Cell Metabolism
Short Article

Loss of Cytochrome *c* Oxidase Promotes RAS-Dependent ROS Production from the ER Resident NADPH Oxidase, Yno1p, in Yeast

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http://dx.doi.org/10.1016/j.cmet.2013.07.005

SUMMARY

Many disease states, including the aging process, are associated with the accumulation of mitochondria harboring respiratory dysfunction. Mitochondrial dysfunction is often accompanied by increased ROS levels that can contribute to cellular dysfunction and disease etiology. Here we use the model eukaryote *S. cerevisiae* to investigate whether reduced cytochrome *c* oxidase (COX) activity, commonly reported in aging organisms and associated with neurodegenerative disorders, leads to ROS production from mitochondria. We provide evidence that although reduced COX complex activity correlates with ROS accumulation, mitochondria are not the major production center. Instead we show that COX-deficient mitochondria activate Ras upon their outer membrane that establishes a pro-ROS accumulation environment by suppressing antioxidant defenses and the ERAD-mediated turnover of the ER-localized NADPH oxidase Yno1p. Our data suggest that dysfunctional mitochondria can serve as a signaling platform to promote the loss of redox homeostasis, ROS accumulation, and accelerate aging in yeast.

INTRODUCTION

Mitochondria are essential for the maintenance, adaptability, and survival of eukaryotic cells and are an important component of diverse signaling pathways. The importance of understanding how mitochondria influence the general health of cells becomes apparent when we consider that many muscular and neurodegenerative diseases have been linked with their dysfunction. Good evidence also exists that demonstrates a loss of mitochondrial function occurs during the progression of normal aging (Santos et al., 2012). A phenotype that is commonly associated with mitochondrial dysfunction, and in fact with many age-related diseases, is the accumulation of damage attributable to the buildup of reactive oxygen species (ROS). Indeed, a vicious cycle of decline in which ROS arising from the mitochondrial electron transport chain (ETC) leads to the damage to mitochondrial DNA and a resultant increase in radical production provides the cornerstone of the much scrutinized free radical theory of aging (Harman, 1956). However, ROS also serve as important signaling molecules that can promote longevity in *C. elegans* (Schulz et al., 2007) and also in yeast (Mesquita et al., 2010). The overall level of ROS in a cell at any given time is a consequence of the sum of the activity of all production and detoxification systems (Murphy, 2009). This complex scenario involves ROS-producing enzymes and processes within a number of different compartments including the mitochondrial electron transport system, fatty acid β-oxidation within peroxisomes (Fransen et al., 2012), membrane-localized NADPH oxidases (Hayes and Knaus, 2012), and the ER protein disulphide resolution system (Tavender and Bulleid, 2010). Despite the range of possible origins of ROS and redox status scenarios that contribute to disease, it is dysfunctional mitochondria that are most routinely cited as the major source. Levels of superoxide production from the mitochondria, thought to arise predominantly from complexes I and III, rely on a number of factors that are highly variable, such as O_2 availability, proton motive force, $NADH/NAD^+$, and $CoQH_2/CoQ$ ratios (Murphy, 2009). This means that results obtained from in vitro experiments may provide only a partial account of ROS production in vivo. In this study we examine whether the loss of COX function, which is found to occur during aging, leads to mitochondria-derived ROS production in vivo in the model eukaryote *S. cerevisiae*. Surprisingly, although our results confirm that ROS accumulation correlates well with the loss of COX function, we provide evidence that mitochondria are unlikely to be the major source. Instead our data support a model in which dysfunctional mitochondria act as a platform from which aberrant *RAS* signaling disrupts central homeostatic control mechanisms to set up a pro-ROS accumulation environment. Constitutive activation of *RAS* upon the surface of dysfunctional mitochondria leads to the suppression of antioxidant defenses and the endoplasmic reticulum-associated degradation (ERAD) pathway. Suppression of the ERAD system in turn leads to the inappropriate activity of the recently identified ER-localized NADPH oxidase, Yno1p (Rinnerthaler et al., 2012), which we found to account for the majority of detectable ROS. Our data therefore support a model whereby ROS production from COX-depleted mitochondria arises not as a result of excessive radical production from the ETC, but rather as a consequence of the resultant loss of homeostatic control. Our

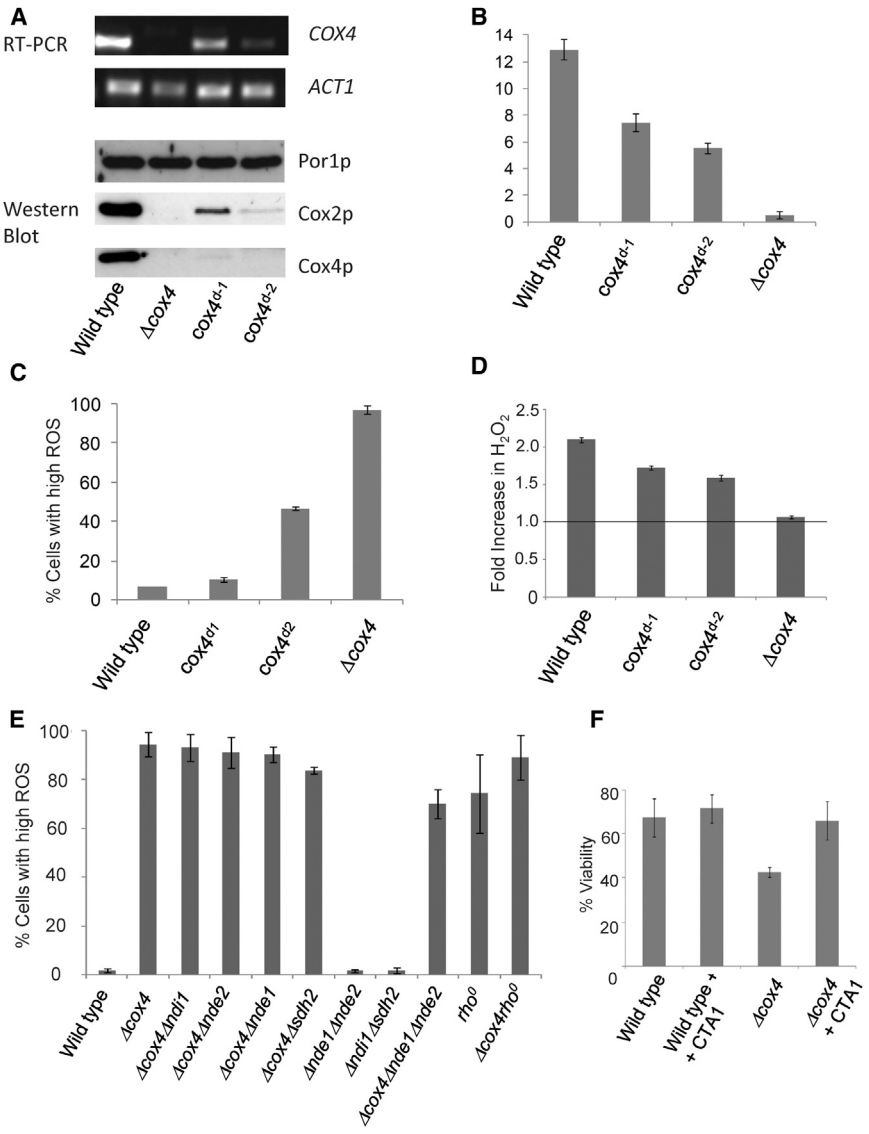

Figure 1. Loss of COX Function Leads to the Accumulation of Nonmitochondrial ROS

(A) COX4 and ACT1 mRNA levels were assessed by RT-PCR and Por1p, Cox1p, and Cox4p protein levels assessed by western blot in extracts prepared from wild-type, *Δcox4*, *cox4*^{d1}, and *cox4*^{d2} strains.

(B) High-resolution respirometry was performed on the same strains grown in either glucose or lactate to 15 hr postdiauxic shift; a representative data set is presented.

(C) Wild-type, *Δcox4*, *cox4*^{d1}, and *cox4*^{d2}strains were grown in glucose to 15 hr postdiauxic shift and assessed for ROS production by flow cytometry. The percentage of cells exhibiting high ROS levels is displayed.

(D) Mitochondria-enriched cell extracts were prepared and activated as described in the Experimental Procedures before the addition of antimycin A to block electron transport at complex III, and assessment of fold increase in H_2O_2 production was conducted using the Amplex Red assay.

(E) Strains were grown in glucose to 15 hr postdiauxic shift and assessed for ROS production by flow cytometry.

(F) Viability of wild-type and *Δcox4* cells expressing the catalase, Cta1p, was conducted at the same stage of growth postdiauxic shift using a colony-forming assay in biological triplicate.

Error bars represent the mean ± standard deviation. See also Figure S1.

work has implications for the understanding of how mitochondria fit within the signaling processes that control redox balance and have significance for our understanding of healthy aging, age-related disease, and the development of therapeutic antioxidants.

RESULTS

Loss of COX Function Leads to Mitochondrial Dysfunction and ROS Accumulation

As yeast cells exhaust their fermentable carbon source supply, in this case glucose, they enter the diauxic shift during which mitochondria undergo biogenesis and respiratory functions are increased as cells switch from a fermentative to an oxidative metabolism. In concert with this, cells upregulate expression of genes whose products protect against oxidative damage such as catalase and superoxide dismutase (Galdieri et al., 2010). Yeast cells that are unable to respire are placed under extreme mitochondrial stress during the diauxic shift phase of fermentative growth. We examined whether yeast mitochondria with reduced, or a lack of, cytochrome *c* oxidase function produce ROS during the diauxic shift. It has been reported that the levels of COX can be regulated by Cox4p at the level of translation (Su and Dowhan, 2006). We exploited this fact to generate strains that exhibited stable and stepwise reductions in COX function using decreased abundance by mRNA perturbation (DAmP) allele technology (Breslow et al., 2008). Briefly, by extending the 3′UTR region, one can destabilize a specific mRNA which in turn leads to more rapid degradation and a reduction in steady-state protein levels. Using this approach, we generated the strains *cox4*$^{d-1}$ and *cox4*$^{d-2}$, which exhibit approximately 60% and 93% reductions in *COX4* mRNA with respect to wild-type and a *COX4* knockout strain (Figure 1A). This approach led to reductions in protein levels and COX complex formation as both Cox4p and mitochondrial DNA-encoded Cox2p levels were significantly reduced in *cox4*$^{d-1}$ and *cox4*$^{d-2}$ cells (Figure 1A). The respiratory capacity of *cox4*$^{d-1}$ and *cox4*$^{d-2}$ cells and mitochondrial membrane potential were found to decrease accordingly (Figure 1B and see Figure S1A online). ROS production increased in *cox4*$^{d-2}$ cultures and dramatically so when *COX4* was deleted (Figure 1C). We next extracted mitochondria from wild-type, *cox4*$^{d-1}$, and *cox4*$^{d-2}$ and *Δcox4* cells and assessed mitochondrial ROS production capability in vitro using an

Amplex Red assay. Isolated mitochondria were activated by the addition of ADP, malate, pyruvate, and the electron donor succinate. ROS production, assessed by detection of H_2O_2, was conducted in the presence or absence of the complex III inhibitor Antimycin A, which blocks electron transfer and leads to ROS production from mitochondria. Wild-type cells could be induced to give rise to a robust 2-fold increase in H_2O_2 when incubated with Antimycin A (Figure 1D). However, assessment of H_2O_2 levels in *cox4*$^{d-1}$ and *cox4*$^{d-2}$ and *Δcox4* cells demonstrated a clear correlation between reduced respiratory capacity and reduced capability of mitochondria to produced ROS (Figure 1D). As COX loss correlates well with ROS accumulation in vivo, it would seem intuitive that a reduction in the number of electrons passing into the transport chain should reduce ROS levels in cells harboring dysfunctional mitochondria. To test this, we generated a series of double and triple mutant strains that lacked both the *COX4* gene and enzymes that supply electrons to the chain (Figure 1E). We found that the loss of single or multiple complex I or II components had little effect upon the oxidative stress phenotype observed in cells lacking *COX4* (Figure 1E). We also found that the ablation of mitochondrial DNA from wild-type or *Δcox4* cells resulted in the accumulation of high levels of ROS (Figure 1E). In accordance with ROS acting as an accelerant of cell death, the loss viability observed in *Δcox4* cultures could be restored by the overexpression of catalase (Figure 1F). Superoxide levels were increased in *Δcox4* cells when compared to wild-type, and this was further exacerbated by deletion of *SOD1* (Figure S2A). The overexpression of *SOD1* or *SOD2* did not alleviate ROS levels as assessed by DCF fluorescence (Figure S2B), nor did they prevent loss of viability (Figure S2C), as was observed for catalase overexpression (Figure 1F). These data suggest that a reduction in COX activity promotes superoxide production which is further converted to hydrogen peroxide that accumulates to toxic levels. However, the data also suggest that the electron transport activity within mitochondria may not represent the major source.

Loss of COX Function Leads to Activation of Mitochondria-Localized RAS Signaling to Promote ROS Accumulation

It has been proposed that defective mitochondria may accumulate the regulatory GTPase RAS upon their surface (Wang and Deschenes, 2006; Leadsham et al., 2009). To examine whether cells lacking COX function accumulate activated RAS at the mitochondria, we made use of a GFP probe that binds specifically to GTP-bound Ras in yeast cells (Leadsham et al., 2009). During log phase, active Ras was found to localize predominantly at the cell membrane and within the nucleus of both dividing wild-type and *Δcox4* cells accordingly (Figure 2A). During diauxic shift RAS signaling is shut down to facilitate cell-cycle exit and activate stress response mechanisms. As would be expected during this phase of growth, the active Ras probe was found to be diffuse within the cytoplasm of wild-type cells (Figure 2A). However, during this time active RAS was found to colocalize with mitochondria in cells lacking COX function (Figure 2A). We also found that the RAS guanine nucleotide exchange factor (GEF) Cdc25p also became aberrantly localized to the mitochondrial compartment of *Δcox4* cells during diauxic shift (Figure 2B). Analysis of purified mitochondria revealed that RAS accumulates on the outer mitochondrial membrane of *Δcox4* cells during diauxic shift (Figures S1B and S1C). As the loss of *COX4* leads to a loss in mitochondrial membrane potential (Figure S1A), we tested whether this could drive the relocalization of RAS to mitochondria by addition of the proton-ionophore FCCP. Within 2 min of FCCP addition, active RAS could be observed to leave the plasma membrane of wild-type cells and relocalize to the mitochondria (Figure 2C). The deletion of *RAS2* led to a complete rescue of ROS accumulation in *Δcox4* cells (Figure 2D). We also assessed the effects of reduced COX activity upon catalase activity within wild-type, *cox4*$^{d-1}$, and *cox4*$^{d-2}$ and *Δcox4* (Figure 2E). A loss of catalase activity was observed that mirrored the increase in ROS production seen in each of the cell lines. Catalse activity could be restored to cells lacking COX activity by the deletion of *RAS2* (Figure 2E). In addition, the deletion of *RAS2* led to a rescue of a reduction in Sod2p activity (Figure 2F) and loss of viability observed in *Δcox4* cells (Figure 2G).

Ras Operates Independently of PKA or Retrograde Signaling at the Mitochondria to Promote ROS and Requires the Bromodomain Transcriptional Regulator *BRD1*

Ras responds to glucose levels by control of cAMP levels and the activation of three PKA subunits (Thevelein and de Winde, 1999). The deletion of any single or combination of PKA subunits, or overexpression of the cAMP phosphodiesterase *PDE2*, did not alleviate ROS accumulation or restore catalase activity in *Δcox4* cells (Figure 3A and Figure S3A). The loss of *COX4* leads to the activation of the mitochondrial retrograde response during log growth (Miceli et al., 2011). However, while wild-type cells maintained retrograde signaling during diauxic shift, cells lacking *COX4* did not (Figure S3B). Removal of *RTG2* signaling capability did not prevent the accumulation of ROS in cells lacking *COX4* (Figure S3C). Excessive RAS signaling has been shown to suppress genes involved in the regulation of stress that contain stress response (STRE) and postdiauxic shift (PDS) elements via the transcription factors *MSN2/4*, *RIM15*, and *GIS1* (Galdieri et al., 2010). The overexpression of these transcription factors led to reduction in ROS accumulation in *Δcox4* cells (Figure S3A). Ras may also act upon STRE and PDS elements via the regulation of the TOR signaling cascade (Longo et al., 2012). However, we could not ascertain differences in the phosphorylation status of Sch9p between wild-type and *COX4* deletion cells that would suggest aberrant TOR signaling (data not shown). ROS accumulation in COX-deficient cells was also not responsive to TOR pathway suppression by addition of rapamycin (data not shown).

To determine new transcriptional regulators that control the promotion of ROS by RAS via mitochondria-based signaling, we generated rho^0 version of 256 strains knocked out for genes currently annotated as transcriptional regulators. We tested whether their loss would impart rescue the H_2O_2 sensitivity and elevated ROS associated with cells lacking mtDNA. From this screen we found that deletion of *BDF1*, which encodes for a bromodomain-containing TFIID binding protein (Matangkasombut et al., 2000), was sufficient to prevent ROS accumulation in rho^0 zero strains (data not shown). Deletion of *BDF1* in wild-type cells led to an increase in ROS accumulation, while ablation

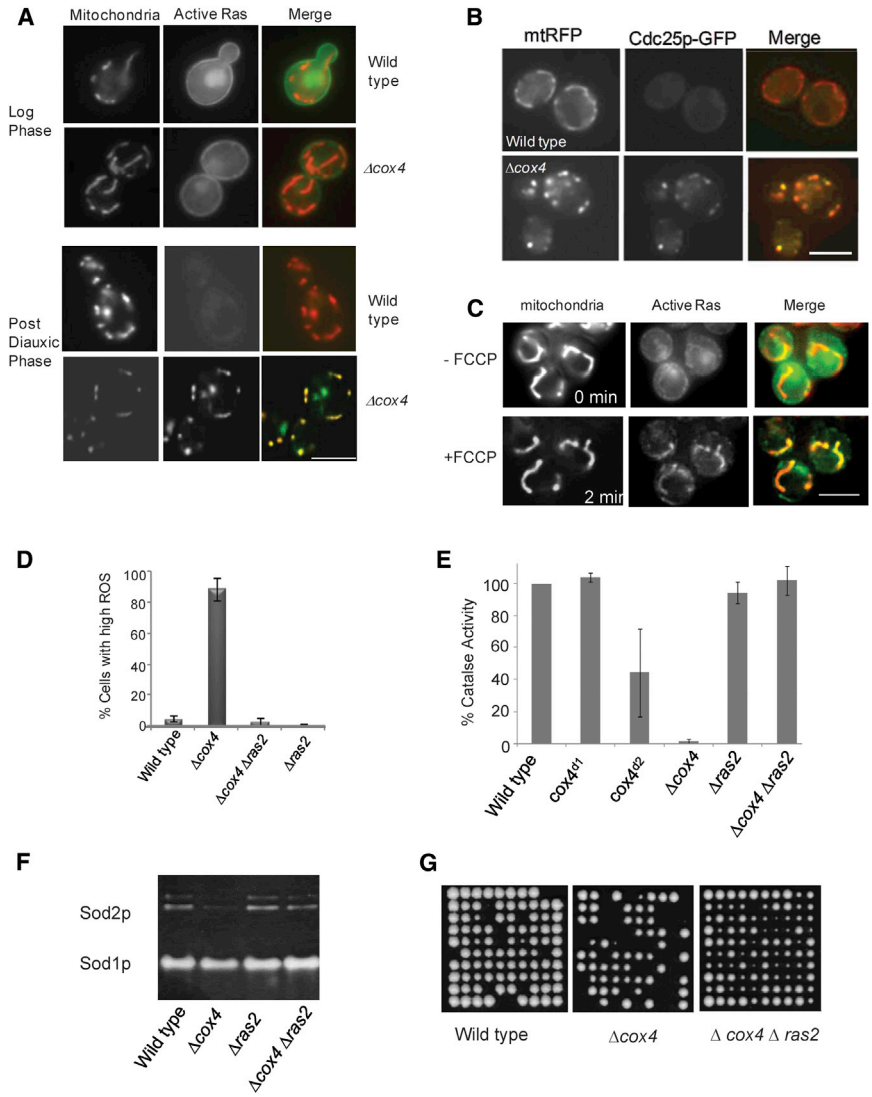

Figure 2. Loss of COX Leads to the Recruitment of Active RAS to Mitochondria that Promotes ROS Accumulation and Cell Death

(A) Active GTP-bound Ras and was visualized using an RBD-GFP reporter and mitochondria with a matrix-targeted RFP within live wild-type and *Δcox4* cells during log and postdiauxic phases of growth.

(B) Cdc25p-GFP was visualized alongside RFP-labeled mitochondria in wild-type and *Δcox4* cells during diauxic shift.

(C) Movement of active Ras during a 2 min treatment with 12 μM FCCP was visualized within a Cellasic microfluidics chamber.

(D) Wild-type, *Δcox4*, *Δras2*, and *Δcox4Δras2* cells were grown in glucose to 15 hr postdiauxic shift and assessed for ROS production by flow cytometry. The percentage of cells exhibiting high ROS levels is displayed.

(E) Catalase activity was assessed in cell extracts prepared from wild-type, *Δcox4*, $cox4^{d1}$ and $cox4^{d2}$, *Δras2*, and *Δcox4Δras2* grown in YPD medium to 15 hr postdiauxic shift as described in the Experimental Procedures.

(F) Superoxide dismutase activity was assessed in extracts prepared from wild-type, *Δcox4*, *Δras2*, and *Δcox4Δras2* on a native polyacrylamide gel as described in the Experimental Procedures.

(G) Viability was assessed in wild-type, *Δcox4*, and *Δcox4Δras2* strains by micromanipulating 100 cells from each culture onto a fresh YPD agar surface and incubating at 30°C for 3 days; representative data set is presented.

Error bars represent the mean ± standard deviation. Scale bar, 10 μm. See also Figure S2.

of this gene in a *Δcox4* background was sufficient to completely prevent ROS accumulation (Figure 3B). Deletion of *BDF1* prevented active-RAS translocation to COX-deficient mitochondria (Figure 3C), restored the full expression of *SOD1* and catalase (Figures S3B and S3C), and led to the degradation of Yno1p in a manner analogous to wild-type or *Δcox4 Δ*ras2 cells (Figure 3D and Figure 4D).

Mitochondria-Based Ras Signaling Suppresses ERAD-Dependent Clearance of the NADPH Oxidase Yno1p and Dampens Antioxidant Capacity to Facilitate ROS Accumulation

We next examined whether a number of nonmitochondrial candidates may be responsible for ROS production in COX-deficient cells: *POX1* (fatty-acyl coenzyme A oxidase, involved in the fatty acid β-oxidation pathway; localized to the peroxisomal matrix), which produces H_2O_2 (Fransen et al., 2012); *ERO1* (thiol oxidase required for oxidative protein folding in the ER), which produces H_2O_2 (Tavender and Bulleid, 2010); *YNO1* (ER localized NADPH oxidase), which produces superoxide (Rinnerthaler et al., 2012); and *ERG11* (ER-localized member of the cytochrome P450 family, a Lanosterol 14-α-demethylase). The knockdown of Erg11p or Ero1p protein levels in *Δcox4* cells did not reduce ROS production (data not shown). A similar result was obtained when we deleted *POX1* in a *Δcox4* background, as ROS levels remained high (Figure 4A). However, the loss of *YNO1* led to a significant reduction in ROS levels in *Δcox4* cells (Figure 4A). Interestingly although ROS were lowered in *Δcox4 Δ*yno1, catalase activity in these cells remained significantly reduced when compared to wild-type cells, also suggesting that Yno1p is required for ROS production in cells lacking COX function (Figure 4B). The deletion of *YNO1* in a *Δcox4* background led to a complete restoration of viability (Figure 4C).

Ras activity has been linked to the control of the activity of the ERAD pathway, which plays a crucial role in ER protein quality control (Umebayashi et al., 2001). As ROS accumulation in cells lacking COX complex activity requires the activity of the ER resident NADPH oxidase Yno1p, we investigated the involvement of ERAD in this mechanism. Yno1p-GFP could be found to localize to ER membranes in dividing cells in wild-type, *Δcox4*, *Δcox4Δras2* cells. The same ER localization could be found in log phase cells lacking *HRD1*, a ubiquitin-protein ligase required

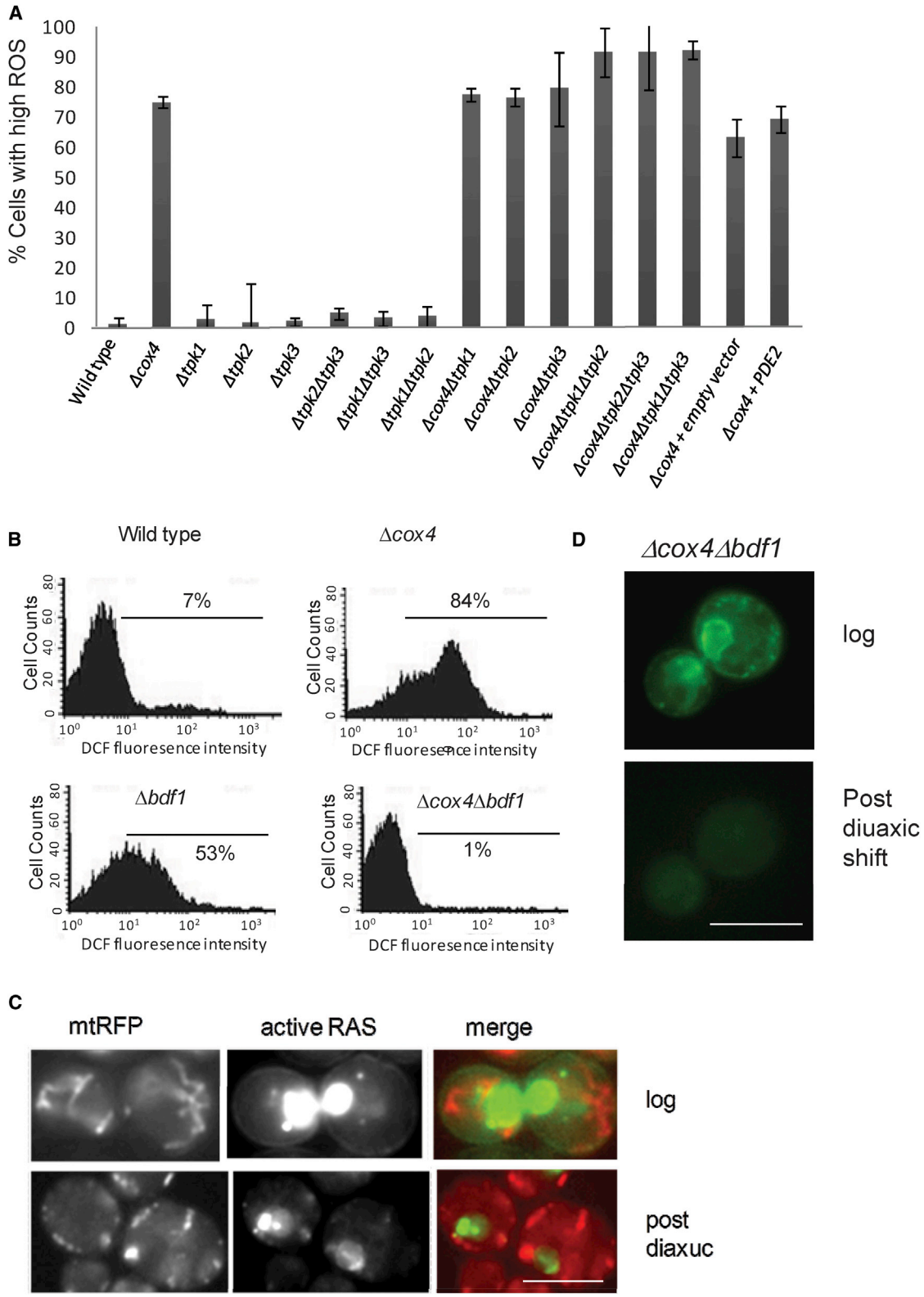

Figure 3. ROS Accumulation in Cells Lacking COX Does Not Utilize cAMP/PKA Signaling but Requires the Bromodomain Protein Brd1p

(A) Strains deleted for *COX4* and either single or double knockouts of PKA subunits *TPK1*, *TPK2*, or *TPK3* or overexpressing *PDE2* were grown in glucose to 15 hr postdiauxic shift and assessed for ROS production by flow cytometry.

(B) ROS accumulation was also assessed in strains lacking *BDF1* or *BDF1* and *COX4*.

(legend continued on next page)

for ERAD function (Figure 3D) (Izawa et al., 2012). During diauxic shift we found that Yno1p-GFP could no longer be detected in wild-type cells, suggesting its downregulation and proteolysis at this stage of growth (Figure 4D). However, a strong ER-localized Yno1p-GFP signal could be observed in *Δcox4* cells during the diauxic shift phase of growth (Figure 4D). The deletion of *RAS2* in *Δcox4* cells led to a loss of Yno1p-GFP, as had been observed for wild-type (Figure 4D). In agreement that Yno1p is a substrate for ERAD activity during the diauxic shift, the *Δhrd1* cells also accumulated Yno1p-GFP (Figure 3D). These data suggest that Yno1p is a target for ERAD-mediated degradation during the diauxic shift phase of growth and that this is suppressed by Ras signaling activity in cells lacking COX activity.

DISCUSSION

The activity of the COX complex has been shown to decline in the human brain (Ojaimi et al., 1999), mouse brain synaptic mitochondria (Martínez et al., 1994), and heart (Paradies et al., 1997) with age. In addition, it has been suggested that the toxic effect of Aβ on COX generates a mitochondrial state that promotes the production of ROS that can be protected against by bolstering mtDNA maintenance (Xu et al., 2009). As aging and degenerative cell populations are often found to contain elevated levels of ROS, it becomes important to identify whether loss of COX activity leads to increased radical generation from the ETC. We find that a reduction of COX complex function beyond 90% correlates with increased ROS levels in vivo. However, our data suggest that although a loss of COX function may trigger ROS production, the increase in radical accumulation rests not with the ETC but with nonmitochondrial sources. We propose that the loss of mitochondrial membrane potential results in RAS localization to the mitochondria, where it signals to inhibit of the ERAD system. This in turn prevents the regulated turnover of the ER-localized NADPH oxidase Yno1p, which produces superoxide inappropriately. Mitochondria-localized RAS suppresses peroxidase capacity, which leads to the buildup of H_2O_2 to toxic levels and accelerates cell death. Our unpublished observations suggest that degradation of unstable respiratory complexes by protease action does not play a role in this process, as deletion of the ATP-dependent Lon protease *PIM1* or AAA ATPases *YTA10*,*YTA12* or *YME1* does not prevent RAS localization to dysfunctional mitochondria or the accumulation of ROS (data not shown). Instead our data suggest that the loss of mitochondrial membrane potential acts as a trigger to relocalize RAS from the plasma membrane to trigger a series of pro-cell-death signaling events. Recent research suggests that unicellular organisms such as yeast possess apoptotic capabilities, and we propose that the localization of RAS to mitochondria lacking membrane potential may represent a mechanism by which yeast cells lacking mitochondrial DNA can be cleared from a population.

A previous study in mammalian cells has demonstrated that K-Ras can become targeted to the mitochondria upon phosphorylation by PKC, where it triggers an apoptotic response (Bivona et al., 2006). Interestingly, a recent large-scale study conducted in yeast identified a number of Ras2p-derived phosphopeptides, suggesting a significant role for kinase activity in its regulation (Bodenmiller et al., 2010). We find that RAS localization to the mitochondria relies on the function of the *BDF1*, a bromodomain-containing protein homologous to human Brd3 that associates with chromatin and the TFIID transcription factor to regulate RNA polymerase II activity (Matangkasombut et al., 2000). It will be of great interest to establish how *BDF1* regulates RAS localization and whether the mitochondrial targeting and phosphocontrol of Ras proteins represent new and conserved signaling mechanisms within eukaryotic cells.

In conclusion, our findings suggest that mitochondria-based Ras signaling in cells lacking COX complex activity leads to a suppression of ERAD activity and the unchecked production of ROS from the NADPH oxidase, Yno1p. We propose that the inappropriate activity of Yno1p in COX-deficient cells, in combination with an abundant cytosolic Sod1p, but absent catalase activity leads to the accumulation of hydrogen peroxide and the acceleration cell death (see the accompanying Graphical Abstract). This scenario highlights the complexity and multifaceted nature of ROS accumulation within living cells. Our findings also suggest that the mitochondrial theory of aging may need to accommodate the role of this organelle as a signaling platform that can coordinate redox homeostasis and ROS levels, and hence the health span of eukaryotic cells.

EXPERIMENTAL PROCEDURES

A full explanation of all methods used can be found within the Supplemental Information.

Yeast Strains, Plasmids, Media, and Growth Conditions

Yeast strains used in this study are listed in Table S2. Unless stated otherwise, cells were grown in a rotary shaker at 30°C in liquid YP medium (1% yeast extract, 2% bactopeptone) containing 2% glucose (YPD). Plasmids and primers used are listed in the Supplemental Information and Table S1.

High-Resolution Respirometry

The protocols used to conduct high-resolution respirometry on intact cells grown in YPD at the indicated stage of cell growth were identical to those previously described (Leadsham and Gourlay, 2010) and detailed within the accompanying the Supplemental Experimental Procedures.

Catalase Activity

Catalase activity was assessed on a total cell lysate, prepared from cells grown to 15 hr postdiauxic shift in YPD using the Amplex Red (AR) catalase assay (Invitrogen).

Extraction and Preparation of Mitochondria

Mitochondrial enriched extracts were isolated from 7.5 × 10^8 cells grown to 15 hr PDS in YPD by differential centrifugation. Highly purified

(C) The percentage of cells exhibiting high ROS levels are displayed in (A) and (B). Active Ras was visualized using an RBD-GFP reporter and mitochondria with a matrix-targeted RFP within cells lacking *BDF1* during the postdiauxic phase of growth.
(D) Yno1p-GFP was visualized in *Δcox4Δbdf1* cells grown to log phase or 15 hr postdiauxic shift.
Error bars represent the mean ± standard deviation. Scale bar, 10 μm. See also Figure S3.

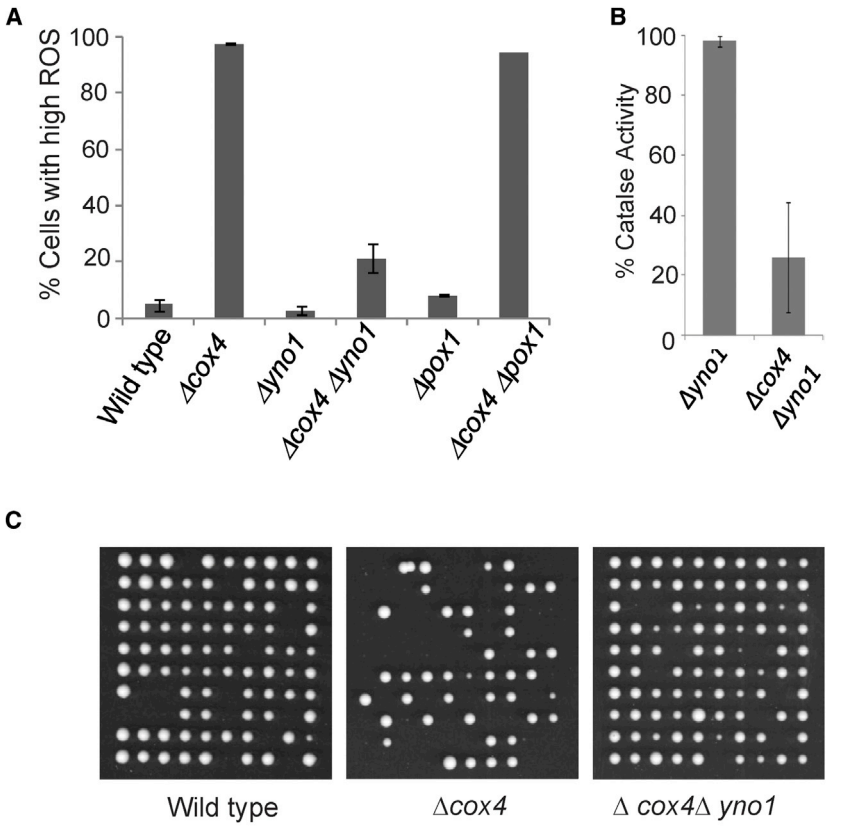

Figure 4. Loss of COX Leads to ROS Production from the ER-Localized NADPH Oxidase Yno1p by the RAS-Mediated Suppression of ERAD

(A) ROS levels were assessed in *Δcox4*, *Δyno1*, *Δcox4Δyno1*, *Δpox1*, and *Δcox4Δpox1* strains. Cells were grown in glucose to 15 hr postdiauxic shift and assessed for ROS production by flow cytometry. The percentage of cells exhibiting a high level of ROS is displayed.

(B) Catalase activity was assessed in extracts prepared from *Δyno1* and *Δcox4Δyno1*strains grown in YPD for 15 hr postdiauxic shift; results are displayed as a percentage of wild-type catalase activity.

(C) Viability was assessed in wild-type, *Δcox4*, and *Δcox4Δyno1* strains by micromanipulating 100 cells from each culture onto a fresh YPD agar surface and incubating at 30°C for 3 days; a representative data set is presented.

(D) Yno1p-GFP was visualized in wild-type, *Δcox4*, and *Δcox4Δras2* and *Δhrd1* cells grown to log phase or 15 hr postdiauxic shift.

Error bars represent the mean ± standard deviation. Scale bar, 10 μm. See also Figure S4.

mitochondria were obtained by centrifugation through a sucrose density gradient. Hydrogen peroxide generation was determined using the Amplex Red (AR) hydrogen peroxide assay (Invitrogen). ROS production from whole cells was assessed using either H_2DCF-DA as previously described (Leadsham and Gourlay 2010) or DHE as previously described (Walter et al., 2010) and fluorescence assessed using a FACS flow cytometer.

Assessment of Cell Viability

Cells were grown to15 hr PDS in YPD and assessed for viability using micromanipulation to array individual cells or by assessment of colony forming units.

Superoxide Dismutase Activity

Cells were grown to15 hr PDS in YPD and SOD activity assayed on nondenaturing 12% polyacrylamide gels by NBT color change.

RTG2 Retrograde Signaling Activity

Retrograde signaling was assessed by the activity of the *CIT2* promoter fused to a LacZ reporter as described in the accompanying the Supplemental Experimental Procedures.

Statistical Analysis

Biological and technical replicates were analyzed for standard deviation, and this is represented in figures presented by inclusion of error bars.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2013.07.005.

ACKNOWLEDGMENTS

This work was supported by a Medical Research Council (MRC) career development grant (78753) to C.W.G.

Received: January 14, 2013
Revised: May 14, 2013
Accepted: June 25, 2013
Published: August 6, 2013

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