The essential iron-sulfur protein Rli1 is an important target accounting for inhibition of cell growth by reactive oxygen species

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ABSTRACT
Oxidative stress mediated by reactive oxygen species (ROS) is linked to degenerative conditions in humans and damage to an array of cellular components. However, it is unclear which molecular target(s) may be the primary “Achilles’ heel” of organisms, accounting for the inhibitory action of ROS. Rli1p (ABCE1) is an essential and highly conserved protein of eukaryotes and archaea that requires notoriously ROS-labile cofactors (Fe-S clusters) for its functions in protein synthesis. In this study, we tested the hypothesis that ROS toxicity is caused by Rli1p dysfunction. In addition to being essential, Rli1p activity (in nuclear ribosomal-subunit export) was shown to be impaired by mild oxidative stress in yeast. Furthermore, prooxidant resistance was decreased by \textit{RLI1} repression and increased by \textit{RLI1} overexpression. This Rli1p dependency was abolished during anaerobicity and accentuated in cells expressing a FeS-cluster-defective Rli1p construct. The protein’s FeS clusters appeared ROS labile during in vitro incubations, but less so in vivo. Instead, it was primarily \textit{{}^{55}\text{FeS-cluster}} supply to Rli1p that was defective in prooxidant-exposed cells. The data indicate that, owing to its essential nature but dependency on ROS-labile FeS clusters, Rli1p function is a primary target of ROS action. Such insight could help inform new approaches for combating oxidative stress-related disease.

INTRODUCTION
Reactive oxygen species (ROS) are a necessary evil of aerobic life, generated continuously during respiration with the potential to cause oxidative deterioration of proteins, lipids, and DNA. ROS damage is linked to serious diseases in humans, including amyotrophic lateral sclerosis, Alzheimer’s disease, Friedreich’s ataxia, and cancer (Roberts et al., 2009). Furthermore, ROS generation is elevated by environmental perturbation, with oxidative stress being common to the effects of diverse natural (e.g., radiation) and anthropogenic (e.g., chemical pollutant) stresses (Avery, 2001; Limon-Pacheco and Gonsebatt, 2009).

The responses invoked by organisms to counter oxidative stress have received considerable research attention over the last two decades. These include the up-regulation of ROS-scavenging proteins, such as peroxidases and superoxide dismutases, or enzymes that reverse oxidative damage, such as methionine sulfoxide reductases. Oxidative stress responses are now well characterized in a diverse range of organisms (Imlay, 2008). However, when such defenses are overwhelmed, a key question remains: What is the principal cellular function(s) targeted by ROS that accounts for their toxicity?

Whereas oxidative damage to cellular macromolecules is very widely reported, just two types of effect are thought potentially to cause ROS toxicity: gain of toxic function or loss of essential cellular function (Avery, 2011). Gain-of-function mechanisms could include accumulation of toxic oxidized-protein aggregates (Holland et al., 2007) or apoptotic cell death (Circu and Aw, 2010). Essential targets may include membrane lipid integrity, via lipid peroxidation, and certain ROS-susceptible proteins (Avery, 2011; Daly, 2012). Recent studies indicate that essential protein targets of ROS include functions required for faithful mRNA translation (Holland et al., 2007;
FeS clusters are protein cofactors that are among the most ROS-sensitive structures in biology, yet they have been conserved through evolution and are required for diverse protein functions (Imlay, 2006; Lill, 2009; Py et al., 2011). Several Fe-S proteins are notoriously ROS labile, although studies of ROS sensitivity to date have focused on nonessential FeS proteins. The first FeS protein identified as essential for eukaryotic cell viability was the multifunctional ABC-family protein, termed Rli1 in the yeast model (ABC1 in humans and other organisms; Kispal et al., 2005; Yarunin et al., 2005). It was suggested that the essential nature of FeS-cluster biosynthesis might reflect the essentiality solely of Rli1p (Kispal et al., 2005). More recently, several essential nuclear proteins involved in DNA replication or repair have also been shown to require FeS metallocenters (Rudolf et al., 2006; Klinge et al., 2007; Netz et al., 2012). Rli1p has roles in ribosome biogenesis and maturation (Kispal et al., 2005; Yarunin et al., 2005), translation initiation (Dong et al., 2004; Chen et al., 2006), translation termination (Koshelevich et al., 2010; Shoemaker and Green, 2011), and ribosome recycling (Barthelme et al., 2011; Pisareva et al., 2011; Shoemaker and Green, 2011; Becker et al., 2012). Integrity of the N-terminal [4Fe-4S]–cluster domain of Rli1p is crucial for its function in protein synthesis. Rli1p is one of the most highly conserved proteins across the eukaryotes and archaea (Barthelme et al., 2007; Becker et al., 2012). This, together with its essentiality in all organisms tested, but functional dependency on ROS-labile FeS clusters, suggested to us that Rli1p could be a primary cellular target of ROS. In this study, we tested that hypothesis.

Previously it was found that methionine sulfoxide reductases (MSRs) help to preserve the integrity of FeS clusters in oxidatively stressed yeast (Sideri et al., 2009). We used MSR-deficient cells alongside other tools to show that Rli1p fulfills the key criteria listed above, as a crucial target on which wild-type cell viability pivots during oxidative stress. Because Rli1p is so central to the essential process of protein synthesis and is so highly conserved, the need to maintain Rli1p function could be an “Achilles’ heel” of many aerobic organisms.

RESULTS
Amelioration of FeS protein activity increases prooxidant resistance
A previous study with MSR-deficient strains suggested a link between toxicity of the prooxidant metal copper and the cluster integrity of FeS proteins in the yeast model (Sideri et al., 2009). This was consistent with work in bacteria (Macomber and Imlay, 2009). To explore this further with wild-type yeast, we tested the effect of enhanced FeS protein activity on Cu(II) resistance. First, we exploited the Mn-superoxide dismutase, Sod2p, which protects FeS clusters from superoxide attack at the mitochondrial location of cluster biogenesis (Irazusta et al., 2006; we confirmed an approximately twofold increase in FeS protein [aconitase] activity in cells overexpressing SOD2). The Sod2p-overexpressing cells had increased Cu resistance (Figure 1A). Atm1p exports a substrate from the mitochondrial FeS biogenesis pathway to the cytosol that is required for assembly of cytosolic FeS-cluster proteins (Kispal et al., 1999). ATM1 overexpression gave a Cu-resistance phenotype (Figure 1A; we confirmed an approximately twofold increase in cytosolic FeS protein [Leu1] activity in cells overexpressing ATM1). This suggested that an extramitochondrial FeS protein(s) affects Cu resistance.

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We confirmed that ATM1 overexpression increased Cu resistance in either rich yeast–peptone–dextrose (YPD) medium or minimal medium, indicating that resistance was not due to rescued production of amino acids that require FeS protein activity for biosynthesis, as these amino acids are supplied in YPD.

The apparent titration of Cu(II) toxicity with FeS-protein activity (discussed above) raised the possibility that Cu may act by decreasing the activity of an essential and extramitochondrial FeS protein function. Essential nuclear FeS proteins are known (Rudolf et al., 2006; Klinge et al., 2007; Netz et al., 2012), but the cellular requirement for FeS-cluster biogenesis was originally suggested to hinge on cytosolic Rli1p, which is essential for protein synthesis (Kispal et al., 2005). We determined whether sensitivity to Cu and other prooxidants could be rescued by elevating cellular RLI1 expression. This was tested first in the MSR-deficient mxrΔ background, as this has FeS-cluster defects and is prooxidant sensitive in yeast–nitrogen base (YNB) medium (a previously reported Cu-resistance phenotype was specific to YPD medium; Sideri et al., 2009). RLI1 was expressed under tetO regulation in the absence of doxycycline, to give >40-fold derepression (Supplemental Figure S1A). RLI1 overexpression fully rescued mild sensitivity of the mxrΔ mutant to the prooxidants Cu, H2O2, and paraquat (Figure 1B). The outcome was similar in a sod2Δ background (Figure 1C), suggesting that the Sod2p dependency of Cu resistance (Figure 1A) may involve an effect on Rli1p activity.

Rli1p-dependent resistance to oxidative stress

The above data indicating resistance with increased Rli1p expression (Figure 1B) were in a mutant predisposed to FeS-cluster defects. We also showed that decreasing Rli1p expression through use of a tet-RLI1 construct produced mild Cu(II) sensitivity (Figure 2A). A heterozygous RLI1/rli1 strain also appeared to be slightly Cu sensitive. It might still be argued that the above phenotypes were all specific to the particular strain defects (Figures 1B and 2A), whereas Rli1p function may normally be protected effectively in wild-type cells. Therefore we overexpressed Rli1p in wild-type cells, as increasing resistance with this approach is diagnostic that function of the normal toxicity target is being preserved (Avery, 2011). RLI1 overexpression improved resistance to all of the test prooxidants, particularly H2O2, Cr(VI), and Cu(II) (Figure 2B); we verified that doxycycline addition abolished the advantage of tet-RLI1 cells, confirming the effect was due to [tet-regulated] RLI1 expression. The effect appeared to be specific, as overexpression of other known essential FeS proteins (Rad3, Pri2, Pol1, Pol2, Pol3; Rudolf et al., 2006; Klinge et al., 2007; Netz et al., 2012) did not increase resistance (Figure S2). Rli1p-overexpressing cells were also resistant to acute short-term killing by Cu (Figure S3). The relatively short (~38 min) half-life of Rli1p (Belle et al., 2006) means that even if copper normally only blocks de novo formation of the active protein (see FeS clusters of Rli1p are ROS labile in vitro, but FeS-cluster supply to Rli1p is the critical target in vivo), this would be sufficient to give a marked loss of Rli1p activity during the above 1-h time course (Figure S3). The resistance to killing was not inconsistent with (Rli1p-dependent) protein synthesis being the critical target, as a protein synthesis inhibitor (cycloheximide) is sufficient to cause viability loss; that effect also was partly rescued by Rli1p overexpression (Figure S3). Collectively the data suggested that prooxidant-sensitive Rli1p function is a pivotal determinant of growth inhibition by prooxidants.

Mild prooxidant stress perturbs Rli1p function

The essential nature of Rli1p function and the fact that prooxidant resistance correlated positively with expression level of the protein (Figure 2) fulfilled two criteria expected of a key protein target of ROS toxicity (Avery, 2011). A third criterion is that the protein function should be susceptible to mild oxidative stress. The principal in vivo assay for Rli1p function is of ribosomal subunit export from the nucleus. Nuclear green fluorescent protein (GFP) accumulation in cells expressing a GFP fusion with the small ribosomal subunit protein Rps2 is a sensitive indicator of defective Rli1p function (Kispal et al., 2006) means that even if copper normally only blocks de novo formation of the active protein (see FeS clusters of Rli1p are ROS labile in vitro, but FeS-cluster supply to Rli1p is the critical target in vivo), this would be sufficient to give a marked loss of Rli1p activity during the above 1-h time course (Figure S3). The resistance to killing was not inconsistent with (Rli1p-dependent) protein synthesis being the critical target, as a protein synthesis inhibitor (cycloheximide) is sufficient to cause viability loss; that effect also was partly rescued by Rli1p overexpression (Figure S3). Collectively the data suggested that prooxidant-sensitive Rli1p function is a pivotal determinant of growth inhibition by prooxidants.

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function, we assayed rescue of the phenotype by RLI1 overexpression; this was achieved with an isolonotic tet-RLI1 strain that gave >15-fold higher RLI1 expression than the wild-type in the absence of doxycycline (Figure S1B). RLI1 overexpression partly rescued nuclear Rps2-GFP export during the mild oxidative stresses (Figure 3B). The fact that export was not fully restored was not unexpected, as potential issues, such as defective FeS-cluster assembly or integrity in Rli1p, would still persist. To test whether FeS-cluster supply may affect Rli1p-dependent nuclear export, we overexpressed Atm1p, a manipulation that increased Cu resistance (Figure 1A). Similar to Rli1p overexpression, Atm1p overexpression suppressed the nuclear Rps2-GFP export defect during mild Cu stress (Figure 3C). This result also corroborated the idea that Atm1-dependent Cu resistance (Figure 1A) was related to an impact on Rli1p.

Oxidative targeting of the FeS clusters required for Rli1p function

The facts that the above stressors were prooxidants and that [4Fe-4S] clusters are notoriously ROS labile pointed to an oxidative mode of action against Rli1p. The above effects were measured over a timescale of hours, which is ample time for oxidative stress caused by the prooxidants to take effect (Avery, 2011). However, nonoxidative [4Fe-4S]-cluster damage is also known (Macomber and Imlay, 2009). Therefore we compared the Rli1p dependence of stressor resistance under aerobic and anaerobic conditions. As in the broth experiments, Rli1p overexpression conferred prooxidant resistance during aerobic growth on agar (Figure 4). However, these cells’ relative resistance was abolished under anaerobic conditions, suggesting an oxidative mode of Rli1p targeting.

The recovery of Rli1p function seen in Atm1-overexpressing cells during mild oxidative stress (Figure 3C) was consistent with FeS clusters being the specific ROS target relevant to Rli1p. To substantiate this, we tested cells expressing RLI1C58A. This labile version of Rli1p lacks one of the protein’s cluster-coordinating cysteine residues (Barthelme et al., 2007). Replacement of wild-type RLI1 with RLI1C58A had little impact on growth in control conditions (Figure 5A). However, the RLI1C58A-expressing cells were stress sensitive, particularly to H2O2 and Cu(NO3)2. This effect was reflected in Rli1p activity, as the proportion of cells exhibiting defective Rps2-GFP export during mild Cu stress was approximately twofold greater in RLI1C58A-expressing cells than in the wild-type (Figure 5B). The results supported the hypothesis that growth inhibition by the oxidants was centered on FeS-cluster integrity in Rli1p.

FeS clusters of Rli1p are ROS labile in vitro, but FeS-cluster supply to Rli1p is the critical target in vivo

To determine the integrity of the protein’s FeS clusters during ROS stress, we monitored the association of 55Fe with hemagglutinin (HA)-tagged Rli1p (Kispal et al., 2005). 55Fe was not detectable in immunoprecipitations from wild-type (non-Rli1-HA–expressing) cells incubated with 55FeCl3. Rli1-HA protein that was immunoprecipitated from Rli1-HA–expressing cells, after preincubation with 55FeCl3, exhibited rapid FeS-cluster turnover (according to 55Fe release) during incubation in vitro with a copper/ascorbic acid system (Macomber and Imlay, 2009) (Figure 6A). This supported previous indications concerning lability of the FeS clusters in Rli1p (Barthelme et al., 2007), but we were conscious that it might not necessarily reflect the physiological situation in vivo. Therefore, after preincubation with 55FeCl3, Rli1-HA was immunoprecipitated before and during exposure of cells to prooxidants. Prooxidant concentrations were just lower than those affecting growth markedly (<10% slowing of growth rate), that is, equivalent to the conditions associated with...
defective Rli1p activity (Figure 3). Under these conditions, FeS-cluster turnover in Rli1p in vivo was not increased by the presence of stressor; $^{55}$Fe retention actually appeared to be improved slightly by the stressors after 1 h (Figure 6B). FeS-cluster turnover in Rli1p in vivo could be detected only at growth-inhibitory stressor doses beyond those needed to affect Rli1p activity. It was concluded that the in vitro FeS-cluster turnover in Rli1p (Figure 6A) had limited physiological relevance in this study. Further exposure assays were performed in vivo. Because FeS clusters are assembled before incorporation to Rli1p (Lill, 2009), targeting of exposed FeS clusters could potentially occur upstream of Rli1p. The influence of Atm1p seen here (Figures 1A and 3C) and recent work on oxidant disruption of the bacterial lsc system (Jang and Imlay, 2010) were consistent with that possibility. Therefore we measured incorporation of $^{55}$Fe to Rli1p during exposure of cells to prooxidants. Cu and paraquat only were used for these experiments, as chromate and H$_2$O$_2$ treatment decreased $^{55}$Fe uptake by cells, as has been described elsewhere (Faulkner and Helmann, 2011), making it difficult to discern any Rli1p-specific incorporation effects. Cellular $^{55}$Fe uptake was not decreased by subinhibitory Cu(NO$_3$)$_2$ and paraquat concentrations, but $^{55}$Fe incorporation to Rli1p was decreased by 50–80% (Figure 6C). The level of Rli1 protein remained unaltered in these experiments (Figure 6C, right panel), and Rli1 apoprotein level is reported elsewhere to be unaffected by loss of FeS-cluster supply (Balk et al., 2004). The results support a model in which ROS targeting of FeS clusters prior to their assembly into Rli1p leads to depletion of essential Rli1p function and, in turn, defective growth during oxidative stress.

**DISCUSSION**

This work points to the evolutionarily conserved protein Rli1 as a novel and primary target of prooxidant toxicity in cells. This study encompassed diverse prooxidants with distinct activities, including H$_2$O$_2$ and superoxide-generating agents, such as paraquat and Cr(VI) (Sumner et al., 2005). Given this, together with the extensive...
FeS cluster turnover and incorporation to Rli1-HA. Assays were with pCM190-tetRLI1-HA–transformed cells cultured in the absence of doxycycline. (A) Rli1-HA was immunoprecipitated from protein extracts of cells preincubated with $^{55}$FeCl$_3$. $^{55}$Fe retention in Rli1-HA was measured before (Un-treated) and 10 min after the indicated treatments. FC, ferricyanide. (B) Cells preloaded with $^{55}$FeCl$_3$ were incubated for 1 or 4 h in YNB in the absence (U.) or presence of 0.8 mM Cu(NO$_3$)$_2$, 0.15 mM CrO$_3$, 1 mM paraquat (PQ), or 0.25 mM H$_2$O$_2$ before Rli1-HA immunoprecipitation and $^{55}$Fe quantification. (C) Cells were incubated in the absence or presence of 0.8 mM Cu(NO$_3$)$_2$ or 1 mM paraquat, and simultaneously with $^{55}$FeCl$_3$. $^{55}$Fe incorporated to Rli1-HA was determined via immunoprecipitations at the indicated intervals. Right, Western blot analysis of Rli1-HA in protein extracts at 4 h. Representative gel from one of four independent experiments and quantification of Rli1-HA band intensities from these experiments. All values are means from at least three replicate determinations ± SEM.

A tape graphic shows FeS-cluster turnover and incorporation to Rli1-HA. The graph on the left illustrates the measurement of $^{55}$Fe retention in Rli1-HA before and after various treatments. The right side of the figure includes a Western blot analysis of Rli1-HA in protein extracts at 4 h, with representative gel images and quantification of band intensities from different experiments. The data indicate that FeS clusters are turnover and incorporated into Rli1-HA, with specific conditions affecting retention and incorporation levels.

Data already available on biological responses to oxidative stress, not least in yeast, might be considered surprising that such a role for Rli1p in ROS resistance has not been reported previously. Several factors may explain this. First, the function of Rli1p is only emerging from recent studies and there remains a general paucity of published work on this protein or its orthologue ABCE1. Second, the protein is essential, it is excluded in homozygous deletion–mutant screens. Third, Rli1p activities are not very easy to assay, particularly in vivo. Last, the relative stability of its FeS clusters in vivo (discussed below), undermines what might otherwise be the most obvious potential mechanism of Rli1p inactivation by ROS. Indeed, the clusters in Rli1p (ABCE1) are not solvent exposed as are other ROS-sensitive FeS proteins (Karcher et al., 2008).

The necessity for Rli1p in protein synthesis dictates that this protein's essential nature is not conditional. This makes Rli1p unique among the FeS proteins reported to have ROS-sensitive function. The bacterial dehydratases are more dispensable. For example, propylmalate isomerase is strongly H$_2$O$_2$ sensitive, but is only required for growth in the absence of leucine (Jang and Imlay, 2010). Furthermore, previous studies of FeS protein inactivation in vivo have mostly been with compromised mutant strains. In contrast, Rli1p function could be identified here as a prooxidant target in wild-type cells not predisposed to ROS action.

Besides loss of protein function, oxidative disruption of FeS clusters can produce a (toxic) gain-of-function effect, as the released Fe(II) may participate in Fenton chemistry and exacerbate oxidative stress (Keyer and Imlay, 1996; Liochev and Fridovich, 1999). Our data do not support the latter model for Rli1p, as increased Rli1p expression rescued rather than exacerbated ROS toxicity (although we cannot rule out the possibility that sequestration of FeS clusters into Rli1p could decrease the cellular pool of ROS-sensitive FeS clusters). A similar argument also counters a previous suggestion that the FeS domain of Rli1p could serve an ROS-sensing function, as part of the (decreased) protein synthesis response to oxidative stress (Yarunin et al., 2005; Barthelme et al., 2011). That response is thought to ameliorate ROS resistance, but Rli1p down-regulation in this study had the opposite effect. A role for Rli1p has not been detected from previous studies of the protein synthesis response (Shenton et al., 2006). Instead, Rli1p dysfunction appears to be a deleterious outcome of oxidative stress.

Previous studies on proteins with solvent-exposed FeS clusters have mostly focused on ROS-mediated cluster turnover as the mechanism of protein inactivation (Flint et al., 1993; Macomber and Imlay, 2009). The two [4Fe-4S] clusters of Rli1p (ABCE1) are predicted to be well shielded from solvent (Karcher et al., 2008). Thus, although there are only one or two reports of successful purification of active Rli1p (Shoemaker and Green, 2011), the protein’s FeS clusters are not elaborately in Rli1-HA immunoprecipitations (Kispal et al., 2005). Our data suggest that FeS clusters already incorporated to Rli1p are not the key ROS targets in vivo. In vitro, using purified Rli1-HA, we observed some $^{55}$FeS turnover. That effect was not reproduced at prooxidant doses that were just subinhibitory in vivo, whereas $^{55}$Fe incorporation to Rli1p was inhibited by 50–80% in the same conditions. Obviously, in vitro assay conditions do not reproduce the complexity of the intracellular environment, and parameters such as subcellular compartmentation, cellular metabolism, and redox status can modulate the in vivo action of the stressors used here.
The indication that $^{55}$Fe incorporation to Rli1p is the primary ROS-sensitive target in vivo resonates with recent work in bacteria, in which cluster assembly on or transfer from scaffold proteins was proposed to underpin oxidant disruption of the Isc system (Jang and Imlay, 2010). FeS clusters are likely to be solvent-exposed (and therefore ROS susceptible) during transfer. This affected FeS-cluster insertion into proteins akin to Rli1p, such as NADH dehydrogenase I, in which FeS clusters are normally buried (Jang and Imlay, 2010). In the case of Rli1p, targeting of upstream FeS-cluster assembly or transfer seems most likely to occur in the cytosol (e.g., at scaffold or transfer proteins such as Cfd1, Nbp35, Nar1, and Gia1), as FeS assembly in mitochondria is partly shielded from external stress. The difficulty of further identifying the step(s) at which such targeting takes place was noted previously (Jang and Imlay, 2010). Moreover, a major biological consequence is that such events may lead to defective FeS delivery to an essential protein. We propose that oxidative disruption of FeS clusters destined for insertion into Rli1p is the principal mechanism of ROS action on Rli1p.

The possibility of FeS-cluster turnover suggested by our in vitro data may be more important for ROS toxicity in cells expressing Rli1CSBA as their sole Rli1p. These cells’ prooxidant sensitivity was in keeping with the predicted lability of Rli1CSBA (Barthelme et al., 2007). Rli1CSBA lacks a [Fe$^{4}$-4S]-coordinating cysteine, yielding a [3Fe-4S]$^{―}$ cluster that nonetheless supports sufficient Rli1p function for cell viability (the other coordinating cysteines are mostly essential; Kispal et al., 2005; Barthelme et al., 2007). Rli1CSBA-expressing cells were especially sensitive to Cu and H$_{2}$O$_{2}$. This fits with the fact that Cu (via Fe displacement; Macomber and Imlay, 2009) and H$_{2}$O$_{2}$ (Jang and Imlay, 2010) can degrade FeS clusters beyond the [3Fe-4S] state in which the CSBA cluster is thought to exist (Barthelme et al., 2007). In contrast, superoxide-generating agents such as paraquat and Cr are not thought to oxidize FeS clusters beyond [3Fe-4S]$^{+}$ in vivo (Varghese et al., 2003; Macomber and Imlay, 2009). The limited paraquat and Cr sensitivity of Rli1 being the protein’s primary frailty during oxidative stress. The limited paraquat and Cr sensitivity of Rli1 accordingly fine for Rli1p in influencing organism fate during ROS stress.

**MATERIALS AND METHODS**

**Strains and plasmids**

*Saccharomyces cerevisiae* BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ural3Δ0), the diploid strain BY4743, and isogenic deletion strains were from Euroscarf (Frankfurt, Germany). BY4741 was the background used for all experiments except those in which an alternative background is specified. An mxr1Δ/mxr2Δ (mxrΔ) double mutant isogenic with BY4741 was constructed previously (Sideri et al., 2009). The wild-type W303 and isogenic tet-RLI1 strain were kind gifts from R. Lill (University of Marburg; Kispal et al., 2005). Strain R1158 and isogenic strains carrying the following constructs were from Open Biosystems (Lafayette, CO): tet-RLI1, tet-RAD3, tet-PRI2, tet-POL1, tet-POL2, and tet-POL3. The native RLI1 gene in BY4741 was replaced with a mutant RLI1CSBA construct by short-flanking homology PCR (Wach et al., 1997), involving amplification of RLI1CSBA together with HIS3 marker from the vector pRS423 (kindly provided by R. Tanpe, University of Frankfurt; Barthelme et al., 2007). An isogenic control strain was constructed by inserting the HIS3 marker alone in strain BY4741. Yeast transformations were by the lithium acetate method (Gietz and Woods, 2002). Appropriate integration of cassettes to the genome was confirmed by diagnostic PCR (Wach et al., 1997). All primer sequences are available on request.

For overexpression of SOD2 and ATM1, a multicopy vector YEp351-KanMX6 that had been constructed by replacement of the LEU2 marker with KanMX6 between the PpuMI and BsrGI sites of YEp351, was digested with Kasi and EcoNI, and the hphNT1 marker was inserted in place of KanMX6. Fragments encompassing the SOD2 and ATM1 open reading frames (ORFs), together with native promoters, were amplified from yeast genomic DNA and ligated between the KpnI-Sall (SOD2) or Sall-SbfI (ATM1) sites of YEp351-hphNT1. Yeast transformants were selected in YPD agar (see Yeast Culture and Toxicity Assays) supplemented with 150 μg/ml hygromycin B (Invitrogen, Carlsbad, CA). Plasmid pRS513-RPS2-EFP was kindly donated by E. Hurt (University of Heidelberg; Milkereit et al., 2005). pCM190-tetATM1 and -tetRLI1 were constructed by PCR amplification of fragments encompassing the relevant ORFs from yeast genomic DNA and ligation between the NotI and PstI sites of pCM190. This placed ATM1 or RLI1 under the control of the tetO promoter. An Rli1 construct that was C-terminally tagged with the HA epitope was described and validated previously (Kispal et al., 2005). An analogous RLI1-HA construct was prepared here by PCR-amplifying the RLI1 gene from pCM190-tetRLI1 with addition of a terminal sequence comprising HA and a PstI restriction site. The amplified ~1.9-kb fragment was cut at the PstI site and at a BgIII site internal to the RLI1 ORF, yielding an ~1-kb fragment encompassing the HA-tagged C-terminal portion of RLI1. This fragment was ligated between the PstI and BgIII sites of pCM190-tetRLI1, replacing the corresponding wild-type RLI1 sequence. All DNA cloning and genetic manipulations were performed in Escherichia coli XL1-Blue cells (Invitrogen). Restriction digests, DNA ligations, sequencing, and PCR were carried out using standard protocols (Ausubel et al., 2007).

**Yeast culture and toxicity assays**

Yeast strains were maintained and grown in YPD broth (Khozoie et al., 2009) or in YNB medium (0.69% yeast-nitrogen base without
amino acids [Formedium], 2% (wt/vol) glucose), supplemented as required for plasmid selection with amino acids, uracil, or hygromycin B (Ausubel et al., 2007). Where necessary, media were solidified with 2% (wt/vol) agar (Sigma-Aldrich, St. Louis, MO). Experimental S. cerevisiae cultures were inoculated from overnight starter cultures grown from single colonies, and cultured to exponential phase (OD600 ~ 2.0) in liquid medium at 30°C, 120 rpm. Samples were diluted to OD600 ~ 0.02, and 300-μl aliquots were transferred to 48-well plates (Greiner Bio-One, Monroe, NC) before addition or not of specified stressors. Cultures were incubated with shaking in a BioTek Powerwave XS microplate spectrophotometer, as previously described (Khozoie et al., 2009). For growth assays on agar, cultures at OD600 ~ 2.0 were diluted in 10-fold series and then inoculated as 5- or 8-μl spots to YPD or YNB agar supplemented with stressors as specified. Plates were incubated at 30°C for 5–7 d before image capture, with anaerobic incubation under H2 and CO2, where indicated. Determination of short-term cell killing in YNB broth was according to loss of colony-forming ability, as described previously (Sumner et al., 2003).

Assay of nuclear Rps2-eGFP export

Cells transformed with plasmid pRS315-RPS2-eGFP were examined for nuclear retention of fluorescence during appropriate treatment with stressors. As described previously (Milkereit et al., 2003; Kispal et al., 2005), fluorescence in individual cells was either visibly colocalized or not with the nucleus, with additional Rps2-eGFP fluorescence dispersed in the cytoplasm. Cell nuclei were stained with 4’6-diamidino-2-phenylindole (DAPI; Pringle et al., 1989). Cells were viewed with a Zeiss Axioscope MS fluorescence microscope fitted with a HBO50 illuminator. Images were captured with a Zeiss Axioscope digital camera (Jena, Germany).

RNA extraction and quantitative RT-PCR (qRT-PCR)

RLI1 mRNA was quantified as described previously (Halliwell et al., 2012). Briefly, cDNA was generated from isolated RNA with Oligo(dT)20 Primer (Invitrogen) and purified (PCR purification kit; Geneflow, Lichfield, Staffordshire, UK) before use as a template for triplicate qRT-PCR reactions, comprising 30 ng cDNA, 100 nM RLI1-specific primers (sequences available on request), 1X Fast SYBR Green Master Mix (Applied Biosystems, Bedford, MA), made up to 10 μl with RNase-free water. Agarose gel electrophoresis and melting-curve analysis confirmed a single PCR product. Reactions performed in sealed MicroAmp 96-well fast-optical plates were monitored with a 7500 Fast Real-Time PCR System (Applied Biosystems). Amplification was quantified from a standard curve constructed from reactions with defined cDNA copy number.

Enzyme assays, 55Fe-labeling studies, and Western blotting

Protein extraction from yeast (Cashikar et al., 2005) and assay of acornitase or isopropylmalate dehydratase (Leu1) activities (Siders et al., 2009) were as described previously. Protein extracts were maintained under nitrogen throughout to protect FeS clusters. For measurement of FeS-cluster turnover in the Rli1-HA protein, exponential phase cultures in YNB broth were labeled by incubation for 3 h with 55FeCl3 (38 μCi/ml). Labeled cells were washed and suspended in prewarmed YNB supplemented with stressor as specified. At intervals during incubation with shaking, OD600 was recorded, and cell samples were pelleted by centrifugation (1500 × g, 5 min), washed, and stored at −20°C. Frozen pellets were resuspended in lysis buffer (300 μl 50 mM phosphate buffer, pH 7.4, 5% [vol/vol] glycerol, EDTA-free protease inhibitor cocktail [Roche, Indianapolis, IN]). Samples were dropped into liquid N2, ground with a mortar and pestle, and thawed, before centrifugation at 16,000 × g for 10 min. Protein in the supernatant was determined with a Bradford assay kit (Bio-Rad, Hercules, CA). Protein (150 μg) was mixed with 10 μl anti-HA beads (A2095; Sigma-Aldrich) for 1 h at 4°C. Beads were washed six times with lysis buffer and were then transferred to 3 ml scintillation fluid (Emulsifier Safe; Perkin Elmer-Cetus, Waltham, MA). Fe55 was measured with a Packard Tri-Carb 2100TR liquid scintillation analyzer (Meriden, CT). The overall procedure was the same for measurement of Fe55 incorporation into Rli1-HA, except that 55FeCl3 was added to cultures at the same time as specified stressors, with samples removed for analysis during subsequent incubation. For in vitro analysis of FeS-cluster turnover, protein was extracted from 500 ml cultures labeled with 55Fe for 1 h (in the absence of stressor). After immunoprecipitation with 150 μl anti-HA beads, aliquots of the beads were incubated for 10 min at room temperature in the absence or presence of 25 or 100 μM Cu(NO3)2 with 350 μM ascorbate and 100 μM histidine (Macomber and Imlay, 2009), or ascorbate and histidine only, or 200 and 500 μM ferricyanide (Barthelme et al., 2007), before centrifugation and analysis of bead-associated 55Fe. For Western blotting, proteins (17.5 μg) were separated by electrophoresis on 12% (wt/vol) NuPAGE Bis-Tris gels (Invitrogen) before transfer to nitrocellulose membrane (Bio-Rad). Immunodetection of Rli1-HA was with a rabbit anti-HA primary antibody (1:1000 dilution; Abcam, Cambridge, MA) and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:1000 dilution; Pierce, Rockford, IL). Rli1-HA was detected with an electrochemiluminescence HRP kit (Pierce) and imaged using a Chemidoc XRS (Bio-Rad).

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