Multiple Roles of the Cox20 Chaperone in Assembly of *Saccharomyces cerevisiae* Cytochrome c Oxidase

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ABSTRACT The *Cox2* subunit of *Saccharomyces cerevisiae* cytochrome c oxidase is synthesized in the mitochondrial matrix as a precursor whose leader peptide is rapidly processed by the inner membrane protease following translocation to the intermembrane space. Processing is chaperoned by *Cox20*, an integral inner membrane protein whose hydrophilic domains are located in the intermembrane space, and *Cox20* remains associated with mature, unassembled *Cox2*. The *Cox2* C-tail domain is exported post-translationally by the highly conserved translocase *Cox18* and associated proteins. We have found that *Cox20* is required for efficient export of the *Cox2* C-tail. Furthermore, *Cox20* interacts by co-immune precipitation with *Cox18*, and this interaction requires the presence of *Cox2*. We therefore propose that *Cox20* binding to *Cox2* on the *trans* side of the inner membrane accelerates dissociation of newly exported *Cox2* from the *Cox18* translocase, promoting efficient cycling of the translocase. The requirement for *Cox20* in cytochrome c oxidase assembly and respiratory growth is partially bypassed by *yme1*, *mgr1* or *mgr3* mutations, each of which reduce *i*-AAA protease activity in the intermembrane space. Thus, *Cox20* also appears to stabilize unassembled *Cox2* against degradation by the *i*-AAA protease. Pre-*Cox2* leader peptide processing by *Imp1* occurs in the absence of *Cox20* and *i*-AAA protease activity, but is greatly reduced in efficiency. Under these conditions some mature *Cox2* is assembled into cytochrome c oxidase allowing weak respiratory growth. Thus, the *Cox20* chaperone has important roles in leader peptide processing, C-tail export, and stabilization of *Cox2*.

CYTOCHROME c oxidase is composed of three subunits encoded in the mitochondrial genome (mtDNA) and eight subunits encoded in the nuclear genome in the budding yeast *Saccharomyces cerevisiae*. In addition to the genes for these subunits, at least 20 other nuclear yeast genes are specifically required for synthesis of the mitochondrially coded subunits and post-translational steps in assembly of the active enzyme (Barrientos *et al.* 2002; Herrmann and Funes 2005; Fontanesi *et al.* 2006).

The second largest subunit of cytochrome c oxidase, *Cox2*, is a mitochondrial gene product whose acidic N-terminal and C-terminal domains are translocated through the inner membrane from the matrix to the intermembrane space (IMS) and flank two transmembrane helices (Tsukihara *et al.* 1996). *Cox2* topogenesis is of particular interest since its hydrophilic domains are the largest known to be exported through the inner membrane. In budding yeast, localized membrane-

bound translation of the *COX2* mRNA, specifically activated by *Pet111* (Green-Willms *et al.* 2001; Naithani *et al.* 2003), produces a precursor, pre-*Cox2*, with a short N-terminal leader peptide (Pratje *et al.* 1983). The pre-*Cox2* N-tail is co-translationally exported by *Oxa1* (He and Fox 1997; Hell *et al.* 1998), a highly conserved inner membrane translocase that is also required for C-tail export (reviewed in Bonnefoy *et al.* 2009). Once in the IMS, the pre-*Cox2* leader peptide is rapidly processed by the inner membrane protease (IMP) (Nunnari *et al.* 1993; Jan *et al.* 2000), in a reaction chaperoned by *Cox20* (Hell *et al.* 2000).

The acidic *Cox2* C-tail is exported to the IMS by a mechanism that is distinct from N-tail export and appears to be post-translational (He and Fox 1997; Fiumera *et al.* 2007). C-tail export depends specifically upon another highly conserved inner membrane translocase, *Cox18* (Saracco and Fox 2002), which is paralogously related to *Oxa1*, as well as to bacterial YidC proteins (Funes *et al.* 2004). In addition, *Cox2* C-tail export requires *Mss2* and is promoted by *Pnt1*, two proteins that interact with *Cox18* (He and Fox 1999; Broadley *et al.* 2001; Saracco and Fox 2002). Interestingly, overproduction of *Oxa1* in a mutant lacking *Cox18* results in some export of the *Cox2* C-tail, although *Cox2* remains

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unassembled and the cells fail to respire (Fiumera *et al.* 2009). This result suggested that *Cox18* has an assembly function in the IMS that overproduced *Oxa1* cannot carry out, in addition to its translocation function. One possibility here is that *Cox18* could promote interaction of the exported *Cox2* C-tail with an assembly factor in the IMS. One candidate for such a factor is *Cox20*.

Cox20 has previously been shown to be a 205-amino-acid integral mitochondrial inner membrane protein. It has two centrally located transmembrane helices flanked by hydrophilic domains in the intermembrane space (Hell *et al.* 2000). *Cox20* interacts directly with pre-*Cox2* and promotes its processing. Since this interaction depends upon export of pre-*Cox2* by *Oxa1*, it appears to involve domains in the IMS (Hell *et al.* 2000). In addition, *Cox20* remains associated with unassembled mature *Cox2*, suggesting that it has roles in cytochrome *c* oxidase assembly downstream of pre-*Cox2* processing (Hell *et al.* 2000; Preuss *et al.* 2001; Herrmann and Funes 2005), possibly including *Cox2* metallation (Rigby *et al.* 2008). *Cox20* has therefore been described as a chaperone, although it has no detectable similarity to other well-characterized chaperones or domains of known function.

In this study we have investigated the role of *Cox20* in the export and assembly of *Cox2*. We find that *Cox20* is required for efficient export of the *Cox2* C-tail and that it interacts with the translocase *Cox18* but only when *Cox2* is present. In addition, *Cox20* stabilizes mature but unassembled *Cox2*.

Materials and Methods

Yeast strains and genetic analysis of pseudorevertants

S. cerevisiae strains used in this study are listed in Table 1. All strains are congenic to D273-10B (ATCC 25657). Nuclear genes were manipulated using standard methods (Guthrie and Fink 1991). Transformation of plasmids and PCR products into yeast was accomplished with the EZ transformation kit (Zymo Research). Complete media (YPA) containing adenine, dextrose (D), ethanol plus glycerol (EG), or raffinose (R) were prepared as previously described (Guthrie and Fink 1991). Complete synthetic media (CSM) and CSM lacking specific growth factors were purchased from Bio101 Systems. *COX20* was modified to encode a protein tagged with three MYC epitope at its C terminus, but with no other changes, by the pop-in pop-out strategy as described (Schneider *et al.* 1995). The plasmid pOXA1-W56R-ADH1 (Supekova *et al.* 2010) was obtained from F. Supek and P. G. Schultz

Independent spontaneous pseudorevertants of the *cox20Δ* strain LEE83 were isolated by plating cells from distinct clonal cultures on YPAEG plates and incubating at 30° for 7 to 10 days. A single pseudorevertant was picked from each clonal culture, purified, and mated to *cox20Δ* (SCS194), *cox20Δmgr1Δ* (LEE145), *cox20Δmgr3Δ* (LEE100), and *cox20Δyme1Δ* (LEE106). The resulting diploids were isolated and their ability to grow on YPAEG was assessed to

determine that the mutations were recessive and to score their ability to complement the known genes.

Analysis of mitochondrial proteins

To examine export of the C terminus of *Cox2*, we employed strains whose mtDNA encodes a version of *Cox2* bearing C-terminal HA-epitopes (Saracco and Fox 2002). Mitoplasts were prepared by osmotic shock from purified mitochondria as described (Glick 1995; Glick and Pon 1995). For each sample of mitoplasts, the equivalent of 75 μg of mitochondrial protein was treated with 20 μg/ml proteinase K, or mock treated, as described (Saracco and Fox 2002), except that after protease treatment samples were directly resuspended in 20 mM HEPES pH 7.4, 0.6 M sorbitol, 10% trichloroacetic acid, and 2 mM PMSF and incubated at 60° for 10 min.

For co-immune precipitation experiments, crude mitochondria were prepared as described (Diekert *et al.* 2001). One milligram of mitochondrial protein was solubilized in 1 ml of 1% digitonin, 100 mM NaCl, 20 mM Tris pH 7.4, and 1 mM PMSF for 10 min on ice and then centrifuged at 16,000 × *g* for 10 min at 4°. Five percent of the clarified supernatant was precipitated using Strataclean resin (StrataGene). The remaining supernatant was incubated with anti-hemagglutinin (anti-HA) antibody conjugated beads (Roche). After incubation for 1 hr at 4°, the beads were washed three times in the digitonin buffer before proteins were eluted in 5× Laemmli SDS sample buffer.

Protein samples to be analyzed by Western blotting were separated on 12% or 16% SDS-PAGE gels as indicated and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore). Antibodies were anti-HA monoclonal antibody clone 3F10 (Roche) (except the experiment of Figure 1, where anti-HA-HRP conjugate (Roche) was used), anti-c-myc (anti-Myc) monoclonal antibody clone 9E10 (Roche), polyclonal anti-*Cit1* (citrate synthase), polyclonal anti-*Yme1*, and monoclonal anti-*Cox2* CCO6. Anti-mouse IgG-HRP (BioRad) or anti-rabbit IgG-HRP (BioRad) secondary antibodies were applied after washing and detected by the ECL and ECL+ detection methods (GE Healthcare).

In vivo pulse-labeling of mitochondrial translation products was carried out as described (Bonney *et al.* 2001) in cells grown on YPAR medium, except that there was no chase incubation, and mitochondria were prepared as described (Diekert *et al.* 2001). Radiolabeled mitochondrial proteins were separated on 16% SDS PAGE gels, which were dried and autoradiographed.

Results

Cox20 is required for Cox2 C-tail export independently of cytochrome c oxidase assembly or Cox2 N-tail processing

Cox20 is known to be required for the N-terminal processing of pre-*Cox2* and to remain associated with mature *Cox2* prior to its assembly into cytochrome *c* oxidase (Hell *et al.* 2000). Before *Cox2* can be assembled, the C-tail must be

Table 1 Strains and plasmids used in this study

Name	Nuclear (mitochondrial) genotype	Source
CAB116	<i>MATa ura3-53 leu2-3,112 lys2 his3ΔHindIII arg8::hisG cox18Δ::KanMX4</i> (p ⁺)	This study
DFS188	<i>MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG</i> (p ⁺)	Steele et al. (1996)
LEE80	<i>MATa ura3-52 leu2-3,112 lys2 his3ΔHindIII arg8::hisG COX20::3xMYC</i> (p ⁺)	This study
LEE83	<i>MATα lys2 ade2 cox20Δ::URA3</i> (p ⁺)	This study
LEE92	<i>MATa ura3-52 leu2-3,112 lys2 his3ΔHindIII COX20::3xMYC COX18::3xHA</i> (p ⁺)	This study
LEE98	<i>MATα leu2-3,112 lys2 his3ΔHindIII cox20Δ::URA3 mgr1Δ::KanMX4</i> (p ⁺)	This study
LEE99	<i>MATα lys2 his3ΔHindIII cox20Δ::URA3 mrg1Δ::KanMX4</i> (p ⁺)	This study
LEE105	<i>MATα leu2-3,112 cox20Δ::URA3 yme1Δ::URA3</i> (p ⁺)	This study
LEE112	<i>MATa ura3-52 leu2-3,112 lys2 his3ΔHindIII arg8::hisG COX20::3xMYC COX18::3xHA</i> (p ⁺ cox2-20)	This study
LEE121	<i>MATa ura3-52 leu2-3,112 lys3 his3ΔHindIII arg8::hisG pOXA1-W56R-ADH1</i> (p ⁺ cox2-10)	This study
LEE122	<i>MATa ura3-52 leu2-3,112 lys2 his3ΔHindIII arg8::hisG cox18Δ::KanMX4 pOXA1-W56R-ADH1</i> (p ⁺)	This study
LEE123	<i>MATα ura3 ade2 oxa1Δ::LEU2 pOXA1-W56R-ADH1</i> (p ⁺)	This study
LEE126	<i>MATa ura3-52 leu2-3,112 pet111-9 pOXA1-W56R-ADH1</i> (p ⁺)	This study
LEE128	<i>MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG imp1Δ::KanMX4 pOXA1-W56R-ADH1</i> (p ⁺)	This study
LEE139	<i>MATa leu2-3,112 lys2 arg8::hisG ade2 trp1-1 cox20Δ::URA3 yme1Δ::URA3 imp1Δ::KanMX4</i> (p ⁺)	This study
LEE145	<i>MATa leu2-3,112 lys2 ade2 cox20Δ::URA3 mgr1Δ::KanMX4</i> (p ⁺)	This study
LEE173	<i>MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG cox20Δ::KanMX4</i> (p ⁺)	This study
LEE174	<i>MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG cox20Δ::KanMX4 pOXA1-W56R-ADH1</i> (p ⁺)	This study
MCC319	<i>MATα ura3 ade2 oxa1Δ::LEU2</i> (p ⁺)	This study
NB40-3C	<i>MATa ura3-52 leu2-3,112 lys2 his3ΔHindIII arg8::hisG</i> (p ⁺ cox2-62)	Bonnefoy and Fox (2000)
NB60	<i>MATa ura3-52 leu2-3,112 lys3 his3ΔHindIII arg8::hisG</i> (p ⁺ cox2-10)	Bonnefoy and Fox (2000)
NB151-9B	<i>MATa ura3-52 leu2-3,112 pet111-9</i> (p ⁺)	This study
SCS101	<i>MATa ura3-2 leu2-3,112 lys2 his3Δ arg8::hisG</i> (p ⁺ COX2::3xHA)	This study
SCS114	<i>MATa ura3-52 leu2-3,112 his3Δ arg8::hisG</i> (p ⁺ cox2-N15I::3xHA)	This study
SCS182	<i>MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG cox20Δ::URA3</i> (p ⁺ COX2::3xHA)	This study
SCS192	<i>MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG</i> (p ⁺ COX2::3xHA cox3Δ::ARG8 ^m)	This study
SCS193	<i>MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG imp1Δ::KanMX4</i> (p ⁺)	Fiori et al. (2003)
SCS218	<i>MATa ura3-52 leu2-3,112 lys2 his3Δ arg8::hisG</i> (p ⁺ COX2::3xHA cox1Δ::ARG8 ^m)	This study

translocated from the matrix to the intermembrane space. We asked whether *Cox20* plays a role in the export of the *Cox2* C-tail. Mitochondria were purified from strains whose mtDNA encodes a variant of *Cox2* with three HA-epitope tags at its C terminus and then converted to mitoplasts lacking the outer membrane. This epitope is accessible to protease added to wild-type mitoplasts, whose *Cox2* C-tail has been translocated through the inner membrane (Saracco and Fox 2002; Fiumera et al. 2007) (Figure 1). Protease digestion of mitoplasts from a *cox20Δ* strain did not destroy the C-terminal epitope, but did shorten the tagged protein by digestion of the exported *Cox2* N-tail domain (Figure 1). The C-tail epitope was digested by protease when the mitoplasts were solubilized by the addition of the mild detergent octyl glucoside. Thus, *Cox20* is required for export of the *Cox2* C-tail domain through the inner membrane, but not for the mechanistically distinct (He and Fox 1997) N-tail export process.

Hell et al. (2000) had previously observed a defect in *Cox2* C-tail export in the *cox20Δ* mutant. However, they attributed this effect to decreased inner membrane potential caused by the lack of cytochrome *c* oxidase in the mutant. We investigated this possibility further by examining the topology of the HA epitope in strains lacking either *Cox1* or *Cox3*, the other two core subunits of cytochrome *c* oxidase. In both cases the C-terminal epitope on *Cox2* was accessible to protease in mitoplasts from the mutants (Figure 1), confirming that *Cox2* C-tail export is not prevented

indirectly by the absence of cytochrome *c* oxidase activity (Saracco and Fox 2002).

Since *Cox20* is required for N-terminal processing of the pre-*Cox2* leader peptide, we next asked whether this processing is required for C-tail export. Pre-*Cox2* is processed by cleavage after residue 15 by the *Imp1* subunit of the inner membrane protease complex (Pratje et al. 1983; Behrens et al. 1991; Nunnari et al. 1993). Neither the *cox2-N15I* processing site mutation (Saracco 2003; Perez-Martinez et al. 2009) nor the *imp1Δ* mutation, both of which prevent pre-*Cox2* leader peptide processing, prevented *Cox2* C-tail

Figure 1 Export of the C terminus of pre-*Cox2* is dependent on *Cox20*, but not on N-terminal processing or cytochrome *c* oxidase assembly. Mitoplasts (MP) from strains expressing *Cox2* with a C-terminal HA tag were either mock treated or treated with 20 μg/ml of proteinase K (pK) (Materials and Methods) as indicated. Samples were solubilized with 1% octyl glucoside (OG) prior to protease treatment where indicated. For wild type, 5 μg of proteins were loaded per lane, and for the mutants, 50 μg of protein were loaded per lane. The resulting blots were probed with α-HA, α-Cit1, and α-Yme1 antibodies and developed with the ECL detection system (Invitrogen). Strains are indicated as follows: WT (SCS101), *cox2-N15I* (SCS114), *imp1Δ* (SCS184), *cox20Δ* (SCS182), *cox1Δ* (SCS218), and *cox3Δ* (SCS192).

Figure 2 Co-immune precipitation of Cox20-Myc with Cox18-HA is dependent on the presence of Cox2. Mitochondria were prepared from strains LEE80 (COX20::Myc), LEE92 (COX20::Myc COX18::HA), and LEE112 (COX20::Myc COX18::HA [*cox2-20*]), solubilized in buffer containing 1% digitonin, and subjected to immune precipitation with anti-HA beads (Materials and Methods). Five percent of total protein from each extract (Total), and the immune precipitates (α -HA IP) were subjected to SDS-PAGE on a 12% acrylamide gel and Western blotting. The blots were decorated with anti-Myc antibody, stripped, and then redecorated with anti-HA antibody. The presence or absence of Cox20-Myc, Cox18-HA, and Cox2 proteins in the extracts are indicated by + and -.

export as measured by protease sensitivity of the Cox2 C-terminal HA epitopes in mitoplasts (Figure 1). (While some HA-reactive Cox2 was detected in the *imp1Δ* mitoplasts treated with protease, this is largely attributable to inefficient mitoplasting, as evidenced by the partial protection of the IMS marker Yme1.)

Taken together, these data show that Cox20 has a role in the export of the Cox2 C-tail that is independent of the requirements for Cox20 in pre-Cox2 processing and cytochrome *c* oxidase assembly.

Cox20 interacts dynamically with Cox18 in a Cox2-dependent manner

Cox18 is the inner membrane translocase responsible for moving the Cox2 C-tail into the intermembrane space (Saracco and Fox 2002; Bonnefoy *et al.* 2009). To further investigate the role of Cox20 in translocation, we tested for physical interaction between Cox18 and Cox20 by co-immune precipitation. Mitochondria were isolated from cells containing epitope-tagged Cox20-Myc and either tagged Cox18-HA or wild-type Cox18. Digitonin solubilized extracts were immunoprecipitated with anti-HA antibody bound to agarose beads, and the precipitates were analyzed by Western blotting (Materials and Methods). Cox20-Myc coprecipitated with Cox18-HA but was not precipitated from extracts containing untagged Cox18 (Figure 2).

Cox20 and Cox18 could either interact in a stable complex or could interact dynamically during the translocation of the Cox2 C-tail. To distinguish between these possibilities we asked whether Cox20-Myc and Cox18-HA co-immune precipitation depends on the presence of Cox2. Cox2 translation was prevented by introducing the *cox2-20* mutation (Torello *et al.* 1997; Bonnefoy *et al.* 2001) into the mtDNA of a strain containing Cox20-Myc and Cox18-HA. A mitochondrial extract from this strain was analyzed as described above, revealing that the interaction between Cox20 and Cox18 was disrupted when Cox2 synthesis was blocked

Figure 3 Loss of *i*-AAA protease activity due to *yme1*, *mgr1*, or *mgr3* mutations allows weak respiratory growth of *cox20Δ* cells. Cells grown in rich glucose medium were diluted and spotted onto YPAEG (Materials and Methods) nonfermentable ethanol plus glycerol medium (Eth + Gly), incubated at 30° for 14 days, spotted onto YPAD (Materials and Methods) glucose (Gluc), and incubated at 30° for 4 days. Strains are indicated as follows: WT (DFS188), *cox20Δ* (LEE83), *cox20Δmgr1Δ* (LEE99), *cox20Δmgr3Δ* (LEE98), *cox20Δyme1Δ* (LEE105), and *cox20Δyme1Δimp1Δ* (LEE139).

(Figure 2). Thus, mitochondrially coded Cox2 appears to bridge the interaction between the translocase Cox18 and the chaperone-like protein Cox20.

The absence of Cox20 can be partially bypassed by disruption of the *i*-AAA protease complex

Cox20 is required for respiratory growth (Figure 3, Hell *et al.* 2000). However, *cox20Δ* mutant cells plated on non-fermentable medium yielded spontaneous weakly respiring pseudorevertants. We suspected that these mutations might affect the activity of the *i*-AAA protease, on the basis of a previous study of mutations that allow overproduced Oxa1 to partially suppress a *cox18Δ* mutation (Fiumera *et al.* 2009). Since Cox20 and Cox18 appear to function together, we tested the phenotype of double mutants containing the *cox20Δ* mutation and *mgr1Δ*, *mgr3Δ*, or *yme1Δ* (Figure 3). In a *cox20Δ* background, deletion of *YME1*, *MGR1*, or *MGR3* caused weak respiratory growth (Figure 3). However, this weak respiratory growth was dependent upon *IMP1*.

We found that 36 independent spontaneous *cox20Δ* pseudorevertants (Materials and Methods) contained recessive nuclear mutations, since they yielded nonrespiring diploids when mated with an otherwise wild-type *cox20Δ* strain. Twenty-three of these pseudorevertants appear to be due to *mgr3* mutations since they produced respiring diploids when mated to *cox20Δ mgr3Δ* strain, indicating failure to complement, but nonrespiring diploids when mated to either *cox20Δ mgr1Δ* or *cox20Δ yme1Δ* strains. Tetrad analysis confirmed that five of these pseudorevertants were indeed caused by mutations tightly linked to *mgr3Δ*. Ten of the remaining spontaneous pseudorevertants failed to complement after being mated with a *cox20Δ mgr1Δ*. The remaining three pseudorevertants produced nonrespiring diploids when mated to *cox20Δ* double mutant strains containing *mgr1Δ*, *mgr3Δ*, or *yme1Δ* mutations, indicating that they complemented all three. The spontaneous mutations in these three pseudorevertants fall into a single complementation group and are linked to each other, indicating that

Figure 4 Pre-Cox2 is processed by Imp1 in the absence of *i*-AAA protease activity, but inefficiently. (A) Crude mitochondrial protein samples (Diekert *et al.* 2001) in the amounts indicated were separated on 16% SDS-PAGE and Western blotted. The blot was decorated with both antibody specific to the C-tail of Cox2 and antibody specific against Cit1, as a loading control. Strains are indicated as follows: WT (DFS188), *cox20Δ* (LEE83), *cox2Δ* (NB40-3C), *cox20Δ mgr1Δ* (LEE99), *cox20Δ mgr3Δ* (LEE98), *cox20Δ yme1Δ* (LEE105), and *cox20Δ yme1Δ imp1Δ* (LEE139). pCox2 indicates pre-Cox2; mCox2 indicates mature Cox2; * indicates a novel Cox2 cross-reacting species. (B) Cells were labeled *in vivo* with [³⁵S]methionine in the presence of cycloheximide for 20 min at 30° (Materials and Methods). Mitochondria were analyzed by SDS-PAGE (16% acrylamide gel) and autoradiography. Strains are indicated as follows: WT (DFS188), *cox20Δ* (LEE83), *cox2Δ* (NB40-3C), *cox20Δ mgr1Δ* (LEE99), *cox20Δ mgr3Δ* (LEE98), *cox20Δ yme1Δ* (LEE105), and *cox20Δ yme1Δ imp1Δ* (LEE139). The identities of major mitochondrial translation products are indicated.

they affect a single, as-yet unidentified, additional gene. This screen is apparently not saturated, since none of the pseudorevertants contained mutations that failed to complement *yme1Δ*.

Imp1-dependent processing of pre-Cox2 in *cox20Δ* mutants occurs in the absence of *i*-AAA protease activity

Processing of the pre-Cox2 N-terminal leader peptide is essential for assembly of cytochrome c oxidase, since mitochondrial mutations that alter the processing site cause a nonrespiratory growth phenotype in strains with wild-type nuclear genomes (Bonney *et al.* 2001; Saracco 2003; Perez-Martinez *et al.* 2009). The fact that one function of Cox20 is to assist in this Imp1-dependent processing event (Hell *et al.* 2000) raised the question of how the pre-Cox2 N terminus is processed in *cox20Δ* strains lacking *i*-AAA protease activity. We first confirmed that mature Cox2 does accumulate at steady state in the weakly respiring *cox20Δ mgr1Δ*, *cox20Δ mgr3Δ*, and *cox20Δ yme1Δ* double mutants by Western blotting of mitochondrial proteins probed with an anti-Cox2 antibody. While processing was much less efficient in the double mutants than in wild type, each of the weakly respiring double mutants contained significant amounts of mature Cox2 (Figure 4A). The generation of mature Cox2 under these conditions is still dependent upon Imp1 activity since a *cox20Δ yme1Δ imp1Δ* triple mutant, which has a nonrespiratory growth phenotype (Figure 3), accumulated the same pre-Cox2 species as an *imp1Δ* single mutant (Figure 4A).

All of the strains deficient in *i*-AAA protease activity also accumulated detectable amounts of a smaller Cox2 species (indicated by * in Figure 4A). Since the *i*-AAA protease is known to be responsible for degradation of unassembled Cox2 (Nakai *et al.* 1995; Pearce and Sherman 1995; Weber *et al.* 1996), the smaller Cox2 species appears to be a breakdown product of pre-Cox2 that accumulates in the absence of *i*-AAA activity. Interestingly, the *cox20Δ* mutant accumu-

lated substantially less pre-Cox2 than either the *imp1Δ* mutant or any of the *cox20Δ* strains deficient in *i*-AAA protease (Figure 4). These data suggest that Cox20 directly stabilizes pre-Cox2 against degradation by the *i*-AAA protease. Alternatively, pre-Cox2 whose C-tail has not been exported may be more labile due to its aberrant topology.

In contrast to the accumulation of immunologically detectable mature Cox2 in weakly respiring *cox20Δ* mutants deficient in *i*-AAA protease, processing of pre-Cox2 in the absence of Cox20 was not observed during ³⁵S-pulse labeling of mitochondrial translation products in the same strains (Figure 4B). Thus, while Imp1-dependent processing of pre-Cox2 occurs in the double mutants (Figure 4A), it is too slow to be detectable during pulse labeling. These data are consistent with the proposal that Cox20 serves as a chaperone to present pre-Cox2 to the inner membrane protease complex, but is not a component of that complex (Hell *et al.* 2000).

Import of a variant form of pre-Cox2 from the cytoplasm does not bypass the requirements for Cox20 and Imp1 in cytochrome c oxidase biogenesis

We attempted to dissect further the activities of Cox20 using a plasmid-borne mutant version of the COX2 gene, that had been recoded for expression from the nucleus. This recoded COX2 gene (denoted N-COX2 in Figure 5), specifying the amino acid substitution W56R and the mitochondrial targeting signal of the Oxa1 protein, was obtained on a high-copy plasmid termed pOXA1-W56R-ADH1 (Supekova *et al.* 2010). We transformed this plasmid into the nuclei of mutant strains (Figure 5). As expected, it strongly complemented the respiratory growth defect of a *cox2Δ* mutation in mtDNA. Furthermore, import of the nuclearly encoded, cytoplasmically synthesized variant of Cox2 also complemented the respiratory defect of a nuclear *pet111Δ* mutant that is specifically unable to translate the mitochondrial COX2 mRNA (Figure 5). In addition, import of this protein bypassed the requirement for the Cox2 C-tail translocase

Cox18, suggesting that the assembly pathway for the imported variant of *Cox2* does not involve import into the matrix and subsequent reexport of the C-tail. However, import of this protein in the absence of *Cox20*, *Imp1*, or *Oxa1* did not promote respiratory growth (Figure 5) or the assembly of active cytochrome *c* oxidase as assayed by reduction of tetramethyl-*p*-phenylenediamine (Mcewen *et al.* 1985; unpublished data).

Discussion

The experiments presented here argue strongly that *Cox20* has an important role in *Cox2* C-tail topogenesis, in addition to its previously established function in pre-*Cox2* leader peptide processing. First, in the absence of *Cox20*, mitochondrially synthesized unassembled pre-*Cox2* accumulates with its N-tail domain in the IMS and its C-tail domain in the matrix, showing that *Cox20* is required for efficient C-tail translocation through the inner membrane. Second, this defect in *Cox2* C-tail export cannot be due to the defect in pre-*Cox2* processing caused by the absence of *Cox20*, since C-tail export was not affected by either *cox2* or *imp1* mutations, which also prevent pre-*Cox2* processing. Finally, *Cox20* co-immune precipitates with the *Cox2* C-tail-specific translocator *Cox18* from mitochondria synthesizing pre-*Cox2*, but not from mitochondria lacking a functional *COX2* gene. Thus, the mitochondrial gene product *Cox2* appears to bridge a dynamic interaction between *Cox20* and *Cox18*.

These data are consistent with the findings of Hell *et al.* (2000), who reported that a significant fraction of the *Cox2* C-tail, newly synthesized in isolated *cox20Δ* mitochondria, remained on the matrix side of the inner membrane after pulse labeling, while the N-tail was efficiently exported. Our findings that the vast majority of *Cox2* molecules accumulated in a *cox20Δ* mutant have an N-out, C-in topology, while both N- and C-tails of *Cox2* are efficiently exported in other mutants that lack cytochrome *c* oxidase activity, argue that the export defect in *cox20Δ* mitochondria is not a secondary effect due to reduced membrane potential, as previously suggested (Hell *et al.* 2000).

On the basis of these results, we propose that *Cox20* binding to *Cox2* accelerates dissociation of newly exported *Cox2* from the *Cox18* translocase on the IMS side of the inner membrane. Thus, while not directly involved in membrane translocation *per se*, *Cox20* is required for efficient cycling of the *Cox18* translocase.

Unassembled *Cox2* is largely degraded by the *i*-AAA protease *Yme1* (Nakai *et al.* 1995; Pearce and Sherman 1995; Weber *et al.* 1996; Graef *et al.* 2007), which is bound to the inner membrane facing the IMS (Leonhard *et al.* 1996). *Yme1* is associated with two other proteins, *Mgr1* and *Mgr3*, that recognize and deliver at least some substrates for degradation by *Yme1* (Dunn *et al.* 2006; Dunn *et al.* 2008). A study of interspecies *Yme1* chimeras comprising combinations of homologous domains of the *S. cerevisiae*

Figure 5 Expression of a recoded nuclear *COX2* gene bypasses the requirement for *Cox18* but not *Cox20*. Cells containing the plasmid pOXA1-W56R-ADH1 (Supekova *et al.* 2010) were grown in CSM-Ura, and untransformed cells were grown in CSM. Cultures were diluted and spotted onto YEAEG nonfermentable (Eth + Gly) plates and incubated at 30° for 4 days or spotted onto YPAD fermentable (Gluc) plates and incubated at 30° for 2 days. Strains are indicated as follows: WT (DFS188), *cox2-60* (NB60), *cox2-60 N-COX2* (LEE121), *pet111Δ* (NB151-9B), *pet111Δ N-COX2* (LEE126), *cox18Δ* (CAB116), *cox18Δ N-COX2* (LEE122), *oxa1Δ* (MCC319), *oxa1Δ N-COX2* (LEE123), *imp1Δ* (SCS193), *imp1Δ N-COX2* (LEE128), *cox20Δ* (LEE173), *cox20Δ N-COX2* (LEE174).

and *Neurospora crassa* proteins identified *Cox20* as a factor influencing the pathways by which unassembled *Cox2* substrate could reach the *i*-AAA proteolytic domain (Graef *et al.* 2007).

Our results strongly suggest that a third critical chaperone function of *Cox20* is to protect as-yet-unassembled *Cox2* from degradation by the *i*-AAA protease complex during the assembly process downstream of export. Furthermore, they indicate a strong dependence of *Cox2* degradation upon the putative substrate recognition factors *Mgr1* and *Mgr3*, at least in the absence of *Cox20*. In cells lacking *Cox20*, the steady-state level of accumulated pre-*Cox2* was extremely low, and those cells exhibited a tight nonrespiratory phenotype. However, in cells lacking *Cox20* as well *Yme1*, *Mgr1*, or *Mgr3*, the steady-state levels of pre-*Cox2* and mature *Cox2* were increased, and those cells exhibited weak respiratory growth reflecting the assembly of low levels of cytochrome *c* oxidase. Consistent with these findings, Hell *et al.*

(2000) observed that virtually all residual unassembled pre-Cox2 or mature Cox2 was associated with Cox20 when cytochrome *c* oxidase assembly was blocked by either an *imp1* Δ or a *cox4* Δ mutation, respectively. The C-terminal domain of Cox20 is basic, which may facilitate its interaction with the acidic N- and C-tails of Cox2 following their export to the IMS.

Cox20 is required for normal rates of pre-Cox2 processing by the Imp1 subunit of the inner membrane protease. However, slow Imp1-dependent processing does occur in the absence of Cox20 if pre-Cox2 is stabilized by loss of the *i*-AAA protease. In contrast, the requirement for Imp1 is not bypassed by elimination of the *i*-AAA protease.

Although Cox20 is also required for normal export of the Cox2 C-tail domain, low levels of export and assembly into cytochrome *c* oxidase are detectable in the absence of Cox20 if *i*-AAA activity is also absent. On the basis of the model that Cox20 promotes dissociation of exported Cox2 C-tail from the Cox18 translocase, we suggest that in the absence of Cox20 the *i*-AAA protease rapidly degrades unprotected Cox2 C-tail that is associated with Cox18. In the absence of both Cox20 and the *i*-AAA protease, slow dissociation of exported Cox2 from Cox18 can occur and leads to limited cytochrome *c* oxidase assembly. Consistent with these ideas, we have found that respiratory growth of a *cox20* Δ *yme1* Δ double mutant is improved by overproduction of Cox18 (unpublished results).

Supekova *et al.* (2010) recently described a recoded and modified version of the *COX2* gene that supports respiratory growth when allotopically expressed from the yeast nucleus in a *cox2* Δ mutant. As expected, this gene, termed here *N-COX2*, bypassed the requirement for Pet111, which normally activates translation of the *COX2* mRNA inside mitochondria. Interestingly, it also bypassed the requirement for the Cox2 C-tail-specific export translocase Cox18. This finding suggests that the cytoplasmically synthesized C-tail domain of allotopically expressed pre-Cox2 remains in the intermembrane space during import into mitochondria, rendering Cox18 superfluous. However, *N-COX2* did not bypass the requirement for Cox20. Since expression of *N-COX2* required Imp1, Cox20 is presumably required to chaperone the cleavage of imported pre-Cox2 by the inner membrane protease. Cox20 may also be required for stabilization of unassembled imported Cox2, and possibly chaperoning its further assembly.

Previous work in this laboratory has shown that overproduced Oxa1 can partially bypass the requirement for Cox18 in export of the Cox2 C-tail domain, but not the requirement for Cox18 in cytochrome *c* oxidase assembly (Fiumera *et al.* 2009). This observation suggested that in addition to translocating the Cox2 C-tail through the inner membrane, Cox18 normally delivers the C-tail in a fashion promoting assembly, possibly by interacting with downstream assembly factors. This study supports this idea, and indicates that Cox20 may be an assembly factor that can acquire the exported Cox2 C-tail from Cox18, but not from

overproduced Oxa1. (On the basis of the experiments with *N-COX2*, we postulate that Cox20 can interact with the Cox2 C-tail imported from the cytoplasm in the absence of Cox18.) Interestingly, *mgr1* Δ or *mgr3* Δ mutations, but not *yme1* Δ , allowed overproduced Oxa1 to promote weak cytochrome *c* oxidase assembly and respiratory growth in the absence of Cox18. These findings suggested that in the absence of the substrate recognition factors Mgr1 and/or Mgr3, the AAA+ protein Yme1 could function as a chaperone for Oxa1-exported Cox2, instead of degrading it (Fiumera *et al.* 2009). In light of these results, it is tempting to speculate that the role of Cox20 *vis-à-vis* the Cox18 translocase is carried out by latent Yme1 chaperone activity when the Cox2 C-tail is aberrantly exported by overproduced Oxa1.

Cox18 is highly conserved. Indeed the homologous proteins from humans (Gaisne and Bonnefoy 2006), fission yeast (Gaisne and Bonnefoy 2006), *N. crassa* (Funes *et al.* 2004), and *E. coli* (Preuss *et al.* 2005) can partially complement a *cox18* Δ in *S. cerevisiae* at the level of respiratory growth. A putative Cox20 homolog (NCBI NP_932342.1) has also been identified in humans (Herrmann and Funes 2005). A mammalian ortholog of Cox20 could not have a role in Cox2 processing since mammalian Cox2 proteins are not synthesized as precursors (Steffens and Buse 1979; Anderson *et al.* 1982). However, a mammalian Cox20 protein could participate with Cox18 in Cox2 C-tail export as well as Cox2 stabilization and assembly into cytochrome *c* oxidase.

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