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 *RLI1* repression and increased by *RLI1* overexpression. This Rlip1 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 ^{55}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.**

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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;

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Abbreviations used: DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescent protein; HA, hemagglutinin; HRP, horseradish peroxidase; MSR, methionine sulfoxide reductase; ORF, open reading frame; qRT-PCR, quantitative RT-PCR; ROS, reactive oxygen species; YNB, yeast–nitrogen base; YPD, yeast–peptone–dextrose.

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Ling and Soll, 2010). Other ROS-susceptible proteins are likely to be enriched for ROS-sensitive amino acids or cofactors (Moller *et al.*, 2011). Moreover, it remains unknown which putative target(s) first accumulates damage of a severity that precludes cell recovery, that is, what target(s) primarily accounts for growth inhibition and/or loss of viability during oxidative stress? There could be more than one such target, the identity of which may depend on the nature of the oxidative stress (Thorpe *et al.*, 2004; Avery, 2011). To establish the identity of an essential protein that is a major ROS target, key criteria that need to be met are that the protein should be modified in an oxidation-dependent manner and exhibit decreased function (which cannot be accounted for by decreased expression) during oxidative stress. Furthermore, knockdown of the relevant protein should produce an ROS-sensitive phenotype and, moreover, overexpression should confer resistance (Avery, 2011).

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 (ABCE1 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 (Khoshnevis *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.

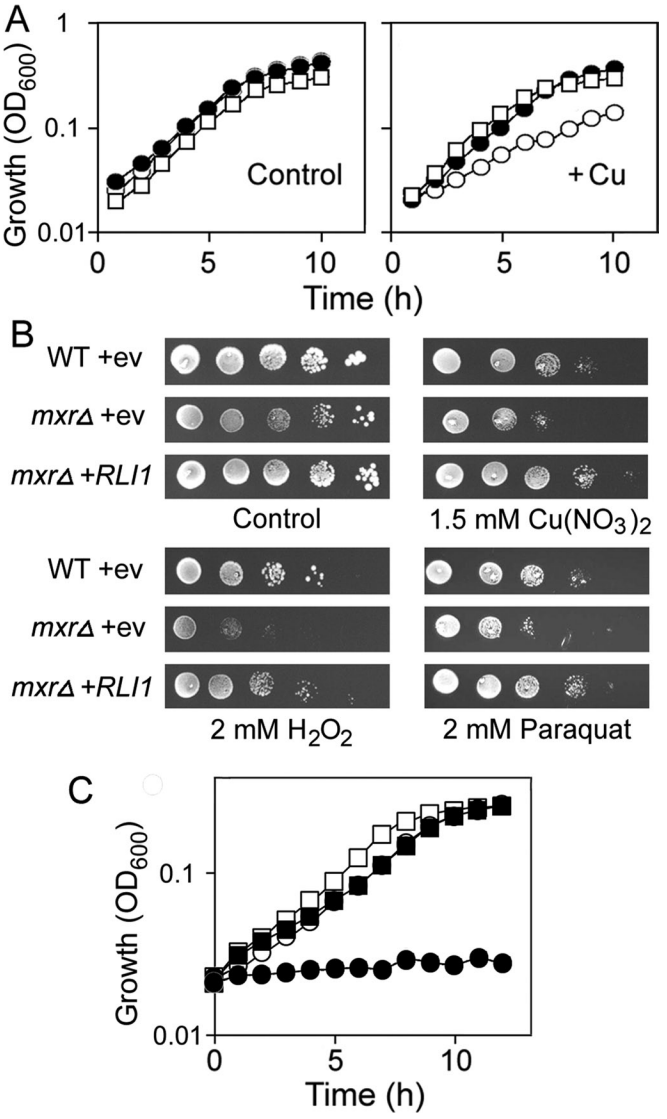

FIGURE 1: Amelioration of FeS protein activity increases prooxidant resistance. (A) Wild-type BY4741 cells transformed with the multicopy vector YEp351-HphNT1 (○), or the same vector containing the *SOD2* (●) or *ATM1* (□) genes were cultured in selective YNB medium in the absence (control) or presence (+Cu) of 0.5 mM $Cu(NO_3)_2$. SEMs from triplicate experiments are smaller than the dimensions of the symbols. (B) Tenfold dilution series of wild-type (WT) or *mxr*Δ cells, transformed with pCM190 (+ev) or pCM190-*tetRLI1* (+*RLI1*), were spotted from left to right on selective YNB agar supplemented with stressors, as indicated, and incubated for 7 d. Typical results from one of several independent experiments are shown. (C) *sod2*Δ cells transformed with pCM190 (○, ●) or pCM190-*tetRLI1* (□, ■) were cultured in the absence (open symbols) or presence (closed symbols) of 1.5 mM $Cu(NO_3)_2$. SEMs from triplicate independent growth experiments are smaller than the dimensions of the symbols.

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 *mxr*Δ 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 *mxr*Δ mutant to the prooxidants Cu, H_2O_2, and paraquat (Figure 1B). The outcome was similar in a *sod2*Δ 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 H_2O_2, 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

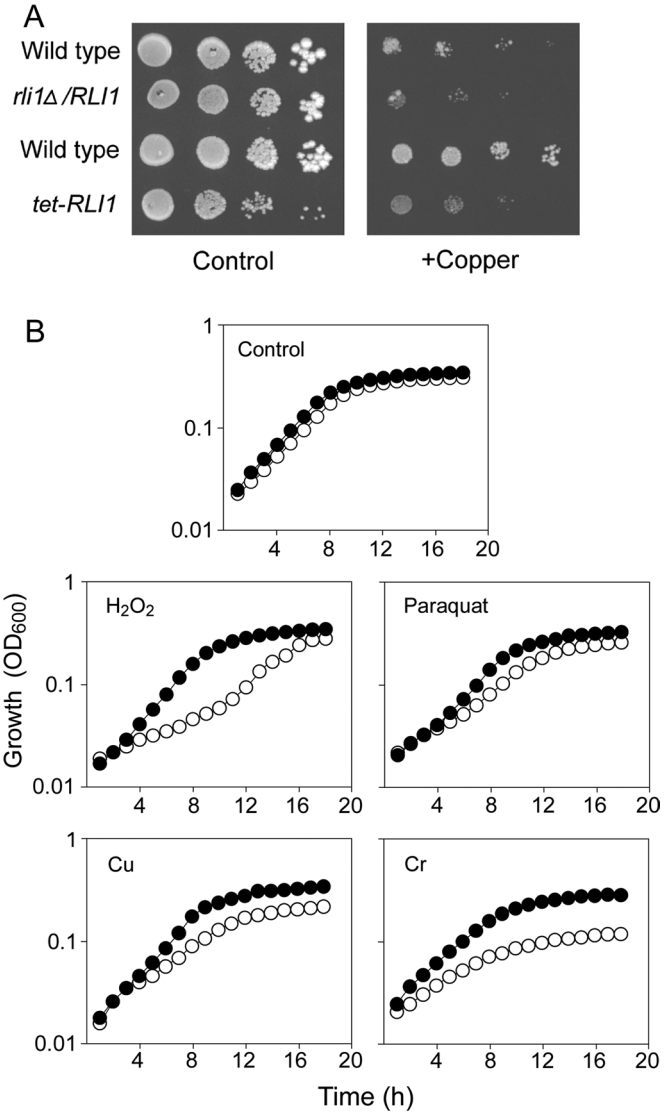

FIGURE 2: Rli1p expression level determines cellular prooxidant resistance. (A) Tenfold dilution series of cultures were spotted from left to right on YPD agar, supplemented or not with 13 mM $Cu(NO_3)_2$ (the use of rich YPD necessitated this high Cu dose for growth inhibition; Avery *et al.*, 1996; Macomber and Imlay, 2009). The strains were: BY4743 wild-type (top row) and the isogenic heterozygote *rli1Δ/RLI1*; R1158 wild-type and isogenic strain *tet-RLI1* (Open Biosystems). Media included 22.5 μM (10 μg/ml) doxycycline, giving a ≥ 90% decrease in *RLI1* expression in *tet-RLI1* cells (Figure S1A). Typical results from one of three independent experiments are shown. (B) BY4741 cells transformed with pCM190 (○) or pCM190-*tetRLI1* (●) were cultured in YNB medium supplemented or not with 1 mM H_2O_2, 4 mM paraquat, 0.35 mM $Cu(NO_3)_2$, or 0.1 mM CrO_3. Doxycycline was excluded to give maximal *RLI1* expression in pCM190-*tetRLI1*–transformed cells. SEMs from triplicate independent growth experiments are smaller than the dimensions of the symbols.

(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

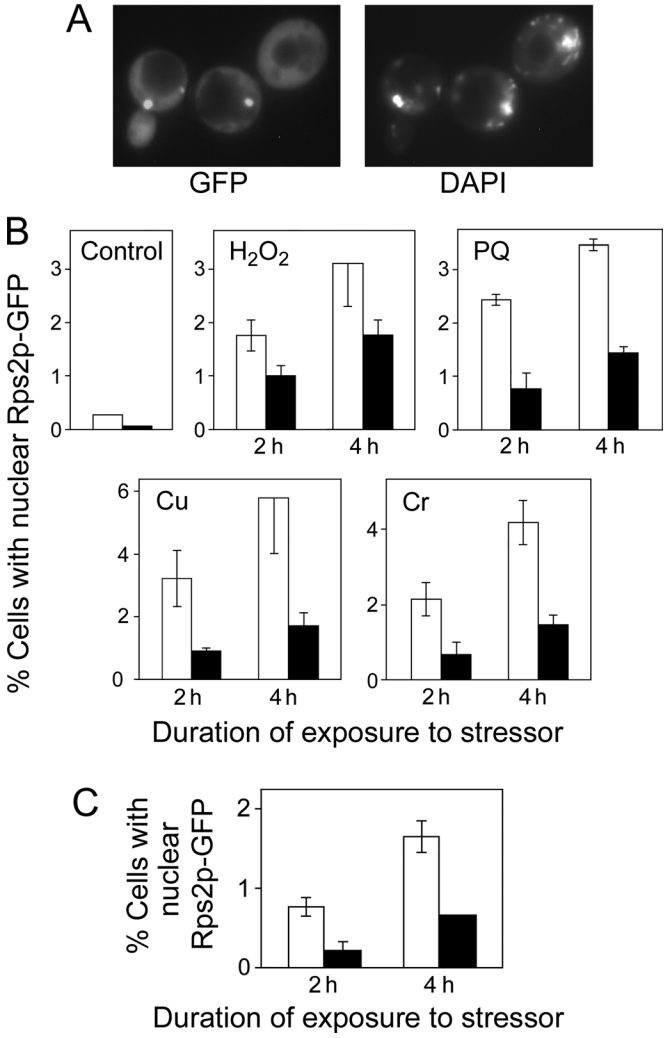

FIGURE 3: Rli1p-dependent activity in ribosomal subunit export is decreased during mild stress. (A) *tet-RLI1* cells transformed with pRS315-RPS2-eGFP were cultured with 22.5 μM (10 μg/ml) doxycycline to down-regulate *RLI1*, promoting nuclear Rps2-GFP accumulation (left). Right, DAPI staining of cell nuclei. (B) Wild-type (W303; white bars) or isogenic *tet-RLI1* (black bars) strains transformed as above were incubated with 0.2 mM H_2O_2, 1 mM paraquat, 0.35 mM $Cu(NO_3)_2$, or 0.1 mM CrO_3. These doses affected cell doubling times <15%. Doxycycline was excluded. Values are means from three independent experiments ± SEM between the experiments, with ≥300 cells counted in each experiment. Similar results were verified for $Cu(NO_3)_2$ stress in a different strain background (BY4741) or with plasmid-borne *tet-RLI1*. (C) As in (B), except that wild-type cells were additionally transformed with pCM190 (white bars) or pCM190-*tetATM1* (black bars) and incubated with 0.25 mM $Cu(NO_3)_2$.

et al., 2005; Yarunin *et al.*, 2005; Figure 3A). The FeS clusters of Rli1p are required for this activity (Kispal *et al.*, 2005). We quantified nuclear Rps2p accumulation in cells exposed to prooxidants supplied at mild doses that affected cell doubling time by <15%. After only 4 h of incubation with any of the stressors, the proportion of cells exhibiting defective nuclear Rps2-GFP export was >10-fold higher than in control incubations (Figure 3B). (Cells with visible nuclear fluorescence were a minority of the population in all cases, suggesting a lower limit to the level of nuclear Rps2-GFP accumulation detectable). To tie the phenotype more specifically to (defective) Rli1p function, we assayed rescue of the phenotype by *RLI1* overexpression; this was achieved with an isogenic *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 $Rli1^{C58A}$. 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 $RLI1^{C58A}$ had little impact on growth in control conditions (Figure 5A). However, the $RLI1^{C58A}$-expressing cells were stress sensitive, particularly to H_2O_2 and $Cu(NO_3)_2$. 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 $RLI1^{C58A}$-expressing cells than in the wild-type (Figure 5B). The results supported the hypothesis that growth inhibition by the prooxidants 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 ^{55}Fe with hemagglutinin (HA)-tagged Rli1p (Kispal *et al.*, 2005). ^{55}Fe was not detectable in immunoprecipitations from wild-type (non-Rli1-HA–expressing) cells incubated with $^{55}FeCl_3$. Rli1-HA protein that was immunoprecipitated from Rli1-HA–expressing cells, after preincubation with $^{55}FeCl_3$, exhibited rapid FeS-cluster turnover (according to ^{55}Fe 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 $^{55}FeCl_3$, 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

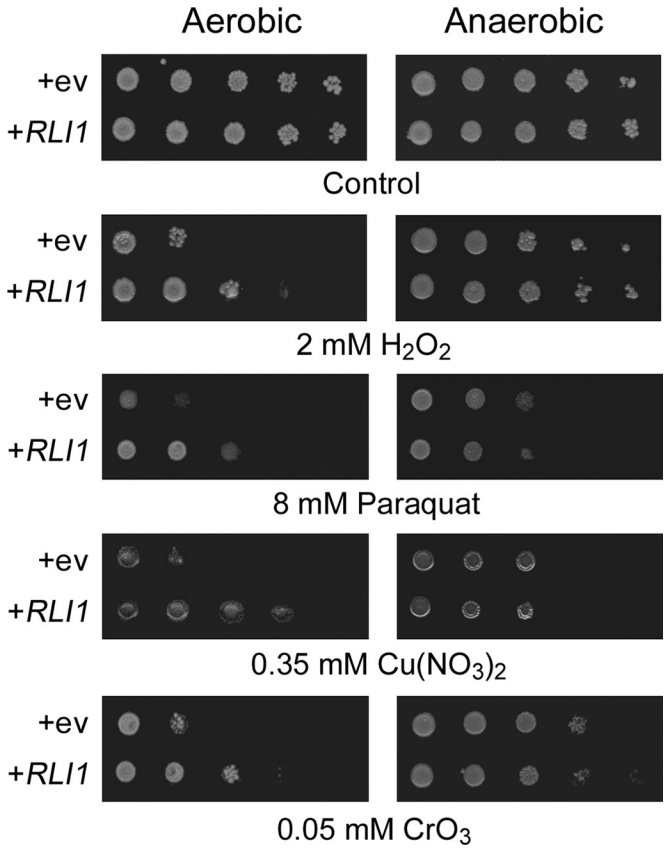

FIGURE 4: Rli1p-dependent resistance to prooxidants requires oxygen. Cells transformed with pCM190 (+ev) or pCM190-*tetRLI1* (+*RLI1*) were 10-fold serially diluted and spotted from left to right on YNB agar supplemented with indicated stressors. Plates were incubated for 5 d, either under ambient air or anaerobically under H_2 and CO_2. Typical data from one of several independent experiments are shown.

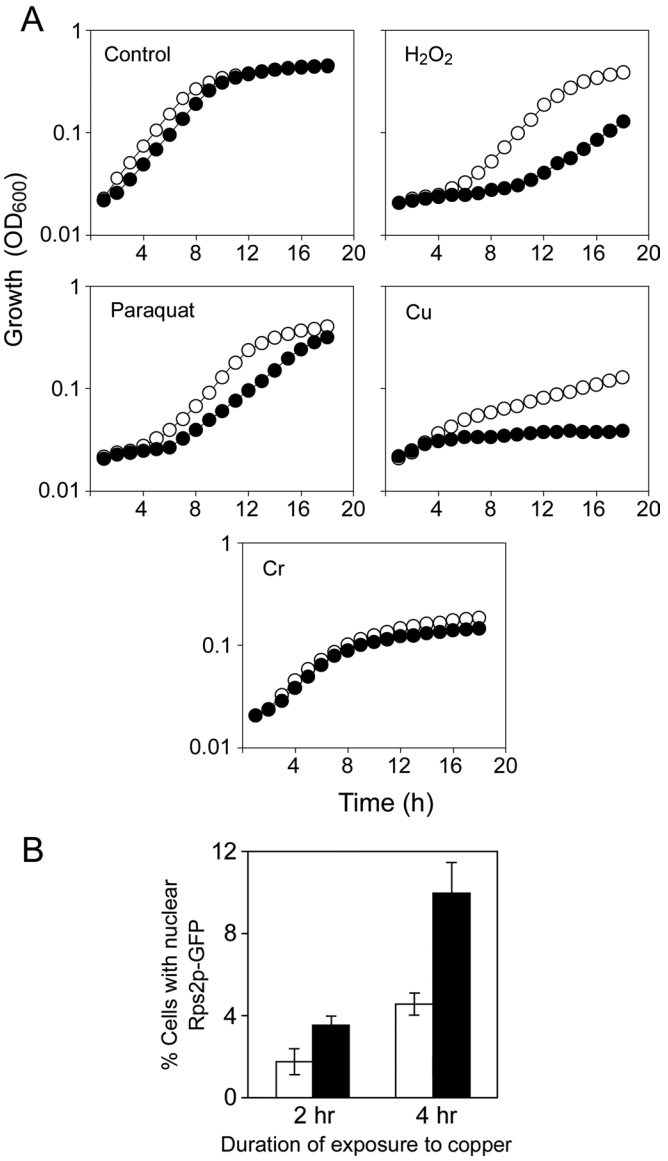

FIGURE 5: Prooxidant sensitivity in $Rli1^{C58A}$-expressing cells. (A) Wild-type (○) or *RLI1*^{C58A}-expressing cells (●) were cultured in YNB medium supplemented or not with 1 mM H_2O_2, 2 mM paraquat, 0.5 mM $Cu(NO_3)_2$, or 0.1 mM CrO_3. SEMs from triplicate independent experiments are smaller than the dimensions of the symbols. (B) Wild-type (□) or *RLI1*^{C58A}-expressing (■) cells transformed with pRS315-RPS2-eGFP were cultured in the presence of 0.25 mM $Cu(NO_3)_2$ (doubling time was increased <3%). Values are means from three independent experiments ± SEM, with ≥300 cells counted in each experiment.

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 Isc 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_2O_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_2O_2 and superoxide-generating agents, such as paraquat and Cr(VI) (Sumner *et al.*, 2005). Given this, together with the extensive

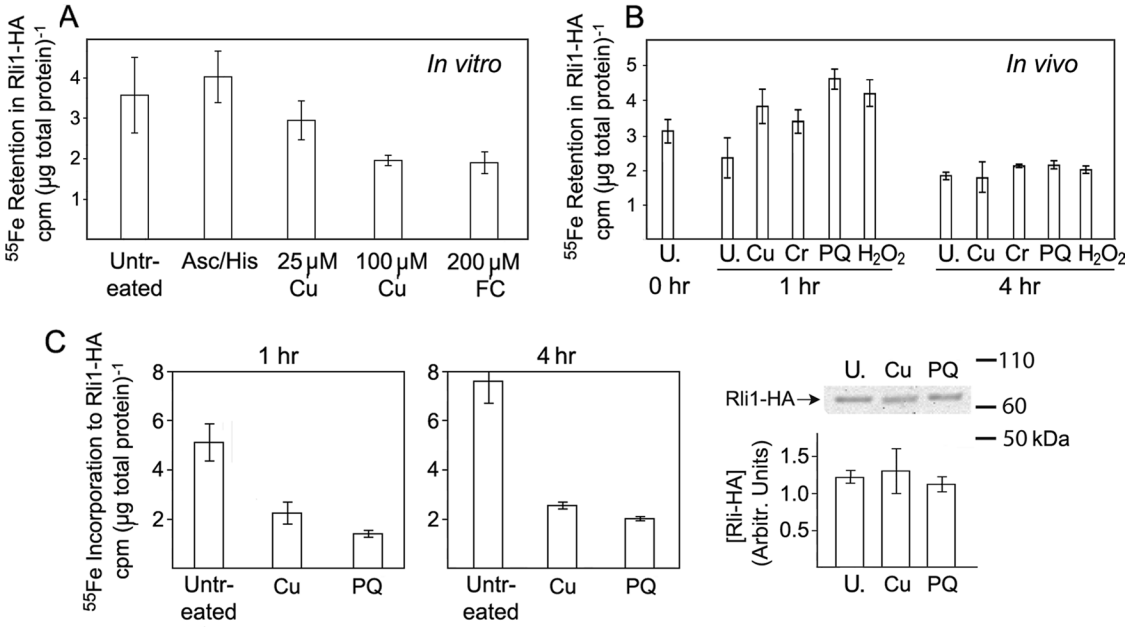

FIGURE 6: FeS-cluster turnover and incorporation to Rli1-HA. Assays were with pCM190-*tetRLI1-HA*–transformed cells cultured in the absence of doxycycline. (A) Rli1-HA was immunoprecipitated from protein extracts of cells preincubated with $^{55}FeCl_3$. ^{55}Fe retention in Rli1-HA was measured before (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_2O_2, before Rli1-HA immunoprecipitation and ^{55}Fe quantification. (C) Cells were incubated in the absence or presence of 0.8 mM $Cu(NO_3)$ 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.

data already available on biological responses to oxidative stress, not least in yeast, it could 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, as 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, isopropylmalate isomerase is strongly H_2O_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 notably stable 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 Cia1), 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 $Rli1^{C58A}$ as their sole Rli1p. These cells' prooxidant sensitivity was in keeping with the predicted lability of $Rli1^{C58A}$ (Barthelme *et al.*, 2007). $Rli1^{C58A}$ lacks a [4Fe-4S]-coordinating cysteine, yielding a $[3Fe\text{-}4S]^+$ 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). $Rli1^{C58A}$-expressing cells were especially sensitive to Cu and H_2O_2. This fits with the fact that Cu (via Fe displacement; Macomber and Imlay, 2009) and H_2O_2 (Jang and Imlay, 2010) can degrade FeS clusters beyond the $[3Fe\text{-}4S]^+$ state in which the C58A 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 $[3Fe\text{-}4S]^+$ in vivo (Varghese *et al.*, 2003; Macomber and Imlay, 2009). The limited paraquat and Cr sensitivity of $Rli1^{C58A}$-expressing cells may reflect that only one of the two clusters of the protein remains partly oxidizable by these agents.

The FeS domain of Rli1p appears to have structural function (Becker *et al.*, 2012) and is not required for Rli1p binding to the eIF3 component Hcr1p (Kispal *et al.*, 2005; Khoshnevis *et al.*, 2010) or Rli1-mediated ATP turnover (Barthelme *et al.*, 2011). However, the FeS domain of Rli1p is required for nuclear export of ribosomal subunits, translation termination, interaction with and stabilization of the eRF1 paralogue Pelota, and ribosome splitting (Kispal *et al.*, 2005; Khoshnevis *et al.*, 2010; Barthelme *et al.*, 2011; Becker *et al.*, 2012). The FeS requirement for ribosomal subunit export is consistent with the observed sensitivity of this process to oxidative stress and its rescue by Rli1p overexpression and with the FeS clusters of Rli1p being the protein's primary frailty during oxidative stress.

Rli1p and its orthologues are among the most sequence-conserved proteins known in biology, with essential function in all organisms tested (Barthelme *et al.*, 2007; Becker *et al.*, 2012). Consequently, preservation of Rli1p function could be a principal determinant of oxidative stress resistance in diverse organisms. This includes chronic and acute oxidative stresses, as mild effects on growth as well as severe short-term effects on viability were Rli1p dependent. If sustenance of Rli1p activity is crucial for surviving ROS stress, and *RLI1* overexpression achieves that, then why is the protein not expressed more highly by cells? One reason could be the counterpressure on cells to decrease protein synthesis activity during oxidative stress, when mRNA translation is thought to be error-prone (Shenton *et al.*, 2006). Consistent with that, expression of the Rli1 orthologue ABCE1 has been correlated positively with the incidence of lung and colorectal cancers in humans (Hlavata *et al.*, 2012; Ren *et al.*, 2012), whereas *ABCE1* gene copy number was elevated in drug-resistant cell lines (Yasui *et al.*, 2004). As with all cellular activities, a balance would be reached. This balance could be particularly fine for Rli1p in influencing organism fate during ROS stress.

MATERIALS AND METHODS

Strains and plasmids

Saccharomyces cerevisiae BY4741 (*MAT***a**; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0), 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 *mxr1*Δ*/mxr2*Δ (*mxr*Δ) 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 $RLI1^{C58A}$ construct by short-flanking homology PCR (Wach *et al.*, 1997), involving amplification of $RLI1^{C58A}$ together with *HIS3* marker from the vector pRS423 (kindly provided by R. Tampe, 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 *Ppu*MI and *Bsr*GI sites of YEp351, was digested with *Kas*I and *Eco*NI, 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 *Kpn*I-*Sal*I (*SOD2*) or *Sal*I-*Sbf*I (*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 pRS315-RPS2-eGFP was kindly donated by E. Hurt (University of Heidelberg; Milkereit *et al.*, 2003; Kispal *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 *Not*I and *Pst*I 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 *Pst*I restriction site. The amplified ~1.9-kb fragment was cut at the *Pst*I site and at a *Bgl*II 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 *Pst*I and *Bgl*II sites of pCM190-*tetRLI1*, replacing the corresponding wild-type *RLII* 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) D-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 (OD_{600} ~ 2.0) in liquid medium at 30°C, 120 rpm. Samples were diluted to OD_{600} ~ 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 OD_{600} ~ 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 H_2 and CO_2, 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 HB050 illuminator. Images were captured with a Zeiss Axiocam 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, ^{55}Fe-labeling studies, and Western blotting

Protein extraction from yeast (Cashikar *et al.*, 2005) and assay of aconitase or isopropylmalate dehydratase (Leu1) activities (Sideri *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 $^{55}FeCl_3$ (38 µCi/l). Labeled cells were washed and suspended in prewarmed YNB supplemented with stressor as specified. At intervals during incubation with shaking, OD_{600} 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 N_2, 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). Fe^{55} was measured with a Packard Tri-Carb 2100TR liquid scintillation analyzer (Meriden, CT). The overall procedure was the same for measurement of Fe^{55} incorporation into Rli1-HA, except that $^{55}FeCl_3$ 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 ^{55}Fe 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 $Cu(NO_3)_2$ 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 ^{55}Fe. 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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