for protein synthesis. For example, translation by the bacterial ribosome is inhibited by oxidation of the rRNA bases within the peptidyl transferase center. Reversible oxidation of cysteine residues in ribosome-associated proteins under conditions of increased ROS generation has been proposed to attenuate translation. Oxidative damage to rRNA has been hypothesized to play a role in neurodegenerative disease. However, despite the central role of the ribosome in protein synthesis, our understanding of the impact of ROS on ribosome functions is still very limited.

In our previous work, we found that low, survivable levels of oxidative stress in the budding yeast Saccharomyces cerevisiae lead to rRNA cleavages in a subset of cytoplasmic ribosomes. One prominent cleavage, located in the ES7 region of 25S rRNA in the large (60S) ribosomal subunit, starts at early stages of the adaptive oxidative stress response that helps cells cope with ROS accumulation. The ES7 region is located on the surface of the 60S subunit and is one of the largest rRNA expansion clusters in the eukaryotic ribosome. Cleaved ribosomes are capable of reinitiating translation, suggesting that this type of rRNA cleavage does not inactivate ribosomes and may instead play a role in modulating their function during stress.

Aside from adaptable oxidative stress conditions, excessive ROS accumulation is a hallmark feature of apoptosis. Induction of programmed cell death in various organisms has been shown to involve degradation of rRNA, presumably carried out by nucleases activated as part of apoptotic mechanisms. The identity of eukaryotic ribonucleases that initiate ribosome degradation during programmed cell death has remained uncertain.

In this study, we took advantage of the rapid yeast rRNA analysis techniques we developed recently to survey genes encoding ribonucleases and proteins with function in redox regulation for possible effects on the ES7 cleavage in 25S rRNA. Unexpectedly, we found that the cellular control of iron but not ROS levels was the major determining factor in rRNA fragmentation during oxidative stress. Genetic and biochemical analyses confirmed that oxidant-induced cleavage of yeast rRNA is a nonenzymatic, iron-dependent process. Our results identify a previously unknown mechanism of site-specific changes in the ribosome that may have a biologically significant role in diverse oxidative stress conditions and during programmed cell death.
rRNA undergoes chemical hydrolysis during oxidative stress

Results

Deletion of the monothiol glutaredoxin gene GRX5 causes severe rRNA degradation in yeast exposed to low-level oxidants

Previously, we identified cleavage in the expansion segment ES7 of the 25S rRNA as one of early molecular responses to elevated ROS levels in the budding yeast *S. cerevisiae* (7). To better understand cellular factors that affect ES7 cleavage, we screened a panel of ~150 yeast strains deficient for antioxidant and oxidative stress–related genes as well as genes that encode various RNases. After growing cells to mid-log phase, we induced oxidative stress with low doses of inorganic peroxide (H₂O₂, 0.25 mM) or the redox-cycling agent menadione (vitamin K₃, 50 μM) and isolated total RNA from the cells (15). Fig. 1A shows a representative set of five strains that were either left untreated or treated with the oxidants and compared with the WT BY4741 control. A number of deletion strains in our test panel showed increased accumulation of stress-induced RNA fragments, including products of ES7 cleavage in 25S rRNA (7), consistent with the involvement of multiple antioxidant systems in the cellular neutralization of ROS. To compare the strength of this phenotype in different strains, we performed Northern hybridizations with the probe y540, which detects both full-length 25S rRNA and the well-resolved 5′ fragment (~600 nt) produced by ES7 cleavage (Fig. 1B) and determined the ES7 fragment/25S ratio in each lane (Fig. 1C). Control measurements in biological replicates showed that using this ratio minimizes variations resulting from loading and strain-specific differences in rRNA content. Among all tested mutants, deletion of the monothiol glutaredoxin gene GRX5 led to a particularly strong loss of rRNA stability in response to both H₂O₂ and menadione (Fig. 1C).

To ensure that the observed 25S rRNA degradation phenotype was due to lack of GRX5 rather than any secondary mutations that may have accumulated in a passaged knockout library strain, we backcrossed grx5Δ with WT BY4741 cells and generated several new grx5Δ strains by tetrad dissection. The newly derived grx5Δ strains were viable; however, they grew noticeably slower than the WT in fermentation medium (Fig. 1D). In agreement with a previous study (16), grx5Δ strains were deficient in respiration (Fig. S1A). Genetic tests and analysis of mitochondrial DNA showed grx5Δ cells to be p0 (Fig. S1, B–D), explaining their respiration deficiency. Testing the new grx5Δ strains confirmed their high sensitivity to oxidants. For example, grx5Δ cells were unable to grow in medium supplemented with 50 μM menadione, a dose that caused only a transient growth delay in the WT (Fig. 1D). Consistent with the screen data (Fig. 1C), Northern hybridization with the probe y540 showed severe 25S rRNA degradation in grx5Δ cells after treatment with either 0.25 mM H₂O₂ or 50 μM menadione (Fig. S2A). The ES7-cleaved fragment was detectable in grx5Δ cells after treatment with as little as 10 μM menadione, whereas 25S rRNA integrity in WT cells was unaffected by this dose (Fig. 1E).

To better define the kinetics of the oxidant-induced 25S rRNA decay, we next analyzed rRNA at different time points after addition of 50 μM menadione to the culture medium (Fig. 2A). In grx5Δ cells, the ES7-cleaved fragment was apparent after a 1-min treatment and further increased in intensity over time, paralleled by decay of the full-length 25S rRNA (Fig. 2). By comparison, menadione treatment in WT cells led to a slower onset of rRNA degradation and affected only a small fraction of ribosomes (Fig. 2). This indicated that cleavage of the ES7 region of 25S rRNA occurs virtually instantaneously upon oxidant exposure of grx5Δ cells, arguing against lengthy mechanisms such as autophagic delivery of ribosomes to the vacuole (17) or induction of the apoptotic program (14).

Northern hybridizations with probes for other RNA types revealed increased degradation of 5S rRNA, 5.8S rRNA, and tRNAs after treatment of grx5Δ cells with menadione or H₂O₂ (Fig. S2, C–F). However, large rRNAs (18S and 25S) were by far the most severely affected RNA species (Fig. S2, A and B), with only ~2% of intact 25S rRNA remaining after 2-h treatment with 50 μM menadione (Fig. S2A). Hybridizations with additional 25S rRNA probes verified extensive degradation of the entire rRNA molecule in oxidant-treated grx5Δ cells (Fig. S3).

Collectively, these data show that Grx5 plays a crucial role in maintaining ribosome integrity during oxidative stress. Lack of Grx5 greatly intensifies the effects of oxidants on ribosomes. This not only increases the number of ES7-cleaved ribosomes, but can also lead to runaway ribosome degradation after treatment with low oxidant levels that are survivable in Grx5-expressing cells.

The increase in intracellular ROS levels is not sufficient to explain the ribosome degradation phenotype in grx5Δ cells

Grx5 belongs to the glutaredoxin family of enzymes, also known as GSH-dependent oxidoreductases, many of which help to maintain the redox state of proteins during oxidative stress (18). Because some glutaredoxins can also function as ROS scavengers (19, 20), we first sought to determine how lack of Grx5 affected the cellular ROS load. We estimated H₂O₂ levels in grx5Δ cells by using for comparison a ctt1Δ strain deficient for catalase, a key H₂O₂-inactivating enzyme. Amplex Red assays performed after treatment with 50 μM menadione or 0.25 mM H₂O₂ indeed revealed elevated H₂O₂ levels in grx5Δ cells as well as in the control ctt1Δ strain (Fig. 3A). However, the observed increases in H₂O₂ did not correlate well with the extent of rRNA degradation (Fig. 3B). For instance, grx5Δ cells exhibited more RNA degradation after treatment with 25 μM menadione than the WT and ctt1Δ cells after 50 μM treatment (Fig. 3B) despite the lack of a comparable H₂O₂ increase (Fig. 3A).

To assess the levels of the O₂⁻ radical in oxidant-treated grx5Δ cells, we used the superoxide-specific probe dihydroethidium (DHE) (21). Oxidized DHE was analyzed by a flow cytometry assay, which revealed moderately increased superoxide levels in grx5Δ cells compared with the WT cells (Fig. 3C). However, the O₂⁻ levels detected in grx5Δ cells were less pronounced than in the control sod2Δ strain lacking superoxide dismutase (Fig. 3C). Importantly, sod2Δ cells displayed significantly lower RNA degradation than similarly treated grx5Δ cells (Fig. 3D). Thus, neither hydrogen peroxide nor superoxide radical levels reflect the severity of ribosome degradation during oxidant treatments of grx5Δ cells, suggesting that additional factor(s) may play a role in this process.
Increased levels of labile iron is a key factor in 25S rRNA degradation in grx5Δ cells

Previous studies have shown that Grx5 participates in iron–sulfur cluster (ISC) protein biogenesis (22–27), whereas the lack of Grx5 function causes iron accumulation in yeast cells (25, 28). Iron can both potentiate ROS production and exacerbate ROS-induced toxicity in the cell (28, 29). We therefore asked whether the disruption of iron homeostasis in grx5Δ cells contributes to the oxidant-induced effects on rRNA.

First, we analyzed the iron content of the grx5/H9004 cells and confirmed that it was significantly increased compared with the WT (Fig. 4A), in agreement with the results of a previous study (25). We also found that treatment of grx5/H9004 cells with the membrane permeable Fe2+ chelator 1,2-phenanthroline (PHL) (30)
Iron-depdenent site-specific cleavage of 25S rRNA in vitro

The above results indicate that the severe degradation of ribosomes in grx5Δ cells results from the combination of two factors: oxidants and high intracellular iron. Transition metal ions, including iron, can enhance ROS-induced damage in biomolecules, at least in part, through the generation of the highly reactive ·OH radicals in a Fenton reaction:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \cdot \text{OH} + \text{OH}^- 
\]

This led us consider the possibility that the rRNA cleavages might be a result of chemical rather than enzymatic hydrolysis. These cleavages might be directed by redox-reactive iron bound at specific ribosomal sites; for example, through interactions with rRNA or ribosomal proteins in the proximity of the RNA backbone. Previous studies have shown that binding of redox-active metals to remove DNA and RNA molecules can give rise to oxidizing species that are not freely diffusible, thereby producing site-specific cleavages (33–36). Studies have also shown that Fe³⁺ can substitute for Mg²⁺ in RNA (37), with highly structured RNA often exhibiting preferred sites for Fe³⁺ binding (33). Examination of the ES7 region in yeast 25S rRNA, where the major iron-dependent cleavage takes place, revealed three Mg²⁺ ions located 6–8 Å away from the ES7 cleavage site (Fig. 5A), providing potential sites for exchange with Fe³⁺ ions.

To test the hypothesis that the site-specific rRNA cleavages in vivo may be driven by redox-active iron bound to ribosomes, we sought to reconstitute the cleavage process in vitro. We devised reaction conditions based on earlier studies showing that ascorbic acid can induce localized redox reactions at the sites of metal binding (38, 39). Cleavage of nucleic acids by ascorbate in the presence of molecular oxygen is thought to occur by a multistage mechanism involving metal-catalyzed oxidation of ascorbic acid with molecular oxygen, leading to H₂O₂ formation (40) and reduction of the ligand-bound metal to the form capable of producing the highly reactive ·OH radicals from H₂O₂ (38):
Ascorbate $+ O_2 \rightarrow$ Dehydroascorbate $+ H_2O$

Ascorbate $+ 2Fe^{3+} \rightarrow$ Dehydroascorbate $+ 2Fe^{2+}$

$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + \cdot OH + H_2O$

We assembled the in vitro reactions with ribosomes that were purified from WT yeast cells using centrifugation through a sucrose cushion to remove soluble cytosolic components. To minimize the generation of hydroxyl radicals in solution, which can attack solvent-accessible regions of nucleic acids with little selectivity (41, 42), we did not add any exogenous $H_2O_2$, thus relying on ascorbate–iron redox cycling as the principal ROS source in the system. Fig. 5B shows the Northern blot analysis of 25S rRNA fragmentation from the same cultures as in C. SYBR Gold staining of the gel is shown in Fig. S4B. The experiment shown in A and B was performed twice with similar results. C, analysis of superoxide levels in WT, grx5Δ, and sod2Δ strains treated with menadione as above. Cells were stained with DHE and analyzed by flow cytometry using FlowJo software. D, Northern hybridization analysis of 25S rRNA fragmentation from the same cultures as in C. SYBR Gold staining of the gel is shown in Fig. S4B.
rRNA undergoes chemical hydrolysis during oxidative stress

![Graph](image)

**Figure 4. Iron overload contributes to oxidant sensitivity and the degradation of ribosomes in grx5Δ cells.** A, the intracellular iron level is increased in grx5Δ cells. Overnight cultures were diluted with YPDA to A∞ = 0.2, grown for 4 h, and treated or not with PHL for 1 h. Data show mean values in three biological replicates; error bars represent S.D. Two-tailed Student’s t test was used for statistical analysis. ***, p < 0.001; **, p < 0.001. B, PHL restores viability in menadione-treated grx5Δ cells. Cultures grown as in A were treated with 50 µM menadione for 30 or 60 min. Where indicated, cultures were pretreated with 80 µM PHL for 30 min prior to oxidant addition. After treatments, cells were washed, and 2 × 10⁶ cells were serially diluted (1:5) and plated on YPDA agar plates. The plates were incubated at 30 °C for 2 days (WT) or 3 days (grx5Δ). C, PHL pretreatment suppresses the 25S rRNA degradation phenotype. RNA was extracted from the cultures shown in B and analyzed by Northern hybridization with probe y540. SYBR Gold staining of the gel is shown in Fig. S4A. D, suppression of rRNA degradation by ferritin expression. Cells transformed with an empty vector (V) or a mammalian ferritin expression construct (Mf1) were treated or not with 25 µM menadione for 2 h. RNA was analyzed as in C. SYBR Gold staining of the gel is shown in Fig. S4D.

rRNA from the ascorbic acid/iron-treated ribosomes (Fig. 5C) confirmed that the ES7 cleavage in vitro corresponds precisely to cleavage induced by redox stress in live cells, including grx5Δ cells and the previously studied thioredoxin peroxidase–deficient tsa1Δ cells (7).

One notable observation in these experiments was that ES7 cleavage could be obtained with ascorbic acid treatment alone (Fig. 5B), indicating that low levels of iron (or another redox-active metal) were already present in ribosomes isolated from cells. Supplementing the ribosome cleavage reaction with Fe²⁺ markedly increased the level of the ES7 cleavage product (Fig. 5B), showing that iron can indeed promote cleavage at this site. Conversely, addition of the iron chelator defereroxamine (DFO) completely blocked rRNA cleavage (Fig. 5B). Similar protective effects were observed with other chelating agents (EDTA, PHL, and BPS; Fig. 5D), consistent with their ability to displace iron from complexes with biomolecules. Because soluble Fe(EDTA) complexes can readily react with H₂O₂ and generate diffusible ·OH radicals (43, 44), this result further supports the idea that the site specificity of rRNA cleavages depends on the iron ion association with rRNA.

Discussion

Site-specific cleavages of rRNA have been observed previously in different organisms that experience various types of stress (12–14, 45–47). Although these cleavages were ascribed to cellular nucleases, in most cases the enzymes responsible for initiating rRNA strand breaks were not conclusively identified. The results presented here provide several lines of evidence that rRNA cleavages associated with oxidative stress in yeast cells can occur through a nonenzymatic mechanism based on rRNA strand scission by redox-active iron bound to the ribosome. These findings implicate intracellular iron as a key determinant of the effects of oxidative stress on ribosomes (Fig. 6).

Iron is an essential nutrient that acts as a co-factor for many enzymes and transport proteins and participates in a wide variety of physiological processes, including respiration, DNA metabolism, lipid biosynthesis, oxygen transport, and others (48–50). Eukaryotes have developed complex systems for iron uptake, utilization, and storage (48–50). Disruption of these systems can result in either iron shortage or overload, with mitochondrial defects often playing a major role in iron homeostasis disturbances (49). For example, defects in the mitochondrial ISC biosynthesis pathways can increase iron uptake, resulting in excessive amounts of iron in the cytoplasm (51). In this study, we found that deletion of the yeast mitochondrial monothiol glutaredoxin gene GRX5, involved in ISC biogenesis (22–27), made ribosomes highly susceptible to ROS-induced damage. This manifested in the extensive degradation of rRNA after exposure of cells to low-dose oxidants (Figs. 1, A–C, and 2 and Figs. S2 and S3) and was accompanied by a rapid decline in cell viability (Fig. 4B). Although grx5Δ cells con-
tain elevated levels of ROS (Fig. 3, A and C), we observed no direct correlation between ROS amounts and the extent of rRNA fragmentation (Fig. 3, B and D). Instead, we found that lowering the iron content of grx5Δ cells through a cell-permeable chemical chelator or expression of the human mitochondrial ferritin MtF1 (32) suppressed cleavage formation in rRNA (Fig. 4, C and D, and Fig. S5) and improved cell survival (Fig. 4B).

Crucially, we found that probing ribosomes for the presence of bound metals using an in vitro ascorbic acid redox cycling reaction recapitulated the 25S rRNA cleavage pattern observed in cells. Supplementing this reaction with inorganic iron was sufficient to increase the efficiency of the ES7 cleavage, whereas iron chelation completely blocked it (Fig. 5, B and D). These data imply that no additional nuclease is required to cleave the rRNA. Consistent with a role of iron in generating rRNA strand breaks, we observed a markedly increased rRNA degradation during oxidative stress in grx5Δ and other mutants with excessive iron content but not in strains with defective ROS scavenging systems (Fig. 1C).3

One significant observation is that the iron-mediated rRNA cleavages both in vivo and in vitro were not random but rather site-specific. This argues for the existence of high-affinity iron binding sites within the ribosome structure. One possibility is that iron may be brought in proximity to the rRNA’s sugar phosphate backbone by a protein capable of coordinating iron ions without inhibiting their redox potential. An alternative possibility is that rRNA itself captures iron ions; for instance, by substitution of Mg²⁺ in permissive and accessible RNA structures. Consistent with this scenario, structural data demonstrate three Mg²⁺ ions inside of the ES7/ES7C region of 25S rRNA and located ~6–8 Å away from the ES7 cleavage site (Fig. 5A). These Mg²⁺ ions are exposed to the solvent side of the large subunit, allowing their potential replacement with Fe²⁺. In support of iron binding by rRNA, previous studies have shown that Fe²⁺, being a divalent cation with similar ionic radii and geometric properties as Mg²⁺, is capable of structural and functional replacement of Mg²⁺ in nucleic acids (33, 37, 52–55). It is clear, however, that not every Mg²⁺ ion bound to the ribosome could be substituted by iron with the same efficiency. The number of Mg²⁺ ions estimated to exist in the ribosome structure by structural studies (8) greatly exceeds the number of rRNA cleavages observed after oxidative treatments (Fig. 5B). The site specificity of iron binding to rRNA could depend on local structural features, by analogy to the previously described differential binding of iron to DNA (34). In addition, sequence- and structure-specific interactions of RNA with iron have been observed in iron-responsive elements within mRNAs (56), group I intron RNAs (33), and, recently, bacterial ribosomes (57).

The reaction between ligand-bound Fe²⁺ and H₂O₂ is capable of generating hydroxyl radicals (·OH) through a Fenton mechanism and higher oxidation states of iron, including highly reactive ferryl (Fe⁴⁺) species (44, 58). Similarly to ·OH, ferryl oxygen [Fe⁴⁺ = O²⁺] is capable of nucleic acid strand cleavages, as seen with DNAsymes (59). The exact nature of the reactive species produced in a given reaction may depend on multiple factors, including pH, the nature of the substrate, and the chelation state of iron. Although discernible in a controlled chemical system, these parameters are often difficult to account for in a cellular setting (discussed in Ref. 44). Given the complex molecular environment of the ribosome, it is possible that different types of chemistry may underlie rRNA hydrolysis at specific ribosomal locations.

The function of iron as an effector for the ribosome in oxidative stress may be significant in different biological contexts. The finding that the ES7 rRNA cleavage occurs during adaptive stages of the oxidative stress response (7) suggests that iron-mediated rRNA cleavages are not purely destructive but may serve a beneficial biological function in healthy cells. For example, these cleavages might temporarily down-regulate protein synthesis or alter some specific aspects of ribosome function during oxidative stress. By contrast, a failure to control intracellular iron levels, such as seen in strains defective in iron metabolism, predisposes ribosomes to rapid degradation even at low oxidant levels (Fig. 6). This may effectively prevent the synthesis of new proteins required for stress damage control and, hence, enforce a program of cellular self-destruction. In previous studies, ribosome degradation has been observed during apoptotic cell death (13, 14, 60). Recently, a form of programmed cell death in mammalian cells, termed ferroptosis (61), has been related to the induction of excessive lipid damage via iron-dependent peroxidation (reviewed in Ref. 62). Understanding the role of iron in ferroptosis is far from complete (48). It is possible that iron-dependent effects on the cell’s ribosomes, analogous to those described here, may provide a convergence point for cell death mechanisms operating in diverse organisms.

**Experimental procedures**

**Yeast media, chemicals, reagents, and treatment conditions**

WT BY4742 (MATα his3–1 leu2–0 met15–0 ura3–0) and its derivative deletion strains (grx5Δ, sod1Δ, sod2Δ, ctt1Δ, cta1Δ, and tsat1Δ) were obtained from Thermo Fisher. New grx5Δ strains were generated by mating BY4741 (MATα his3–1 leu2–0 met15–0 ura3–0) with grx5Δ, followed by subsequent tetrad dissection as described previously (63). A p0 strain (MATα leu2–3, 112 trpl–1 can1–100 ura3–0 ade2–1 his3–11,15 [phi +]) was kindly provided by Randy Strich.

We used standard recipes forYPD (1% yeast extract, 2% peptone, 2% dextrose, and 10 mg/l adenine), YPAc (1% KOAc, 2% peptone, and 1% yeast extract), and synthetic drop-out media. Unless indicated otherwise, overnight yeast cultures were diluted with fresh YPD to A₆₀₀ ~ 0.2 and grown for an additional 4 h at 30 °C before each experiment.

Cells were treated with H₂O₂ (Avantor Performance Materials), menadione (Enzo), and PHL (VWR Life Science). For in vitro reactions, we used Fe(NH₄)₂(SO₄)₂ (Sigma), ascorbic acid (Alfa Aesar), DFO (BioVision), 4,7-diphenyl-1,10-phenanthroline disulfonic acid (BPS, Alfa Aesar), and EDTA (Sigma).
The human mitochondrial ferritin gene *MTF1* was cloned from human complementary DNA into the pN6 vector using standard PCR-based techniques. The pN6 vector was created by replacing the GALI-GAL10 promoter region within PESC-his (Stratagene) with a 1.5-kb *ADH1* promoter region from the PUAD plasmid (a kind gift from Randy Strich) (64).

**RNA extraction, Northern blotting, and primer extension**

Total RNA was isolated from cells using one-step extraction with formamide–EDTA as described previously (15). To analyze large rRNA species (25S and 18S rRNAs), RNA was separated on 1.2% agarose gels containing 1.3% formaldehyde (65). Small rRNAs and tRNAs were separated on 8% polyacrylamide gels containing 8 M urea as described previously (66). To visualize total RNA, agarose gels were stained with SYBR Gold (Invitrogen) at room temperature for 30 min, and the fluorescent signal was scanned using a Typhoon 9200 imager (GE Healthcare) at 532 nm. RNA was transferred to nylon membranes (Hybond N, GE Biosciences), and individual rRNA and tRNA species were visualized by Northern hybridizations using 32P-labeled oligonucleotide probes (67). The sequences of all probes used in this study are presented in Table S1. All hybridizations were analyzed using Typhoon 9200 in phosphorimaging mode and ImageQuant software (GE Healthcare). For quantification, the volume of the hybridization signal corresponding to the fragment(s) of interest was converted to phosphorimaging signal metrically, and 5 μg was used for one reaction. The reaction volume was adjusted to 100 μl with buffer K. Solutions of ascorbic acid, Fe(NH4)2(SO4)2, DFO, PHL, BPS, and EDTA were freshly prepared as 100 mM stock solutions. 1 μl of each reaction component was placed on a tube wall, and the tubes were briefly centrifuged to start all reactions simultaneously and incubated on ice for 10 min. The reactions were stopped with 50 μl of 0.1 M thiourea. RNA was precipitated by isopropanol, washed with 80% ethanol, and dissolved in 6 μl of water.

**ROS detection**

To measure H2O2 levels, Amplex Red assays were performed with a kit from ThermoFisher as described previously (7) with a few minor modifications. Briefly, 2 × 107 mid-log phase cells were collected, washed with PBS, and resuspended in 300 μl of the reaction buffer supplied with the kit. Cells were incubated for 30 min in a 30 °C shaker and centrifuged, and 50 μl of supernatant was taken into an Amplex Red assay reaction performed in triplicate. The fluorescence of the reaction product resorufin was measured at 590 nm using a Synergy HT microplate reader (BioTek).

For analysis of the mitochondrial superoxide, we used a technique described previously (71). Briefly, 2 × 107 cells were harvested by centrifugation, resuspended in 500 μl of PBS, and stained with 2.5 μg/ml DHE (Molecular Probes) for 30 min in the dark at room temperature. Fluorescence was measured using a BD Accuri C6 flow cytometer (excitation at 488 nm, emission at 585/40 nm), and histograms were analyzed in FlowJo version 10.

**ribosome isolation**

Cells were pelleted by centrifugation, washed, and lysed by glass bead shearing in 30 mM HEPES–NaOH (pH 7.4), 100 mM KOAc, 3 mM MgOAc, 200 μg/ml heparin, and 100 μM phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation, and the amount corresponding to 50–100 A260 units was loaded onto 500 μl of 50% (w/v) sucrose cushion prepared in 70 mM NH4Cl, 4 mM MgCl2, and 10 mM Tris–HCl (pH 7.4). Lysates were centrifuged at 55,000 rpm at 4 °C for 1 h 20 min in a TLA-55 rotor (Beckman). Ribosomal pellets were washed with buffer K (20 mM HEPES–NaOH (pH 7.4), 40 mM KCl, and 4 mM MgCl2) and frozen at −80 °C.

**in vitro redox cycling reaction**

Pellets of purified ribosomes (see above) were resuspended in 100 μl of buffer K supplemented with RNaseLock (Thermo Fisher). The RNA concentration was estimated spectrophotometrically, and 5 μg was used for one reaction. The reaction volume was adjusted to 100 μl with buffer K. Solutions of ascorbic acid, Fe(NH4)2(SO4)2, DFO, PHL, BPS, and EDTA were freshly prepared as 100 mM stock solutions. 1 μl of each reaction component was placed on a tube wall, and the tubes were briefly centrifuged to start all reactions simultaneously and incubated on ice for 10 min. The reactions were stopped with 50 μl of 0.1 M thiourea. RNA was precipitated by isopropanol, washed with 80% ethanol, and dissolved in 6 μl of FAE (98% formamide and 10 mM EDTA) (15). Reaction products were resolved on agarose gels and analyzed by Northern hybridizations as described above.

**Structural analysis**

PDB file 4V88 (8) was obtained from the Protein Data Bank and visualized with PyMOL 2.1 (Schrödinger).

**Cell viability and growth assays**

For growth assays, yeast cultures were adjusted to A600 ~0.1 with appropriate media (with or without 50 μM menadione), 200 μl/well was inoculated into 96-well plates in three replicates, and the cultures were grown for 36 h at 30 °C with shaking. A600 measurements were taken every 5 min and automatically recorded using a BioTek Synergy HT microplate reader. For viability assays, overnight cultures were diluted with YPDA to A600 ~0.2, grown for 4 h, and incubated with 80 μM PHL for 1 h or left untreated. For oxidant treatment, cells were incubated with 50 μM menadione for 30 min to 1 h at 30 °C with shaking. Cells were washed, resuspended in fresh YPDA, and adjusted to the same concentration of 2 × 106 cells/ml. Six serial dilutions for each culture were plated on YPDA agar plates and incubated at 30 °C for 3–5 days.

Measurement of iron levels

To determine levels of total cellular iron, we used a technique described previously (72). Briefly, cells were collected and counted, and the same amounts of cells (5 × 108) were washed and resuspended in 3% nitric acid. Cells were incubated for 16 h at 98 °C, followed by iron chelation with BPS. The absorbance was measured at 535 nm (73).
rRNA undergoes chemical hydrolysis during oxidative stress

Author contributions—D. G. P., and N. S. conceptualization; J. A. Z., A. G., B. M. T., and N. S. data curation; J. A. Z., A. G., and E. B. M. T. formal analysis; N. S. funding acquisition; J. A. Z., A. G., B. M. T., D. S., D. G. P., and N. S. investigation; B. M. T., A. G., and N. S. methodology; D. G. P. resources; D. G. P. and N. S. writing-review and editing; N. S. supervision; D. G. P. and N. S. validation; N. S. writing-original draft; N. S. project administration.

Acknowledgments—We thank Randy Strich, Katrina Cooper, and the members of their laboratories for critical evaluation of this work. We also thank Russell Sapio for help with cloning of the mammalian ferritin gene and constructive comments on the manuscript.

References
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Iron-dependent cleavage of ribosomal RNA during oxidative stress in the yeast
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doi: 10.1074/jbc.RA118.004174 originally published online July 18, 2018

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