Published in final edited form as:

Mol Microbiol. 2008 May ; 68(4): 1061–1072. doi:10.1111/j.1365-2958.2008.06216.x.

Loss of tafazzin in yeast leads to increased oxidative stress during respiratory growth

Shuliang Chen, Quan He, and Miriam L. Greenberg*

Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA

Summary

The tafazzin (*TAZ*) gene is highly conserved from yeast to humans, and the yeast *taz1* null mutant shows alterations in cardiolipin (CL) metabolism, mitochondrial dysfunction and stabilization of supercomplexes similar to those found in Barth syndrome, a human disorder resulting from loss of tafazzin. We have previously shown that the yeast tafazzin mutant *taz1*, which cannot remodel CL, is ethanol-sensitive at elevated temperature. In the current report, we show that in response to ethanol, CL mutants *taz1* as well as *crd1*, which cannot synthesize CL, exhibited increased protein carbonylation, an indicator of reactive oxygen species (ROS). The increase in ROS is most likely not due to defective oxidant defence systems, as the CL mutants do not display sensitivity to paraquat, menadione or hydrogen peroxide (H₂O₂). Ethanol sensitivity and increased protein carbonylation in the *taz1* mutant but not in *crd1* can be rescued by supplementation with oleic acid, suggesting that oleoyl-CL and/or oleoyl-monolysocardiolipin enables growth of *taz1* in ethanol by decreasing oxidative stress. Our findings of increased oxidative stress in the *taz1* mutant during respiratory growth may have important implications for understanding the pathogenesis of Barth syndrome.

Introduction

Barth syndrome (BTHS) is a severe X-linked inherited disorder characterized by short stature, cardioskeletal myopathy, neutropenia, abnormal mitochondria and respiratory chain dysfunction (Barth *et al.*, 1999). The disease is caused by mutations in the tafazzin (*TAZ*) gene G4.5 (Bolhuis *et al.*, 1991; Bione *et al.*, 1996). Tafazzin is a phospholipid acyltransferase that has been shown to remodel cardiolipin (CL) (Neuwald, 1997), the signature lipid of the mitochondrial inner membrane. As a result, BTHS patients exhibit defects in CL metabolism, including aberrant CL fatty acyl composition, accumulation of monolysocardiolipin (MLCL) and reduced total CL levels (Vreken *et al.*, 2000; Valianpour *et al.*, 2005). How these metabolic defects lead to the variety of symptoms characteristic of BTHS is not understood.

The *TAZ* gene is highly conserved from yeast to humans. The *Saccharomyces cerevisiae* gene YPR140w (*TAZI*) is homologous to the human *TAZ* gene, and the yeast *taz1* null mutant shows alterations in CL metabolism and mitochondrial dysfunction similar to those found in BTHS patients (Vaz *et al.*, 2003; Gu *et al.*, 2004; Ma *et al.*, 2004). Therefore,

*For correspondence. mlgreen@sun.science.wayne.edu; Tel. (+1) 313 577 5202; Fax (+1) 313 577 6891.

elucidating mechanisms underlying defects associated with tafazzin deficiency in the yeast model is expected to shed light on BTHS. The *TAZI* gene is essential for growth of yeast in ethanol at elevated temperature, and loss of tafazzin leads to ethanol sensitivity. Ethanol is a powerful inhibitor of the growth, viability and fermentation rate of yeast cells (Brown *et al.*, 1981; Jones and Greenfield, 1985). A proposed target of ethanol damage is the mitochondrion, as ethanol causes loss of mitochondrial DNA and inactivation of some mitochondrial enzymes (Jimenez and Benitez, 1988; Jimenez *et al.*, 1988; Ibeas and Jimenez, 1997). Increasing evidence suggests that ethanol toxicity is correlated with the production of acetaldehyde and reactive oxygen species (ROS) in the mitochondria during ethanol metabolism (Costa *et al.*, 1997; Du and Takagi, 2007). Ethanol is preferentially oxidized to acetaldehyde by cytosolic alcohol dehydrogenase, forming NADH in the process (Wills, 1990). The NADH is then utilized to synthesize ATP by the mitochondrial electron transport chain, which is the main endogenous source of ROS.

Reactive oxygen species are potentially toxic by-products of oxygen metabolism in aerobic organisms. The reaction of ROS with cellular components, including lipids, proteins and nucleic acids, leads to loss of function and cell death. Excess ROS is associated with a variety of diseases and with the process of ageing (Ames *et al.*, 1993; Shigenaga *et al.*, 1994; Costa and Moradas-Ferreira, 2001). Under normal physiological conditions, cells contain antioxidant defence mechanisms to protect against the toxic effects of ROS, including catalase, glutathione peroxidase and superoxide dismutase (SOD), and non-enzymic molecules such as glutathione (Jamieson, 1998). However, during some stress conditions, the levels of ROS exceed the antioxidant capacity of the cell, resulting in oxidative damage. In yeast, the major site of intracellular ROS generation is the mitochondrial electron transport chain. Several mitochondrial respiratory complexes and individual enzymes (NADH dehydrogenase, glycerol-3-phosphate dehydrogenase) have been identified as sources of ROS (Turrens, 2003), and complex III is considered to be the principal site of ROS generation (Chance *et al.*, 1979; Chen *et al.*, 2003). In the yeast *S. cerevisiae*, normal mitochondrial function is required for resistance to oxidative stress (Grant *et al.*, 1997), and the maintenance of a stable respiratory chain strongly prevents the generation of mitochondrial ROS (Barros *et al.*, 2003).

Cardiolipin, an anionic phospholipid in the mitochondrial inner membrane, plays an important role in the stabilization of electron transport chain complexes. The interactions of CL with membrane proteins are required for optimal function (Hoch, 1992; Schlame *et al.*, 2000). CL has been found to bind to inner membrane proteins, such as the ADP/ATP carrier, complex III and complex IV (Yu and Yu, 1980; Drees and Beyer, 1988; Robinson *et al.*, 1990; Schagger *et al.*, 1990; Hayer-Hartl *et al.*, 1992; Beyer and Nuschler, 1996; Gomez and Robinson, 1999; Sedlak and Robinson, 1999). CL binding is essential for the optimal function of mitochondrial enzymes and for supercomplex stability. In yeast, CL synthase mutant cells (*crd1*), which lack CL, exhibit decreased stability of supercomplexes (Zhang *et al.*, 2002; Pfeiffer *et al.*, 2003). Similarly, mitochondrial supercomplex stability is also decreased in BTHS patients and in the yeast *taz1* mutant (Brandner *et al.*, 2005; McKenzie *et al.*, 2006). Phospholipid analysis of cells of BTHS patients and the yeast *taz1* mutant shows reduced CL and altered CL acyl composition (Vreken *et al.*, 2000; Schlame *et al.*, 2003; Vaz *et al.*, 2003; Gu *et al.*, 2004). In the yeast *taz1* mutant, CL defects result in

dissociation of supercomplex III₂IV₂, releasing a complex IV monomer (Brandner *et al.*, 2005). Similar to yeast *taz1* cells, lymphoblasts from BTHS patients exhibit destabilization of the supercomplex by weakening the interactions between complexes I, III and IV (McKenzie *et al.*, 2006). This suggests that reduced levels and/or altered fatty acyl incorporation in CL leads to destabilization of the mitochondrial supercomplex, which may account for the mitochondrial dysfunction observed in the yeast *taz1* mutant and in BTHS patients (Ma *et al.*, 2004; Xu *et al.*, 2005).

Because CL mutants exhibit electron transport chain defects and supercomplex instability, we hypothesized that CL mutant cells produce excessive ROS in the presence of ethanol. Consistent with the hypothesis, we found that *crd1* and *taz1* mutants exhibited increased protein carbonylation, an indicator of ROS, in response to ethanol. The excessive production of ROS is most likely not due to defective oxidant defence systems, as the CL mutants do not display sensitivity to paraquat, menadione or hydrogen peroxide (H₂O₂). Ethanol sensitivity and increased protein carbonylation in the *taz1* mutant but not in *crd1* can be rescued by supplementation with oleic acid (C18:1), suggesting that oleoyl CL/MLCL enables growth of *taz1* in ethanol by decreasing oxidative stress. These findings suggest that the loss of tafazzin leads to chronic and excessive ROS accumulation, which may contribute to cell or tissue damage.

Results

The *taz1* mutant exhibits increased protein carbonylation during growth in ethanol at elevated temperature

We showed previously that during growth in ethanol as a sole carbon source, the yeast *taz1* mutant exhibited decreased growth at elevated temperature (Gu *et al.*, 2004), similar to the *crd1* mutant, which lacks CL synthase and has no detectable CL (Jiang *et al.*, 1997; Chang *et al.*, 1998). Yeast cells grown in aerobic conditions are continuously exposed to ROS generated during oxygen consumption. Under normal conditions, cells use antioxidant defence mechanisms to prevent ROS build-up, maintaining low levels of ROS. However, under some conditions, the levels of ROS exceed the antioxidant capacity, and cells face oxidative stress (Costa and Moradas-Ferreira, 2001). Ethanol has been associated with ROS production in yeast (Jimenez and Benitez, 1988; Costa *et al.*, 1993; Costa *et al.*, 1997; Bailey *et al.*, 1999; Quintans *et al.*, 2005; Du and Takagi, 2007). Therefore, a possible explanation for the growth defect of the *taz1* mutant in ethanol is that the mutant is subject to increased oxidative stress. To examine this possibility, we measured cellular protein carbonylation, which is a sensitive indicator of oxidative stress (Reznick and Packer, 1994). Carbonyl groups (aldehydes or ketones) result from the oxidation of acids and serve as markers for protein oxidation (Dirmeier *et al.*, 2004). As shown in Fig. 1, the level of protein carbonylation in *taz1* and *crd1* is double that of wild-type cells in media containing ethanol, while in glucose media, protein carbonylation levels are not affected. This suggests that *taz1* and *crd1* mutants are subject to oxidative stress when cultured in media containing ethanol at high temperature. When transformed with a plasmid-borne copy of the *TAZ1* or *CRD1* gene, the *taz1* and *crd1* mutants, respectively, did not show increased protein carbonylation.

To determine whether increased protein carbonylation in *taz1* and *crd1* resulted from defective antioxidant defence against oxidative stress versus excessive generation of ROS, we examined *taz1* and *crd1* cells for sensitivity to other ROS-generating agents, including paraquat, menadione and H₂O₂. As shown in Fig. 2, *taz1* and *crd1* mutants were not sensitive to paraquat, menadione or H₂O₂, in contrast to *sod1* and *sod2* mutants, which cannot detoxify ROS. This suggests that the antioxidant defences of *taz1* and *crd1* are not defective. Consistent with this, the expression of *SOD1* and *SOD2* mRNAs (Fig. 3) and cellular and mitochondrial SOD activities (Fig. 4) were not affected in *taz1* and *crd1* in response to ethanol. Therefore, the increased protein carbonylation levels in *taz1* and *crd1* mutants during growth in ethanol are most likely due to excessive ROS generation. These results imply that growth in media with ethanol as carbon source leads to ROS overproduction in CL mutants, which exceeds the antioxidant capacity and causes defective growth. During growth on other non-fermentable carbon sources, including glycerol and lactate, we observed similar growth defects and increased protein carbonylation in the *crd1* and *taz1* mutants (Fig. 1). Therefore, increased oxidative stress occurs in CL mutant cells during respiratory growth conditions, but not during fermentative growth on glucose (or in the presence of ethanol when glucose is also present).

The *taz1* mutant exhibits increased MLCL and decreased CL in response to ethanol

We wished to determine if increased protein carbonylation and ethanol sensitivity of the *taz1* mutant are correlated with aberrant remodelling of CL. We therefore examined the steady-state CL/MLCL levels in the *taz1* mutant. In media containing glucose as the carbon source, the CL level in *taz1* was 30% less than that of the wild type, and the MLCL level was increased by 10-fold (Fig. 5). Despite these perturbations of CL levels, the *taz1* mutant did not show decreased growth in glucose (data not shown). In media with ethanol as the sole carbon source, CL levels were reduced by about 70%, and MLCL was increased by 17-fold (Fig. 5). Abnormalities were also seen in phosphatidylethanolamine (PE) and phosphatidylcholine (PC) composition. Similar to *crd1*, the *taz1* mutant showed increased PE and reduced PC in response to ethanol (Fig. 6, oleic acid). These findings are consistent with the study of Chi and Arneborg (1999), who reported that ethanol sensitivity was associated with decreased CL and PC levels. These results suggest that PE and PC levels are co-ordinately regulated with CL synthesis and remodelling.

Ethanol sensitivity of the *taz1* mutant is rescued by supplementation with oleic acid

Yeast CL has a unique fatty acid composition compared with other mitochondrial phospholipids. It is mainly composed of mono-unsaturated acyl chains of 16–18 carbons in length, such as palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0) and oleic acid (C18:1). Of these, oleic acid and palmitoleic acid are predominant in CL, resulting in a much higher unsaturation index than in other phospholipids. Several studies have documented the alteration of cellular phospholipid composition in response to ethanol exposure (Mishra and Prasad, 1988; Chi and Arneborg, 1999). The degree of fatty acid unsaturation is thought to play a major role in the ethanol sensitivity of yeast (Tuller *et al.*, 1999; You *et al.*, 2003). Consistent with this hypothesis, the *taz1* mutant exhibits a marked decrease in CL molecular species containing unsaturated fatty acids (Gu *et al.*, 2004). We wished to determine if fatty acids with a greater degree of unsaturation could support growth

of the mutants in ethanol. To this end, we examined the effect of supplementation of different fatty acids on growth of the *taz1* mutant. In the presence of 1 mM oleic acid (C18:1), growth of *taz1* was significantly improved in YPE media at 37°C (Fig. 7). In contrast to oleic acid, supplementation with the other fatty acids tested (C18:0, C16:1 and C16:0) did not alleviate ethanol sensitivity of the *taz1* mutant (data not shown). Oleic acid did not rescue the growth defect of *crd1*, which lacks CL and MLCL, suggesting that unsaturated CL or MLCL species are required to alleviate the ethanol sensitivity of *taz1*.

We analysed the phospholipids of wild type and *taz1* cells using one-dimensional thin-layer chromatography (TLC) to determine whether phospholipid levels were affected by supplementation with oleic acid. As shown in Fig. 8A, supplementation with oleic acid did not affect CL or MLCL levels in the *taz1* mutant. In cells supplemented with oleic acid, CL and MLCL migrated faster than those lipids from cells cultured without oleic acid (Fig. 8B), consistent with incorporation of oleic acid into the lipids. The migration of other phospholipids was also slightly increased. Interestingly, supplementation of oleic acid restored PC and PE levels in the *taz1* mutant to those of wild-type cells (Fig. 6, +oleic acid), which is consistent with previous reports that PE levels and the PE/PC ratio tend to decrease when yeast is supplemented with unsaturated fatty acids of longer chain length and higher degree of unsaturation (Pilkington and Rose, 1991; de Kroon, 2007). None of the other fatty acids, including palmitic acid, palmitoleic acid and stearic acid, restored PC and PE levels or growth on ethanol (data not shown). These results suggest that the steady-state levels of PE and PC in the *taz1* mutant are regulated by fatty acid composition of CL/MLCL. However, the presence of wild-type levels of PC and PE are not sufficient to alleviate ethanol sensitivity, as restoration of PC and PE levels in the *crd1* mutant did not lead to growth in ethanol (Figs 6 and 7). These findings suggest that the presence of CL or MLCL is necessary for growth in ethanol. If oleoyl CL/MLCL enabled growth in ethanol by reducing the generation of ROS, we would expect that protein carbonylation would be decreased in *taz1*, but not *crd1* cells supplemented with oleic acid. Consistent with this, in the presence of oleic acid, the protein carbonylation level was decreased by 25% in *taz1*, but not in *crd1* (Fig. 9). This suggests that oleoyl CL/MLCL enables growth of *taz1* in ethanol by decreasing oxidative stress.

Discussion

In this study, we addressed the hypothesis that the *taz1* mutant produces excessive ROS in response to ethanol, resulting in ethanol sensitivity. Consistent with the hypothesis, we showed that: (i) the *taz1* and *crd1* mutants exhibit increased protein carbonylation in the presence of ethanol, which most likely cannot be attributed to a defective oxidant defence system and (ii) ethanol sensitivity and ROS production in *taz1*, but not *crd1*, can be alleviated by supplementation with oleic acid, suggesting that CL/MLCL plays an important role in modulating oxidative stress during respiration.

In yeast, ethanol sensitivity is correlated with the production of ROS (Costa *et al.*, 1997; Du and Takagi, 2007), which is produced primarily in the mitochondrial respiratory chain (Chance *et al.*, 1979; Chen *et al.*, 2003; Turrens, 2003). CL plays multiple roles in mitochondrial structure and function. The increased oxidative stress on CL mutants shown

here reflects the importance of CL in mitochondrial function. The interactions between CL and mitochondrial membrane proteins are important for the stabilization and optimal functions of these proteins, including those that comprise the supercomplexes in the respiratory chain (Schlame *et al.*, 2000). Consistent with this, the loss of CL (in *crd1*) or aberrant remodelling of CL (in *taz1*) leads to destabilization of respiratory chain supercomplexes (Brandner *et al.*, 2005; Zhang *et al.*, 2005). Complex IV is dissociated from complex III in *crd1* and *taz1* mutants. (Zhang *et al.*, 2002; Pfeiffer *et al.*, 2003; Brandner *et al.*, 2005). Mitochondrial complex III is the major source of superoxide in mitochondria (Chance *et al.*, 1979; Chen *et al.*, 2003). The redox centres of complex III may leak electrons to oxygen, constituting the primary source of superoxide anion (Turrens, 2003). It is likely that the observed increased protein carbonylation in *crd1* and *taz1* in response to ethanol consumption (Fig. 1) stems from the destabilization of the respiratory complexes. Increased protein carbonylation was also seen during respiration in the presence of other respiratory carbon sources, including glycerol and lactate (Fig. 1), indicating that oxidative stress is not limited to growth in ethanol, but rather is a by-product of respiration in the absence of CL. An alternative explanation that the CL mutants have defective antioxidant defence mechanisms is unlikely for several reasons. First, CL mutants are not sensitive to paraquat, menadione or H₂O₂ (Fig. 2). Second, SOD is the major ROS scavenger in ethanol tolerance and respiratory growth (Costa *et al.*, 1993; Costa *et al.*, 1997; Lushchak *et al.*, 2005). CL mutants did not exhibit altered expression of *SOD1* and *SOD2* (Fig. 3) or decreased cellular or mitochondrial SOD activities (Fig. 4). Stabilization of complex III₂IV₂ by CL/MLCL may play an important role in transporting electrons along protein complexes to the ultimate acceptors, and prevent leakage of electrons from the respiratory chain. The results presented here are consistent with studies linking functional mitochondria to resistance to oxidative stress (Grant *et al.*, 1997; Barros *et al.*, 2003).

Our finding that oleic acid rescues increased protein carbonylation and ethanol sensitivity in the *taz1* mutant, which contains MLCL and CL, but not in the *crd1* mutant, which lacks both of these lipids, suggests that CL and/or MLCL plays a key role in modulating oxidative stress during respiration. These results are consistent with previous studies that demonstrated a relationship between lipid composition and ethanol sensitivity. Ethanol-tolerant yeast strains contain a higher CL content and a higher proportion of oleoyl species (C18:1) compared with ethanol-sensitive strains (Chi and Arneborg, 1999). Oleic acid (C18:1) was the most effective fatty acid in overcoming ethanol toxicity, while palmitoleic acid (C16:1) did not confer ethanol tolerance (You *et al.*, 2003). When yeast cells were shifted from glucose to ethanol as the carbon source, incorporation of C18:1 into the plasma and mitochondrial membranes increased significantly while incorporation of C16:0 into the phospholipids decreased, and there was no significant difference in C16:1 and C18:0 incorporation (Tuller *et al.*, 1999). The abundance of (C18:1)₄-CL and (C18:1)₃-(C16:1)₁-CL comprises more than 70% of total CL species (Schlame *et al.*, 2005). In contrast, the *taz1* mutant contains markedly decreased (five- to sixfold) (C18:1)₄-CL and (C18:1)₃-(C16:1)₁-CL, while (16:1)₄-CL is increased (fourfold) (Valianpour *et al.*, 2001). In the current study, CL levels were reduced by 70% in the *taz1* mutant during growth in ethanol versus glucose (Fig. 5), and supplementation with C18:1 in the growth media rescued ethanol sensitivity. Supplementation with oleic acid also alleviated protein carbonylation in

the *taz1* mutant. These results indicate that the fatty acid species composition of phospholipids, especially the incorporation of oleic acid, plays an important role in the maintenance of functional mitochondria during ethanol stress. The effect of oleic acid on ethanol sensitivity and protein carbonylation is CL- and/or MLCL-dependent, as ethanol sensitivity and protein carbonylation in the CL-lacking mutant *crd1* cannot be rescued by supplementation with oleic acid. The inability of other fatty acids to rescue ethanol sensitivity suggests that C18:1 species of CL/MLCL are required to lower oxidative stress. The observation that *taz1*, which lacks CL remodelling capability, may incorporate exogenous fatty acids into CL/MLCL suggests that substrate specificity by enzymes that contribute to CL synthesis plays a role in determining the acyl species of CL.

In summary, we have shown that the loss of tafazzin in yeast leads to increased oxidative stress during respiratory growth at high temperature. The yeast *taz1* mutant has been shown to be a good genetic/biochemical model for BTHS. Similar to BTHS cells, *taz1* mutant cells exhibit increased MLCL, decreased CL and aberrant fatty acyl species of this lipid (Gu *et al.*, 2004). Mitochondria from the *taz1* mutant exhibit decreased coupling and destabilization of supercomplexes (Ma *et al.*, 2004; Brandner *et al.*, 2005). Our findings of increased oxidative stress in the *taz1* mutant during respiratory growth may have important implications for understanding the pathogenesis of BTHS.

Experimental procedures

Yeast strains and growth media

The *S. cerevisiae* strains and plasmids used in this work are listed in Table 1. Synthetic complete medium contained adenine (20.25 mg l⁻¹), arginine (20 mg l⁻¹), histidine (20 mg l⁻¹), leucine (60 mg l⁻¹), lysine (200 mg l⁻¹), methionine (20 mg l⁻¹), threonine (300 mg l⁻¹), tryptophan (20 mg l⁻¹), uracil (20 mg l⁻¹), yeast nitrogen base w/o amino acids (Difco) and glucose (2%) (SD). Synthetic drop-out medium (Leu-) contained all of the above ingredients except leucine. Complex media contained yeast extract (1%), peptone (2%) and glucose (2%) (YPD), ethanol (0.5–1.0%) (YPE), glycerol (3%) (YPG) or lactate (2%) (YPL). YPE/FA was YPE supplemented with specific fatty acids, 1.0 mM oleic acid, 1.0 mM palmitoleic acid, 1.0 mM palmitic acid or 1.0 mM stearic acid. Fatty acids were prepared as 1 M stock solutions in 100% ethanol. In the media, fatty acids were solubilized with Tergitol (1%). The solid medium contained agar (2%) in addition to the above-mentioned ingredients.

The *sod1* and *sod2* mutants were constructed as follows. The entire open reading frame of the *SOD1* or *SOD2* gene was replaced by *KanMX4* using polymerase chain reaction (PCR)-mediated homologous recombination in the FGY3 wild-type strain. The *KanMX4* cassette was amplified from pUG6 using primers consisting of 50 nucleotides identical to the *SOD1* or *SOD2* flanking regions at the 5' end and 21 nucleotides for the amplification of the *KanMX4* gene at the 3' end. The PCR product was transformed into FGY3, and transformants were selected on YPD media containing G418. Disruption of *SOD1* and *SOD2* was confirmed by the absence of the target PCR product using primers against the *SOD1* and *SOD2* coding sequences respectively.

Plasmid construction

A 1525 bp *CRDI* sequence containing promoter and coding sequences was amplified from yeast genomic DNA using BamHI-tagged primer CRD15' (5'-GGATCCATGCTTAACTGATCCTGGCGT-3') and ClaI-tagged primer CRD13' (5'-ATCGATGCATTCAAGCCGTTAACATGG-3'). The PCR reaction products were cloned into the pRS200 vector. The resulting recombinant plasmid, pRS200-*CRDI*, was digested with MfeI and SalI. The pYPGK18 vector was digested with EcoRI and SalI. The DNA fragment containing *CRDI* was cloned into the pYPGK18 vector, downstream of the yeast *PGK1* promoter.

Growth conditions

For ethanol sensitivity studies, cultures were started by inoculating 10 ml of YPE liquid medium to a cell density of $A_{550} = 0.5$. Cultures were agitated at 250 r.p.m. at 30°C or 37°C, and growth was followed by measuring A_{550} . For paraquat, menadione and H₂O₂ sensitivity studies, yeast cells were grown in YPD media at 30°C to the early stationary phase. Cultures were then serially diluted and spotted onto YPD or YPE plates with added paraquat (0.5 mM), menadione (0.1 mM) or H₂O₂ (2.5 mM) and incubated for 3–5 days at 37°C.

Superoxide dismutase activity assay

Activities of SOD were determined using the Bioxytech SOD-525 kit (Oxis Health Products). Cell lysate (4 mg protein) in 0.9% NaCl was centrifuged at 10 000 r.p.m. for 60 min in a microcentrifuge at 0°C and the supernatant was used to determine the activity of cytosolic SOD activity. Mitochondrial SOD activity was assayed in mitochondrial extracts. Isolation of mitochondria was performed as previously described (Koshkin and Greenberg, 2000). The assay method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzofluorene as a substrate in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm (Nebot *et al.*, 1993).

Quantification of protein carbonyl content

The content of carbonyl groups in proteins was measured by determining the amount of 2,4-dinitrophenylhydrazine (DNPH) formed upon reaction with 2,4-dinitrophenyl hydrazine (DNPH). The protein carbonyl content was measured as described with minor modifications (Reznick and Packer, 1994; Dirmeier *et al.*, 2004). Yeast cells were grown at 37°C to the early stationary phase in glucose or ethanol. Harvested cells were subjected to mechanical breakage with glass beads. Cell extracts were separated from glass beads and cells by centrifugation, and nucleic acids were removed from the cell extracts with 1.0% streptomycin sulphate. The resulting protein samples (> 1.5 mg protein) were incubated with 10 mM DNPH in 2 M HCl at room temperature for 60 min in the dark. Proteins were precipitated by addition of trichloroacetic acid to a final concentration of 10%, and the pellets were washed with ethanol : ethyl acetate (1:1) to remove the free DNPH. The final protein pellets were dissolved in 6 M guanidine hydrochloride solution containing 20 mM potassium phosphate, pH 2.4. The carbonyl content was calculated from the absorbance maximum of DNP measured at 370 nm.

Phospholipid determination

Yeast cells were grown in the presence of $^{32}\text{P}_i$ ($10 \mu\text{Ci ml}^{-1}$) at 30°C or 37°C to the early stationary phase. Cells were then washed and digested by zymolyase to yield spheroplasts. Total lipids were extracted from spheroplasts with chloroform : methanol (2:1) (v/v). The extracted lipids were applied to a boric acid-treated TLC plate, which was developed in the one-dimensional solvent system chloroform/triethylamine/ethanol/water (30:35:35:7). Developed chromatograms were quantified by phosphorimaging (Vaden *et al.*, 2005).

Real-time PCR

Yeast cultures (10 ml) were grown to the early stationary phase, cells were harvested and total RNA was isolated using the RNeasy Mini Kit (Qiagen). The RNA samples were treated with DNase from a DNA-free Kit (Ambion) to remove contaminating genomic DNA. cDNAs were synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche) according to the manufacturer's protocol. Real-time PCR reactions were performed in a 50 μl volume using Brilliant SYBR Green QPCR Master Mix (Stratagene) in a 96-well plate. Duplicates for each sample were included for each reaction. The real-time PCR primers used in this work are listed in Table 2. *ACT1* was used as the internal control and the RNA level of the gene of interest was normalized to *ACT1* levels. PCR reactions were initiated at 95°C for 10 min for denaturation followed by 40 cycles consisting of 30 s at 95°C and 60 s at 57°C .

Acknowledgments

We thank Dr Zhiming Gu for helping to initiate this study and for valuable discussion, and Dr Quan Zhong, Dr Vishal Gohil and Rania Deranieh for helpful advice. This work was supported by a grant from the Barth Syndrome Foundation and by Grant HL62263 from the National Institutes of Health.

References

- Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci USA*. 1993; 90:7915–7922. [PubMed: 8367443]
- Bailey SM, Pietsch EC, Cunningham CC. Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III. *Free Radic Biol Med*. 1999; 27:891–900. [PubMed: 10515594]
- Barros MH, Netto LE, Kowaltowski AJ. H_2O_2 generation in *Saccharomyces cerevisiae* respiratory pet mutants: effect of cytochrome *c*. *Free Radic Biol Med*. 2003; 35:179–188. [PubMed: 12853074]
- Barth PG, Wanders RJ, Vreken P. X-linked cardioskeletal myopathy and neutropenia (Barth syndrome)-MIM 302060. *J Pediatr*. 1999; 135:273–276. [PubMed: 10484787]
- Beyer K, Nüscher B. Specific cardiolipin binding interferes with labeling of sulfhydryl residues in the adenosine diphosphate/adenosine triphosphate carrier protein from beef heart mitochondria. *Biochemistry*. 1996; 35:15784–15790. [PubMed: 8961941]
- Bione S, D'Adamo P, Maestrini E, Gedeon AK, Bolhuis PA, Toniolo D. A novel X-linked gene, G4.5, is responsible for Barth syndrome. *Nat Genet*. 1996; 12:385–389. [PubMed: 8630491]
- Bolhuis PA, Hensels GW, Hulsebos TJ, Baas F, Barth PG. Mapping of the locus for X-linked cardioskeletal myopathy with neutropenia and abnormal mitochondria (Barth syndrome) to Xq28. *Am J Hum Genet*. 1991; 48:481–485. [PubMed: 1998334]
- Brandner K, Mick DU, Frazier AE, Taylor RD, Meisinger C, Rehling P. Taz1, an outer mitochondrial membrane protein, affects stability and assembly of inner membrane protein complexes: implications for Barth Syndrome. *Mol Biol Cell*. 2005; 16:5202–5214. [PubMed: 16135531]

- Brown SW, Oliver SG, Harrison DEF, Righelato RC. Ethanol inhibition of yeast growth and fermentation: differences in the magnitude and complexity of the effect. *Eur J Appl Microbiol Biotechnol.* 1981; 11:151–155.
- Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev.* 1979; 59:527–605. [PubMed: 37532]
- Chang SC, Heacock PN, Mileyskova E, Voelker DR, Dowhan W. Isolation and characterization of the gene (CLS1) encoding cardiolipin synthase in *Saccharomyces cerevisiae*. *J Biol Chem.* 1998; 273:14933–14941. [PubMed: 9614098]
- Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. *J Biol Chem.* 2003; 278:36027–36031. [PubMed: 12840017]
- Chi Z, Arneborg N. Relationship between lipid composition, frequency of ethanol-induced respiratory deficient mutants, and ethanol tolerance in *Saccharomyces cerevisiae*. *J Appl Microbiol.* 1999; 86:1047–1052. [PubMed: 10389252]
- Costa V, Amorim MA, Reis E, Quintanilha A, Moradas-Ferreira P. Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase. *Microbiology (Reading, England).* 1997; 143:1649–1656.
- Costa V, Moradas-Ferreira P. Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases. *Mol Aspects Med.* 2001; 22:217–246. [PubMed: 11679167]
- Costa V, Reis E, Quintanilha A, Moradas-Ferreira P. Acquisition of ethanol tolerance in *Saccharomyces cerevisiae*: the key role of the mitochondrial superoxide dismutase. *Arch Biochem Biophys.* 1993; 300:608–614. [PubMed: 8434941]
- Dirmeier R, O'Brien K, Engle M, Dodd A, Spears E, Poyton RO. Measurement of oxidative stress in cells exposed to hypoxia and other changes in oxygen concentration. *Methods Enzymol.* 2004; 381:589–603. [PubMed: 15063700]
- Drees M, Beyer K. Interaction of phospholipids with the detergent-solubilized ADP/ATP carrier protein as studied by spin-label electron spin resonance. *Biochemistry.* 1988; 27:8584–8591. [PubMed: 2851329]
- Du X, Takagi. N-Acetyltransferase Mpr1 confers ethanol tolerance on *Saccharomyces cerevisiae* by reducing reactive oxygen species. *Appl Microbiol Biotechnol.* 2007; 75:1343–1351. [PubMed: 17387467]
- Gomez B Jr, Robinson NC. Phospholipase digestion of bound cardiolipin reversibly inactivates bovine cytochrome bc1. *Biochemistry.* 1999; 38:9031–9038. [PubMed: 10413476]
- Grant CM, MacIver FH, Dawes IW. Mitochondrial function is required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* 1997; 410:219–222. [PubMed: 9237633]
- Gu Z, Valianpour F, Chen S, Vaz FM, Hakkaart GA, Wanders RJ, Greenberg ML. Aberrant cardiolipin metabolism in the yeast *taz1* mutant: a model for Barth syndrome. *Mol Microbiol.* 2004; 51:149–158. [PubMed: 14651618]
- Hayer-Hartl M, Schagger H, von Jagow G, Beyer K. Interactions of phospholipids with the mitochondrial cytochrome-c reductase studied by spin-label ESR and NMR spectroscopy. *Eur J Biochem.* 1992; 209:423–430. [PubMed: 1327777]
- Hoch FL. Cardiolipins and biomembrane function. *Biochimica Biophysica Acta.* 1992; 1113:71–133.
- Ibeas JI, Jimenez J. Mitochondrial DNA loss caused by ethanol in *Saccharomyces flor* yeasts. *Appl Environ Microbiol.* 1997; 63:7–12. [PubMed: 8979333]
- Jamieson DJ. Oxidative stress responses of the yeast *Saccharomyces cerevisiae*. *Yeast.* 1998; 14:1511–1527. [PubMed: 9885153]
- Jiang F, Rizavi HS, Greenberg ML. Cardiolipin is not essential for the growth of *Saccharomyces cerevisiae* on fermentable or non-fermentable carbon sources. *Mol Microbiol.* 1997; 26:481–491. [PubMed: 9402019]
- Jimenez J, Benitez T. Yeast cell viability under conditions of high temperature and ethanol concentrations depends on the mitochondrial genome. *Curr Genet.* 1988; 13:461–469. [PubMed: 3042168]

- Jimenez J, Longo E, Benitez T. Induction of petite yeast mutants by membrane-active agents. *Appl Environ Microbiol.* 1988; 54:3126–3132. [PubMed: 3066293]
- Jones RP, Greenfield PF. Replicative inactivation and metabolic inhibition in yeast ethanol fermentations. *Biotechnol Lett.* 1985; 7:223–228.
- Koshkin V, Greenberg ML. Oxidative phosphorylation in cardiolipin-lacking yeast mitochondria. *Biochem J.* 2000; 347(Part 3):687–691. [PubMed: 10769171]
- de Kroon AI. Metabolism of phosphatidylcholine and its implications for lipid acyl chain composition in *Saccharomyces cerevisiae*. *Biochim Biophys Acta.* 2007; 1771:343–352. [PubMed: 17010666]
- Lushchak V, Semchyshyn H, Mandryk S, Lushchak O. Possible role of superoxide dismutases in the yeast *Saccharomyces cerevisiae* under respiratory conditions. *Arch Biochem Biophys.* 2005; 441:35–40. [PubMed: 16084798]
- Ma L, Vaz FM, Gu Z, Wanders RJ, Greenberg ML. The human TAZ gene complements mitochondrial dysfunction in the yeast taz1Delta mutant. Implications for Barth syndrome. *J Biol Chem.* 2004; 279:44394–44399. [PubMed: 15304507]
- McKenzie M, Lazarou M, Thorburn DR, Ryan MT. Mitochondrial respiratory chain supercomplexes are destabilized in Barth Syndrome patients. *J Mol Biol.* 2006; 361:462–469. [PubMed: 16857210]
- Mishra P, Prasad R. Role of phospholipid head groups in ethanol tolerance of *Saccharomyces cerevisiae*. *J Gen Microbiol.* 1988; 134:3205–3211. [PubMed: 3077635]
- Nebot C, Moutet M, Huet P, Xu JZ, Yadan JC, Chaudiere J. Spectrophotometric assay of superoxide dismutase activity based on the activated autooxidation of a tetracyclic catechol. *Anal Biochem.* 1993; 214:442–451. [PubMed: 8109732]
- Neuwald AF. Barth syndrome may be due to an acyltransferase deficiency. *Curr Biol.* 1997; 7:R465–R466. [PubMed: 9259571]
- Pfeiffer K, Gohil V, Stuart RA, Hunte C, Brandt U, Greenberg ML, Schagger H. Cardiolipin stabilizes respiratory chain supercomplexes. *J Biol Chem.* 2003; 278:52873–52880. [PubMed: 14561769]
- Pilkington BJ, Rose AH. Incorporation of unsaturated fatty acids by *Saccharomyces cerevisiae*: conservation of fatty-acyl saturation in phosphatidylinositol. *Yeast.* 1991; 7:489–494. [PubMed: 1897314]
- Quintans LN, Castro GD, Castro JA. Oxidation of ethanol to acetaldehyde and free radicals by rat testicular microsomes. *Arch Toxicol.* 2005; 79:25–30. [PubMed: 15526191]
- Reznick AZ, Packer L. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol.* 1994; 233:357–363. [PubMed: 8015470]
- Robinson NC, Zborowski J, Talbert LH. Cardiolipin-depleted bovine heart cytochrome c oxidase: binding stoichiometry and affinity for cardiolipin derivatives. *Biochemistry.* 1990; 29:8962–8969. [PubMed: 2176838]
- Schagger H, Hagen T, Roth B, Brandt U, Link TA, von Jagow G. Phospholipid specificity of bovine heart bc1 complex. *Eur J Biochem.* 1990; 190:123–130. [PubMed: 2163831]
- Schlame M, Rua D, Greenberg ML. The biosynthesis and functional role of cardiolipin. *Prog Lipid Res.* 2000; 39:257–288. [PubMed: 10799718]
- Schlame M, Kelley RI, Feigenbaum A, Towbin JA, Heerdt PM, Schieble T, et al. Phospholipid abnormalities in children with Barth syndrome. *J Am Coll Cardiol.* 2003; 42:1994–1999. [PubMed: 14662265]
- Schlame M, Ren M, Xu Y, Greenberg ML, Haller I. Molecular symmetry in mitochondrial cardiolipins. *Chem Phys Lipids.* 2005; 138:38–49. [PubMed: 16226238]
- Sedlak E, Robinson NC. Phospholipase A(2) digestion of cardiolipin bound to bovine cytochrome c oxidase alters both activity and quaternary structure. *Biochemistry.* 1999; 38:14966–14972. [PubMed: 10555978]
- Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. *Proc Natl Acad Sci USA.* 1994; 91:10771–10778. [PubMed: 7971961]
- Tuller G, Nemec T, Hrastnik C, Daum G. Lipid composition of subcellular membranes of an FY1679-derived haploid yeast wild-type strain grown on different carbon sources. *Yeast.* 1999; 15:1555–1564. [PubMed: 10514572]

- Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol.* 2003; 552:335–344. [PubMed: 14561818]
- Vaden DL, Gohil VM, Gu Z, Greenberg ML. Separation of yeast phospholipids using one-dimensional thin-layer chromatography. *Anal Biochem.* 2005; 338:162–164. [PubMed: 15707948]
- Valianpour F, Vreken P, Nijtmans LG, van Roermund CWT, Grivell LA, Wanders RJ, Barth PG. Investigation of defective cardiolipin remodeling in Barth syndrome using yeast as study model (Abstract). *J Inherit Metab Dis.* 2001; 24:83. [PubMed: 11286389]
- Valianpour F, Mitsakos V, Schlemmer D, Towbin JA, Taylor JM, Ekert PG, et al. Monolysocardiolipins accumulate in Barth syndrome but do not lead to enhanced apoptosis. *J Lipid Res.* 2005; 46:1182–1195. [PubMed: 15805542]
- Vaz FM, Houtkooper RH, Valianpour F, Barth PG, Wanders RJ. Only one splice variant of the human *TAZ* gene encodes a functional protein with a role in cardiolipin metabolism. *J Biol Chem.* 2003; 278:43089–43094. [PubMed: 12930833]
- Vreken P, Valianpour F, Nijtmans LG, Grivell LA, Plecko B, Wanders RJ, Barth PG. Defective remodeling of cardiolipin and phosphatidylglycerol in Barth syndrome. *Biochem Biophys Res Commun.* 2000; 279:378–382. [PubMed: 11118295]
- Wills C. Regulation of sugar and ethanol metabolism in *Saccharomyces cerevisiae*. *Crit Rev Biochem Mol Biol.* 1990; 25:245–280. [PubMed: 2171877]
- Xu Y, Sutachan JJ, Plesken H, Kelley RI, Schlame M. Characterization of lymphoblast mitochondria from patients with Barth syndrome. *Lab Invest.* 2005; 85:823–830. [PubMed: 15806137]
- You KM, Rosenfield CL, Knipple DC. Ethanol tolerance in the yeast *Saccharomyces cerevisiae* is dependent on cellular oleic acid content. *Appl Environ Microbiol.* 2003; 69:1499–1503. [PubMed: 12620835]
- Yu CA, Yu L. Structural role of phospholipids in ubiquinol-cytochrome c reductase. *Biochemistry.* 1980; 19:5715–5720. [PubMed: 6257287]
- Zhang M, Mileyskoykaya E, Dowhan W. Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation in the inner mitochondrial membrane. *J Biol Chem.* 2002; 277:43553–43556. [PubMed: 12364341]
- Zhang M, Mileyskoykaya E, Dowhan W. Cardiolipin is essential for organization of complexes III and IV into a supercomplex in intact yeast mitochondria. *J Biol Chem.* 2005; 280:29403–29408. [PubMed: 15972817]

Fig. 1.

CL mutants exhibit increased protein carbonylation in response to non-fermentable carbon source at 37°C. Cells were grown at 37°C to the early stationary phase in glucose (2%), ethanol (0.75%), glycerol (3%) or lactate (2%) as indicated. Protein was extracted and protein carbonylation was measured as described under *Experimental procedures*. Data represent the average of three independent experiments.

Fig. 2.

CL mutants are not sensitive to paraquat, menadione or H₂O₂. Cells were cultured at 30°C in rich media to the early stationary phase, serially diluted and spotted on YPD (A) and YPE (1% ethanol) (B) plates supplemented with 0.5 mM paraquat, 0.1 mM menadione or 2.5 mM H₂O₂. The plates were incubated at 37°C. While *crd1* and *taz1* cells are sensitive to 1% ethanol in liquid media, sensitivity on solid media is detectable at concentrations greater than 2%.

Fig. 3.

SOD1 and *SOD2* expression in CL mutants. Cells were grown in YPE (0.75% ethanol) plus or minus 1 mM oleic acid at 37°C to the early stationary phase. Total RNA was extracted, and *SOD1* and *SOD2* mRNA levels were determined by real-time PCR as described under *Experimental procedures*. The expression of *SOD1* and *SOD2* was normalized to the RNA levels of the internal control *ACT1*. Data are the average of three independent experiments.

Fig. 4.

Activities of SOD in *CL* mutants at 37°C. Cells were grown in YPE (0.75% ethanol) plus or minus 1 mM oleic acid at 37°C to the early stationary phase. Activities of cellular and mitochondrial SOD were determined using cell lysate or mitochondrial extract respectively. Data represent the average of three independent experiments.

Fig. 5.

The *taz1* mutant exhibits increased MLCL and decreased CL in response to ethanol. Cells were grown in YPD or YPE (0.75% ethanol) at 37°C to the early stationary phase. Cells were harvested by centrifugation, and lipids were extracted and separated as described in *Experimental procedures*. Phospholipids were separated by one-dimensional TLC and visualized by phosphorimaging. CL and MLCL were quantified using ImageQuant software. The relative amount of ^{32}P in CL and MLCL is presented as a percentage of the ^{32}P incorporated into total phospholipids. Data represent the average of three independent experiments.

Fig. 6.

Supplementation with oleic acid restores PE and PC in CL mutants to wild-type levels. Cells were grown in YPE (0.75% ethanol) plus or minus 1 mM oleic acid at 37°C to the early stationary phase. Phospholipids were extracted and quantified as described in the legend for Fig. 5. Data represent the average of three independent experiments.

Fig. 7. Supplementation of oleic acid rescues ethanol sensitivity of the *taz1* mutant at 37°C. Cells were grown in YPE (1% ethanol) plus or minus 1 mM oleic acid at 37°C. Cell growth was monitored by A₅₅₀ at the indicated times.

Fig. 8.

Supplementation of oleic acid affects migration of CL and MLCL on TLC.

A. Cells were grown in YPE (0.75% ethanol) plus or minus 1 mM oleic acid. Phospholipid levels were quantified as described in Fig. 5.

B. Analysis of phospholipids by one-dimensional TLC identifies altered migration of CL and MLCL in the presence of oleic acid. Data represent the average of three independent experiments.

Fig. 9.

Supplementation with oleic acid partially rescues accumulation of protein carbonylation in the *taz1* mutant. Cells were grown in YPE (0.75% ethanol) plus or minus 1 mM oleic acid at 37°C to the early stationary phase. Protein was extracted and protein carbonylation was measured as described under *Experimental procedures*. Data represent the average of three independent experiments.

Table 1

Plasmids and yeast strains used in this study.

Plasmid/strains	Characteristics or genotype	Source or reference
pYPGK18	2 μ m, <i>LEU2</i>	Vaz <i>et al.</i> , 2003)
pYPGK18CRD1	Derivative of pYPGK18, expresses <i>CRD1</i> from PGK1 promoter	This study
pTAZ1	Derivative of pYPGK18, expresses <i>TAZ1</i> from PGK1 promoter	Vaz <i>et al.</i> (2003)
FGY3	<i>MAT</i> α , <i>ura 3-52</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1 1</i> , <i>his3 200</i> , <i>leu2 1</i>	Jiang <i>et al.</i> (1997)
FGY2	<i>MAT</i> α , <i>ura 3-52</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1 1</i> , <i>his3 200</i> , <i>leu2 1</i> , <i>crd1 ::URA3</i>	Jiang <i>et al.</i> (1997)
ZGY1	<i>MAT</i> α , <i>ura 3-52</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1 1</i> , <i>his3 200</i> , <i>leu2 1</i> , <i>taz1 ::KanMX4</i>	Ma <i>et al.</i> (2004)
FGY3 <i>sod1</i>	<i>MAT</i> α , <i>ura 3-52</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1 1</i> , <i>his3 200</i> , <i>leu2 1</i> , <i>sod1 ::KanMX4</i>	This study
FGY3 <i>sod2</i>	<i>MAT</i> α , <i>ura 3-52</i> , <i>lys2-801</i> , <i>ade2-101</i> , <i>trp1 1</i> , <i>his3 200</i> , <i>leu2 1</i> , <i>sod2 ::KanMX4</i>	This study

Table 2

The real-time PCR primers used in this study.

Gene	Primers	Sequence	Product length (bp)
ACT1	Forward	TCGTGCTGTCTTCCCATCTATCG	218
	Reverse	CGAATTGAGAGTTGCCCCAGAAG	
SOD1	Forward	TAACGCAGAACGTGGGTTC	314
	Reverse	CTGGTAATGCCGGTCCAAGAC	
SOD2	Forward	CACAGCAAGGAGAACCAAAGTCAC	374
	Reverse	ACTTAATCAGCTCGTCCAGACTGC	