Altered sterol metabolism in budding yeast affects mitochondrial iron–sulfur (Fe-S) cluster synthesis

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Ergosterol synthesis is essential for cellular growth and viability of the budding yeast Saccharomyces cerevisiae, and intracellular sterol distribution and homeostasis are therefore highly regulated in this species. Erg25 is an iron-containing C4-methyl sterol oxidase that contributes to the conversion of 4,4-dimethylzymosterol to zymosterol, a precursor of ergosterol. The ERG29 gene encodes an endoplasmic reticulum (ER)-associated protein, and here we identified a role for Erg29 in the methyl sterol oxidase step of ergosterol synthesis. ERG29 deletion resulted in lethality in respiring cells, but respiration-incompetent (Rho0) cells survived, suggesting that Erg29 loss leads to accumulation of oxidized sterol metabolites that affect cell viability. Down-regulation of ERG29 expression in aer29 cells indeed led to accumulation of methyl sterol metabolites, resulting in increased mitochondrial oxidants and a decreased ability of mitochondria to synthesize iron–sulfur (Fe-S) clusters due to reduced levels of Yfh1, the mammalian frataxin homolog, which is involved in mitochondrial iron metabolism. Using a high-copy genomic library, we identified suppressor genes that permitted growth of Δerg29 cells on respiratory substrates, and these included genes encoding the mitochondrial proteins Yfh1, Mmt1, Mmt2, and Pet20, which reversed all phenotypes associated with loss of ERG29. Of note, loss of Erg25 also resulted in accumulation of methyl sterol metabolites and also increased mitochondrial oxidants and degradation of Yfh1. We propose that accumulation of toxic intermediates of the methyl sterol oxidase reaction increases mitochondrial oxidants, which affect Yfh1 protein stability. These results indicate an interaction between sterols generated by ER proteins and mitochondrial iron metabolism. Mitochondria house two iron-consuming biosynthetic pathways, heme and iron–sulfur (Fe-S)2 cluster synthesis. In yeast, Fe-S synthesis is the essential iron-consuming process, as yeast can survive without heme or respiration but not without Fe-S cluster synthesis (1, 2). Many of the genes involved in Fe-S cluster synthesis and particularly in the mitochondrial synthesis and export of Fe-S clusters are highly conserved in all eukaryotes. Yfh1, the yeast equivalent of mammalian frataxin, plays an important role in the early steps of Fe-S cluster synthesis (3–13). Whereas the exact role has not been defined, loss of Yfh1 results in severely compromised Fe-S cluster synthesis and decreased mitochondrial function. Loss of Yfh1, as with mutations in genes in the early steps of mitochondrial Fe-S cluster synthesis, results in increased deposits of insoluble iron, “nanoparticles,” within mitochondria (11). The generation of these nanoparticles results in concomitant oxidant radical formation, leading to loss of mitochondrial DNA. YFHI was initially identified through a genetic screen for yeast mutants that were unable to grow on low-iron medium (4). Babcock et al. (4) generated a mutant, bm-8, which was suppressed by a high-copy plasmid that contained YFHI. YFHI was acting as a high-copy suppressor and was not allelic to the mutation in bm-8. The bm-8 mutant allele was a missense mutation in YMR134W, which encoded for a protein of unknown function. Moretti-Almeida et al. (14) reported that a temperature-sensitive allele of YMR134W showed low mitochondrial iron accumulation at the restrictive temperature along with defects in ergosterol synthesis. Here, we report a detailed description of YMR134W, herein referred to as ERG29. We determined that deletion of ERG29 resulted in lethality in respiring cells, but cells that had lost the ability to respire (Rho− or Rho0) could survive and that ERG29 encodes an endoplasmic reticulum (ER) protein that plays a role in sterol synthesis at the methyl sterol oxidase step. We determined that in the absence of ERG29, methyl sterol metabolites accumulate and result in a decreased ability of mitochondria to synthesize Fe-S clusters. We further show that decreased Fe-S cluster synthesis is due to a dramatic reduction in Yfh1. The effects of ERG29 loss can be exacerbated by increased mitochondrial iron import or decreased by overexp%22The abbreviations used are: Fe-S, iron–sulfur; ER, endoplasmic reticulum; 4,4-DMZ, 4,4-dimethylzymosterol; BPS, bathophenanthroline sulfonate; GE, glycerol-ethanol.
pression of mitochondrial iron exporter gene MMT1 or MMT2 or by overexpression of YFH1 or PET20, a nuclear gene that encodes a mitochondrial protein involved in protecting mitochondria from oxidant damage (15).

Results

Deletion of ERG29 affects sterol synthesis

Erg25 is a methyl sterol oxidase, which is an essential oxodiiron–containing enzyme located in the ER (16–18). In a complex set of reactions, Erg25 along with two other enzymes (Erg26 and Erg27) catalyzes the demethylation of 4,4-dimethylzymosterol (4,4-DMZ) to zymosterol. Preliminary studies showed that when cells with a missense mutation in YMR134W/ERG29 were placed on low iron, they had an altered sterol pattern, similar to that seen in mutants in ERG25 (data not shown). This finding led us to examine the effects of deletion of ERG29. Previous studies showed that ERG29 was lethal in respiring cells (19). To determine the function of Erg29, we generated a ERG29 deletion strain that contained a plasmid expressing carboxyl-terminally His-tagged Erg29 whose expression was under the control of either the β-estradiol promoter (turned on in the presence of β-estradiol, pGEV) or the GAL1 promoter (in the presence of galactose) (Table 1). This two-plasmid system allows for independent regulation of expression via growth in glucose with the addition of β-estradiol (no carbon source change) or via growth in galactose to permit expression and then shifted to glucose to suppress expression (20). This system also allows for tightly regulated β-estradiol–titrated expression from low to extremely high levels. Cells with a deletion in ERG29 grown in β-estradiol were viable (Fig. 1A, middle), whereas the absence of β-estradiol resulted in a severe growth defect (Fig. 1A, right, top left quadrant), which over time led to the generation of small colonies. These small colonies were able to grow but were shown to be respiration-incompetent, as they were unable to grow on YPGE (Fig. 1A, right, top right quadrant compared with left, top right quadrant). We noted growth of Δerg29 β-estradiol-ERG29 cells on YPGE without β-estradiol (Fig. 1A, left, top left quadrant). As those cells were unable to grow on YPD (– β-estradiol (Fig. 1A, right, top left quadrant), we attribute the growth on YPGE to the absence of glucose-mediated repression of the GAL1 promoter in the pGEV system (20). We utilized galactose regulation of the Δerg29pβ-estradiolGAL1ERG29 strain to examine the effects of loss of Erg29 on sterol synthesis. Δerg29pGAL1ERG29 cells grown in galactose had some increase in the levels of 4,4-DMZ and 4-methyl fecosterol and decreased levels of ergosterol compared with WT cells; however, in glucose-containing medium, Δerg29pGAL1ERG29 cells showed an increase in intermediate sterols and a corresponding decrease in zymosterol and ergosterol (Fig. 1B). We confirmed that growth in glucose resulted in the loss of Erg29-His protein (Fig. 1C). We note that WT cells grown in glucose accumulated more zymosterol than when grown in galactose. We do not know the reason for this observation. We hypothesize that the altered sterols seen in galactose-grown Δerg29pGAL1ERG29 cells reflect decreased function of the epitope-tagged Erg29. We confirmed this hypothesis by transforming the Δerg29pβ-estradiolGAL1ERG29 strain with a plasmid containing non-tagged ERG29 under its endogenous promoter. Sterol analysis revealed lower levels of methyl sterol intermediates and increased zymosterol and ergosterol in cells expressing nontagged Erg29 (Fig. 1D). This supports the hypothesis that the epitope tag reduced the function of Erg29. We also determined that the untagged Erg29 functioned better in complementing the loss of Erg29 (Fig. 1D, + pYcp ERG29 + β-estradiol (hatched gray bar) compared with + β-estradiol alone (gray bar)).

The sterol pattern observed in ERG29 “shut-off” cells was similar to that seen in cells with mutations in ERG25 (18). To more closely examine the similarities in sterol profiles, we generated a ERG25 deletion strain expressing a β-estradiol pGAL1ERG25 similar to the “ERG29 shut-off” system. Δerg25pGAL1ERG25 cells grown in galactose had some increase in the levels of 4,4-DMZ and 4-methyl fecosterol and decreased levels of ergosterol compared with WT cells; however, in glucose-containing medium Δerg25pGAL1ERG25 cells showed a dramatic increase in 4,4-DMZ and a corresponding decrease in zymosterol and ergosterol (Fig. 1E). We confirmed that growth in glucose resulted in the loss of Erg25 protein (Fig. 1C). The sterol pattern seen in glucose-grown Δerg25pGAL1ERG25 cells appears similar to that in Δerg29pGAL1ERG29 cells, although we consistently observed higher levels of the methyl sterol intermediates, such as 4-methyl fecosterol (see Fig. 1B) and 4-carboxyzymosterol in Δerg29pGAL1ERG29 cells (data not shown). That the loss of ERG29 affects the methyl sterol oxidation reaction is supported by the observation that overexpression of ERG25, through transformation with a high-copy plasmid permitted Δerg29 cells to survive (Fig. 2A). In contrast, transformation with a high-copy ERG26 plasmid, which encodes the subsequent enzyme in the methyl sterol oxidation reaction (21), did not. Together, the results support the hypothesis that the loss of ERG29 affects the methyl sterol oxidation reaction in which Erg25 is the first enzyme in the reaction. These results suggest that the “shut-off” of ERG29 specifically affects the Erg25 reaction and not another enzyme in the methyl sterol oxidation reaction. Erg25 is localized to the endoplasmic reticulum (18). We generated a MET3-regulated ERG29-GFP plasmid and transformed it into WT or Δerg29pGAL1ERG29 cells. We confirmed that the carboxyl-tagged Erg29-GFP complemented the loss of ERG29 (Fig. 2B) and confirmed that Erg29-GFP is localized to...
Increased sterol intermediates affect Fe-S cluster synthesis

As described in Fig. 1, loss of respiration can permit Δerg29 cells to survive as Rho0 cells. Based on this result, we examined whether there were changes in the sterol pattern in Rho0 cells. We used ethidium bromide to generate Rho0 WT cells and then made cells with deletion of Erg29 or Erg25 to Dpm1 was determined using Bio-Rad ImageLab™ software. Dpm1, an ER protein, was used as a load control. To determine whether iron limitation affected sterol synthesis, WT Rho0 and Δerg29 Rho0 cells were grown in medium made iron-limited by the iron chelator bathophenanthroline sulfonate (BPS). Δerg29 Rho0 cells grew poorly (BPS with 0–4 μM iron (BPS(0) to BPS(4)) but equal to WT Rho0 cells with increased iron (BPS(100)) (Fig. 3B). As Δerg29 Rho0 cells grew poorly if grown on iron-limited medium for extended times, we grew WT Rho0 and Δerg29 Rho0 cells in iron-limited medium for 4 h to examine whether limiting iron affected sterol synthesis. Δerg29 Rho0 cells still showed increased intermediate sterols (4,4-DMZ and 4-methyl fecosterol) and reduced zymos-
Increased sterol intermediates affect Fe-S cluster synthesis

Loss of Erg29 affects the methyl sterol oxidase reaction

Δerg29pΔβ-estradiolGAL1ERG29 cells were much more sensitive to conditions that affected the methyl sterol oxidase reaction than WT Rho⁰ cells. Incubation of cells with cobalt has been shown to inhibit the enzyme activity of oxo-diiron-containing Erg25 in both Saccharomyces cerevisiae and Cryptococcus neoformans, leading to a hyperaccumulation of 4,4-DMZ and decreased levels of zymosterol (23). The addition of 1.0 mM cobalt to WT Rho⁰ cells had little effect on viability, whereas Δerg29 Rho⁰ cells showed a loss of viability (Fig. 3D). Furthermore, we utilized the Erg25 inhibitor 6-amino-2-n-pentylthiobenzothiazole (APB) to assess growth of Δerg29 Rho⁰ cells. WT Rho⁰ cells showed some toxicity at 60 μM APB, whereas Δerg29 Rho⁰ cells showed toxic effects at 20 μM (Fig. 3E). These results support the view that loss of ERG29 increases the sensitivity of Erg25 to agents known to affect the Erg25 reaction. Together, these results strongly support the hypothesis that Erg29 functions in the Erg25 methyl sterol oxidase reaction and that mitochondrial respiration affects ergosterol synthesis.

Identification of genetic suppressors of ERG29

We took a genetic approach to examine the mechanism of toxicity due to loss of ERG29. As loss of ERG29 results in death when cells are grown in respiratory medium, we used a high-copy genomic library (LEU12) to identify suppressors that would permit growth of Δerg29 cells on glycerol-ethanol (GE). We transformed our Δerg29YCP1L1ERG29(URA3) strain with a genomic library. We then selected for loss of YCP1ERG29 on 5-fluoroorotic acid and identified surviving colonies that could grow on GE. As expected, several recovered plasmids that allowed Δerg29 Rho⁺ cells to grow on GE contained the ERG29 gene (data not shown). In addition, we found MMT2, YFH1, and PET20 as high-copy suppressors. That we did not recover ERG25 as a high-copy suppressor suggests that our screen was not saturated. We confirmed that overexpression of MMT2, YFH1, and PET20 permitted Δerg29 to grow on glucose (Δerg29pΔβ-estradiolGAL1ERG29) cells to grow on glucose (Fig. 4A). Babcock et al. (4) initially identified YFH1 as a high-copy suppressor of the missense mutant Erg29 (bn-8) (4). Our results show that YFH1 can also partially suppress the growth defect of Δerg29 cells. MMT2 encodes a mitochondrial iron exporter, which is a member of the family of cation diffusion facilitators (24, 25). MMT2 has a parologue, MMT1. We did not find MMT1 in our screen, but we confirmed that overexpression of MMT1 would permit Δerg29 cells to grow as Rho⁺ (Fig. 4A). PET20 is a nuclear gene that encodes a mitochondrial protein, which when deleted leads to increased sensitivity to oxidant stress (15). All of the plasmids that permitted growth of Δerg29 cells also affected the Δerg29 sterol pattern (Fig. 4B). Overexpression of these genes did not normalize the sterol pattern of ERG29 shut-off cells but did reduce the levels of 4,4-DMZ and 4-methyl fecosterol and concomitantly increased the levels of zymosterol and ergosterol.

The finding that the overexpression of mitochondrial iron exporter genes MMT1 and MMT2 preserved the respiratory activity of a Δerg29 led us to examine the effect of either deletion or overexpression of the mitochondrial iron importer genes MRS3 and MRS4. Deletion of MRS3 and MRS4 together but not singly permitted Δerg29 cells to grow on GE medium (Fig. 5A, right plate, bottom panels). We observed that overexpression of MRS3 in Δmrs3Δmrs4 cells did not affect viability (Fig. 5A, left plate, top panels), whereas overexpression of MRS3 in Δmrs3Δmrs4Δerg29 cells resulted in a loss of viability on GE medium (Fig. 5A, left plate, bottom panels). Indeed, overexpression of MRS3 in Δerg29 Rho⁰ cells led to increased levels of 4,4-DMZ and 4-methyl fecosterol and decreased zymosterol and ergosterol (Fig. 5B). The effects of MRS3 were suppressed if the mitochondrial iron exporter MMT1 was overexpressed at the same time. These results suggest a connection between mitochondrial iron levels, loss of viability, and high levels of methyl sterol intermediates.

Loss of ERG29 affects Fe-S cluster synthesis

Increased MRS3 expression affects cell growth of Δerg29 cells, whereas overexpression of MMT1 or deletion of MRS3/
MRS4 protects these cells. Similar findings have been reported in cells with a deletion in YFH1. Most notably, the growth of \( \text{H9004}^{yfh1} \) cells was reduced by overexpression of MRS3 or MRS4 but protected by deletion of these genes (26–29). We confirmed that overexpression of MMT1 permitted \( \text{H9004}^{yfh1} \) cells to grow in medium supplemented with iron or \( \text{H}_2\text{O}_2 \) (Fig. 6A).

Loss of Yfh1 is known to decrease Fe-S cluster synthesis, which leads to the accumulation of iron in mitochondria as ferric iron deposits (5, 29–31). Together, these findings suggest that the accumulation of methyl sterol intermediates may also lead to loss of mitochondrial Fe-S cluster synthesis. We tested this hypothesis by measuring the activity of the Fe-S–containing mitochondrial aconitase in \( \text{H9004}^{erg29pGAL1ERG29} \) cells. In the presence of glucose, \( \text{ERG29} \) shut-off, there was a dramatic decrease in aconitase activity in \( \text{H9004}^{erg29pGAL1ERG29} \) cells compared with WT cells (Fig. 6B). Overexpression of the high-copy suppressor YFH1, MMT1, MMT2, or PET20 in \( \text{H9004}^{erg29pGAL1ERG29} \) cells resulted in a partial recovery of aconitase activity (Fig. 6C). Together, these results confirm that loss of ERG29 affects mitochondrial Fe-S cluster synthesis.

**Loss of ERG29 in respiratory cells affects Yfh1 levels**

Yfh1 affects an early step in the synthesis of Fe-S clusters within mitochondria. The observation that loss of ERG29 can be suppressed by overexpressed YFH1 led us to examine the levels of Yfh1 in respiring cells when ERG29 was “shut off.” Isolation of mitochondria from \( \text{H9004}^{erg29pGAL1ERG29} \) cells grown in glucose showed a decrease in the level of Yfh1 (Fig. 7A, lane 6), which was not due to a reduction in YFH1 mRNA levels (Fig. 7B). In fact, YFH1 transcripts were up-regulated slightly in response to loss of Erg29, whereas ISU1 transcripts were unaltered. The decrease in Yfh1 protein was specific to Yfh1, as little or no decrease was observed in other Fe-S cluster synthesis proteins, including Nfs1, the cysteine desulfurase involved in generating sulfide (37, 38); Isu1, a Fe-S scaffold protein (39); and Ssq1, a chaperone involved in the assembly of mitochondrial Fe-S clusters (40, 41). Overexpression of MMT1 or PET20

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**Figure 3. Loss of respiration affects the sterol pattern of \( \text{H9004}^{erg29} \) cells.** A, WT Rho0 and \( \text{H9004}^{erg29} \) Rho0 cells were grown in CM glucose medium for 18 h, and sterol analysis was performed as described. The data are expressed as the percentage of total sterols. Error bars, S.D. of four separate experiments. B, cells as in A were plated onto iron-limited medium (BPS (0–100 \( \mu \text{M} \) iron)) and grown at 30 °C for 2 days. C, sterol analysis was performed on cells grown in iron-limited medium (BPS(0)) for 4 h. The data are expressed as the percentage of total sterols. Error bars, S.D. of two separate experiments. D, cells as in A were plated onto YPD with or without 1 mM \( \text{CoCl}_2 \) or YPGE and grown at 30 °C for 3 days. E, cells as in A were plated onto CM medium containing varying concentrations (\( \mu \text{M} \)) of ABP and grown at 30 °C for 2 days.
in Δerg29pGAL1ERG29 cells permitted partial rescue of Yfh1 levels. We note that overexpression of PET20 affected the levels of Isu1 in both galactose- and glucose-grown cells. We do not know the reason for this reduction but note that the reduction in Isu1 did not affect aconitase activity.

Loss of ERG29 leads to increased mitochondrial oxidants

Under normal conditions, increased pools of free mitochondrial iron do not impose a growth deficit in cells (42). Under conditions of stress or specific gene deletions, however, pools of free mitochondrial iron exacerbate the generation of oxidant radicals (43, 44). Based on those findings, we examined whether loss of ERG29 increased mitochondrial oxidants and that altering mitochondrial iron either through increased iron export or by increasing Fe-S cluster synthesis reduces mitochondrial oxidants.

Loss of ERG25 affects mitochondrial activities

The finding that loss of ERG29 affects mitochondrial activities prompted us to test the effects of loss of ERG25 on mitochondrial activity, because both genes affect the methyl sterol oxidase reaction. Δerg25pGAL1ERG25 cells grown in glucose showed decreased aconitase activity compared with WT cells (Fig. 8A), decreased Yfh1 levels (Fig. 8B), and increased mitochondrial oxidants (Fig. 8C). Neither the growth phenotype (data not shown) nor changes in mitochondrial activity due to “shut-off” of ERG25 could be suppressed by overexpression of MMT1, MMT2, or YFH1. As Erg25 is an essential enzyme, these results suggest that the effects of complete Erg25 loss...
Increased sterol intermediates affect Fe-S cluster synthesis

**Discussion**

The results reported here suggest that Erg29 is involved in sterol metabolism and that loss of Erg29 results in the generation of 4-methyl sterol metabolites. Data suggesting that Erg29 is involved in sterol metabolism include the following: 1) Erg29 is localized to the ER, where methyl sterol synthesis occurs; 2) loss of *ERG29* affects the methyl sterol oxidase reaction, resulting in increased levels of 4,4-DMZ and 4-methyl sterol intermediates and correspondingly decreased levels of zymosterol and ergosterol; and 3) *Δerg29* Rho⁰ cells are more sensitive than WT cells to agents that affect the methyl sterol oxidase reaction. These data, most notably the accumulation of 4,4-DMZ, the loss of zymosterol and ergosterol, and suppression by overexpression of *ERG25* point to the methyl sterol oxidase reaction being affected in *Δerg29* cells, although the exact role of Erg29 is unclear. The finding that Erg29 affects the sterol pathway is supported by data presented in large chemogenomic studies.
We were unable to suppress the loss of ERG29 through the addition of ergosterol even in cells overexpressing the Sut1 transcription factor that up-regulates sterol uptake genes (47, 48) (data not shown). Similarly, Lee et al. (46) reported that the addition of ergosterol did not rescue the growth defect associated with Erg29-specific inhibitors. These results suggest that it is not the loss of ergosterol that results in toxicity but rather the accumulation of intermediate sterols. A similar finding was reported for loss of ERG26 or a temperature-sensitive allele of erg26-1 in which ergosterol supplementation could not overcome toxicity (21, 49). The exact role of Erg29 in the methyl sterol oxidase reaction remains to be identified. We hypothesized that Erg29 may be acting to deliver iron to Erg25 or that it may be a chaperone for Erg25, although extensive experimentation has not shown iron bound to Erg29, and we have been unsuccessful in co-immunoprecipitating Erg29 and Erg25 (data not shown).

We have not identified the specific toxic sterol intermediates generated when ERG29 is lost, but our data suggest that their levels are affected by oxidants. Reductions in mitochondrial respiration (Rho0), or reduction of mitochondrial iron-based oxidants led to suppression of toxicity and at least a partial restoration of sterol metabolism. The genetic suppressors did not return the sterol profile to normal but did decrease the accumulation of 4,4-DMZ and 4-methyl fecosterol. Loss of ERG25 led to a similar constellation of phenotypes (decreased Fe-S synthesis, increased mitochondrial oxidants, and loss of Yfh1). These phenotypes, however, could not be suppressed by overexpression of those genes that suppressed the erg29 phenotype. These results suggest that loss of Erg29 makes the methyl sterol oxidase reaction sensitive to oxidized sterol intermediates. Most of the oxidation of the sterols is due to reactive oxygen present in the mitochondria either as a result of respiration or the interaction of reactive sterol metabolites with iron, leading to a further increase in reactive oxygen. In the absence of mitochondrial respiration (Rho0), however, other conditions that lead to increased oxidants, such as decreased mitochondrial iron transport (43) or increased cobalt (23), may result in more oxidized sterols and an attack on Fe-S clusters. Many of the enzymes involved in Fe-S cluster synthesis are themselves

**Figure 6. Loss of YFH1 or ERG29 affects Fe-S cluster synthesis.** A, ∆yfh1pMET3YFH1 cells containing empty vector or pMMT1/2 were grown on CM plus (OFF) or minus 10μ methionine (ON) with or without 0.00125% H2O2 or 3.0 mM iron (Fe) for 3 days. B, WT 1457 and ∆erg29 pGAL1ERG29 cells with empty vector, pYFH1, pMMT1, pMMT2, or pPET20 were grown in glucose for 1.8 h, and aconitate activity was measured as described under “Materials and methods.” n = 3. C, lysates from B were analyzed for lipoic acid protein modification by Western blotting as described previously (35). n = 3. PDH, pyruvate dehydrogenase; KDH, α-ketoglutarate dehydrogenase. The ratio of lipoic acid modification/porin was determined using Bio-Rad ImageLab™ software. Error bars, S.D.
Fe-S cluster–containing enzymes. The nature of the genetic suppressors identified in this study supports the model that exporting iron through Mmt1 and Mmt2 will reduce oxidant generation. Yfh1 may be the target for reactive oxidants, which results in its degradation. It is also possible that YFH1 translation may be decreased, resulting in decreased levels of Yfh1 protein. We have not formally addressed this possibility in the current studies. We found that overexpression of YFH1 results in increased Yfh1 in mitochondria, yet it only showed mild suppression of the sterol intermediate accumulation seen in Δerg29 cells. It may be that oxidized Yfh1 is functionally inactive or that Yfh1 suppresses the Δerg29 phenotype by chelating iron, as Yfh1 has been suggested to be an iron-binding protein (8, 11, 50). The function of Pet20 is unclear except that its loss renders respiratory active yeast susceptible to a wide variety of conditions, including H2O2 and the antifungal drug fluconazole (15).

In otherwise normal cells, increases in the mitochondrial free iron pool do not affect cell growth. Indeed, overexpression of MRS3 or MRS4 can rescue the cytosolic iron lethality due to loss of the vacuolar iron importer Ccc1 (43). To our knowledge, it is only under conditions in which mitochondrial Fe-S cluster synthesis is diminished that increased mitochondrial iron is toxic. Loss of Fe-S synthesis results in the formation of iron

Figure 7. Loss of ERG29 results in decreased levels of Yfh1 protein and increased mitochondrial oxidants. A, mitochondrial preparations from WTpYEp, Δerg29pGAL1ERG29pYEp, pMMT1, or pPET20 grown in galactose or glucose were analyzed for Yfh1, porin, Nfs1, Isu1, and Ssq1 by Western blotting. n = 2. Gels were quantified using Bio-Rad ImageLab™ software with Yfh1 levels normalized to porin in each sample. B, mRNA was isolated from WTpYEp or Δerg29 pGAL1ERG29 cells grown in glucose for 16 h, and quantitative PCR was performed for YFH1 or ISU1 and ACT1 for normalization. C, cells as in B were incubated with 20 nm Mitosox dye and washed, and Mitosox fluorescence was determined by flow cytometry in duplicate as described under “Materials and methods.” n = 2 separate experiments. Error bars, S.D.
nanoparticles, presumably by an oxidation step. Reducing the generation of iron nanoparticles by restricting the free iron pool reduces oxidant generation and the deleterious effects of those oxidants (11). The findings reported here all support the idea that generation of methyl sterol metabolites results in mitochondrial oxidation linked to iron, which then affects the essential Fe-S cluster biosynthetic process. The generation of sterol metabolites precedes the loss of Fe-S cluster activity, as decreasing Fe-S cluster activity by reducing the levels of Yfh1 or Nfs1 does not result in altered sterols (51).

Other reports show a relationship between altered sterol biosynthesis and mitochondrial Fe-S cluster synthesis. Fluconazole is an azole derivative that affects the reaction catalyzed by Erg11 (52), an ER-localized sterol synthesis enzyme. The toxicity of fluconazole is due to the generation of sterol intermediates, which can be affected by mitochondrial respiration, resulting in the generation of toxic 4-methyl sterol intermediates. Most recently, Demuyser et al. (53) identified the mitochondrial chaperone protein Mge1 as a high-copy suppressor of fluconazole toxicity. Mge1 is a chaperone involved in Fe-S cluster metabolism and protein import into the mitochondria. The authors showed that Mge1 acted as a suppressor by increasing ergosterol upon fluconazole treatment and that the suppressive effect of Mge1 overexpression was lost in cells that had a deletion in the Fe-S chaperone encoded by SSQ1, pointing again to involvement of Fe-S synthesis in the toxicity of fluconazole.

The finding that Yfh1 and Fe-S cluster synthesis may be a target for mitochondrial oxidants may extend to agents other than sterol metabolites and to species other than yeast. It is well established that doxorubicin-induced cardiac toxicity is due to the generation of iron-related oxidants (54, 55). Expression of mitochondrial ferritin can protect HeLa cells from doxorubicin toxicity (56), and mice with a targeted gene deletion in mitochondrial ferritin are much more sensitive to doxorubicin-mediated cardiac toxicity than normal mice (57). Ardehali and colleagues (58) reported that overexpression of Abcb8, a mitochondrial ATP transporter, reduced doxorubicin-mediated toxicity. The substrate transported out of the mitochondria by Abcb8 was a Fe-GSH conjugate, although the exact compound was unclear. Recently, Mouli et al. (59) reported that doxorubicin induced the loss of Fe-S cluster synthesis by reducing the levels of frataxin in cultured cardiac myocytes due to increased degradation. They also showed that overexpression of frataxin prevents doxorubicin toxicity. Overexpression of frataxin in transgenic flies suppressed the effects of iron, H$_2$O$_2$, and paraquat on lifespan (60). Further studies demonstrated that a purified recombinant cell-permeable frataxin (PEP1-frataxin) protected neuronal cells from oxidant-mediated cell death (61). The effects of the Parkinson's disease-inducing neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which targets complex I in the electron transport chain, can be suppressed by overexpression of ferritin or through increased levels of frataxin due to administration of Tat-frataxin (62). These results underscore that mitochondria are highly susceptible to iron-mediated toxicity and suggest that this toxicity may target Fe-S cluster formation. The data also suggest that frataxin may be a central target for oxidants either due to its specific role in Fe-S cluster synthesis or as an iron-binding molecule. Based on these findings, we speculate that other compounds that result in mitochondrial toxicity, through generation of reactive oxygen radicals, might also attack Fe-S cluster synthesis and be suppressed by either removal of mitochondrial iron or increased levels of frataxin.

![Figure 8. Loss of ERG25 results in decreased Fe-S synthesis and decreased Yfh1.](image)

**A.** WT and Δerg25pGAL1ERG25 cells were grown in glucose-containing medium for 16 h, and aconitate activity was measured. n = 2 with two separate clones per experiment. **B.** Mitochondrial lysates from cells as in A grown in galactose or glucose were analyzed for Yfh1, porin, Nfs1, Isu1, and Ssq1 by Western blotting. Gels were quantified using Bio-Rad ImageLab™ software with Yfh1 levels normalized to porin in each sample. C, Δerg25pGAL1ERG25 containing pYEp, pMMT1, pMMT2, or pYFH1 grown in galactose or glucose for 16 h was incubated with 20 nM Mitosox dye and washed, and Mitosox fluorescence was determined by flow cytometry in duplicate as described under “Materials and methods.” n = 2.
Increased sterol intermediates affect Fe-S cluster synthesis

Table 2

| Yeast strains used in this study |
|----------------------------------|
| **Strain** | **Genotype** | **Source/Reference** |
| DY1457 | ura3-52, leu2-3,112, trp1-1, his3-11, 15, ade6, can1-100(oc) | Ref. 42 |
| DY150 | ura3-52, leu2-3,112, trp1-1, ade2-1, can1-100(oc) | Ref. 42 |
| Δerg29pβ-estradiol (pGEV)pGAL1ERG29 | Δerg29::KanMX4 pβ-estradiol-pGAL1ERG29–6XHIS | This study |
| Δerg25pβ-estradiol (pGEV)pGAL1ERG25 | Δerg25::KanMX4 pβ-estradiol-pGAL1ERG25–6XHIS | This study |
| ΔYFH1 | ura3-52, leu2-3,112, trp1-1, his3-11, 15, ade6, can1-100(oc) | ΔYFH1::HIS3 |
| Δmrs3Δmnr4 | Δmrs3Δmnr4::KAN | Refs. 5 and 51 |
| Δmrs3Δmnr4Δerg29 | Δmrs3Δmnr4Δerg29 | Refs. 42, 67, and 68 |

Materials and methods

**Yeast, plasmids, and growth medium**

Genotypes of strains employed in this study are listed in Table 1. The WT strains employed for these experiments were from the W303 background. Most deletion strains were created by either PCR amplifying the KanMX cassette from the homozygous diploid deletion collection (Research Genetics) or fusion PCR (63). Cells were grown in YPD (1% yeast extract, 2% peptone, 2% dextrose), YPGE (2% glycerol/ethanol), CM (0.67% yeast nitrogen base, 0.12% drop-out amino acid mixture, 2% dextrose), CM with 2% galactose or 2% glycerol/ethanol. Media were made iron-deficient by the addition of 8 μM BPS, and specific concentrations of ferrous sulfate (μM) were added back. The concentration of added iron in μM is denoted as BPS(x). Plasmids used in this study are listed in Table 2. Strains with β-estradiol–regulated expression plasmids consist of a Gal4 fused to an estrogen-responsive VP16 activation domain expressed from the MR77 promoter as described (20) and a plasmid with a GAL1 promoter driving expression of ERG29 or ERG25. To induce ERG25 or ERG29 expression, cells were grown in 50 nm β-estradiol-containing (Sigma) or galactose-containing medium. AP8 was added to growth medium at various concentrations, or cobalt chloride (CoCl2, 1.0 mm) was added to growth medium. Cells were grown for 2–4 days on plates and overnight in liquid medium.

**Suppressor library screen**

Δerg29 cells containing pYCpERG29 URA3 were transformed with a high-copy YEp-LEU2 genomic library. Colonies were selected on uracil–leucine. Colonies were replicated onto 5-fluoroorotic acid to allow loss of the URA3 ERG29 plasmid, and colonies were plated onto YPGE. Colonies positive for growth on YPGE were identified, genomic preparations were made, and rescued plasmids were sequenced and identified genes subcloned to determine the gene(s) responsible for suppression. To confirm suppression of the poor growth due to loss of ERG29 Δerg29pGAL1ERG29 (TRP1), cells were transformed with pYFH1, pMMT1, pMMT2, or pPET20 (LEU2 plasmids), and growth on CM–trp–leu–his galactose or glucose was assessed.

**Sterol analysis**

Cells were grown in galactose-containing medium overnight, shifted to glucose-containing medium for 18 h, pelleted, washed, and frozen at −80 °C. Lipid extractions were performed on frozen cell pellets as described (64). All GC/MS sterol analysis was performed with an Agilent 7200 gas chromatograph–mass spectrometer fit with an Agilent 7693A autosampler (Agilent Technologies, Santa Clara, CA). Dried samples were suspended in 40 μl of pyridine, followed by transfer to autosampler vials, and 80 μl of N-methyl-N-tri-methylsilyltrifluoracetamide (Thermo Fisher) was added automatically via the autosampler and incubated for 30 min at 37 °C with shaking. After incubation, 1 μl of the prepared sample was injected into the gas chromatograph inlet in the split mode with the inlet temperature held at 250 °C. A 25:1 split ratio was used for analysis. The gas chromatograph had an initial temperature of 60 °C for 1 min followed by a 10 °C/min ramp to 325 °C and a hold time of 2 min. A 30-m Agilent Zorbax DB-5MS with a 10-m Duraguard capillary column was employed for chromatographic separation. Helium was used as the carrier gas at a rate of 1 ml/min. Data were collected using MassHunter software (Agilent). Metabolites were identified, and their peak area was recorded using MassHunter Quant. These data were transferred to an Excel spreadsheet (Microsoft Corp., Redmond, WA).

**MitoSOX flow cytometry**

Δerg29pGAL1ERG29 plus or minus suppressor-identified plasmids was grown in galactose-containing medium overnight, shifted to glucose-containing medium for 16–18 h, and incubated with 20 nm MitoSOX according to the manufacturer’s instructions (Thermo Fisher), and fluorescence was detected using a BD FACSCanto running FACSDiva version 8.0 software.

**Other procedures and reagents**

Quantitative PCR primers for YFH1, ISU1, and ACT1 were YFH1 forward GTCCAGCTGTAACAAATAAA and reverse GTGCAATTCTTACGAAACCC, ISU1 forward CAAGACACTGACTGCTATTA and reverse GTCCAAGGTTCATCCTCTGTA, and ACT1 forward TGTCAACCACTTGGAACGATA and reverse GGCTTGAGTGGAAACCGTAGA. Differential interference contrast and epifluorescence images were captured using an Olympus BX51 microscope with a ×100, 1.3 numerical aperture oil immersion objective and Pictureframer software. Aconitase activity was determined as described previously (42). For mitochondrial isolations, cells were digested and homogenized, crude mitochondrial pellets were obtained by centrifugation at 10,000 × g for 10 min, mem-
branes were solubilized, and protein determinations were performed as described previously (65). Proteins were analyzed by 4–20% SDS-PAGE Tris/glycine followed by Western blot analysis using Western Lightning (PerkinElmer Life Sciences). Antisera used for probing Western blots included rabbit anti-His6 (1:1000), rabbit anti-Erg25 (1:500), rabbit anti-Yfh1 (1:1000), rabbit anti-Nfs1 (1:1000), rabbit anti-Isu1 (1:1000), rabbit anti-Ssq1 (1:1000), or mouse anti-porin (1:1000). Secondary antibodies were either peroxidase-conjugated goat anti-rabbit IgG or peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories; 1:5000). Western blots were quantified using Bio-Rad ImageLab™ software. Cells were made Rho0 by incubating them in the presence of ethidium bromide overnight. Statistical analyses were performed using a two-tailed Student’s t test with significance set at p = 0.05 (*), 0.01 (**), 0.001 (***) and 0.0001 (****).

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