Identification of Genes Required for Alternative Oxidase Production in the Neurospora crassa Gene Knockout Library

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ABSTRACT
The alternative oxidase (AOX) of Neurospora crassa transfers electrons from ubiquinol to oxygen. The enzyme is not expressed under normal conditions. However, when the function of the standard electron transport chain is compromised, AOX is induced, providing cells with a means to continue respiration and growth. Induction of the enzyme represents a form of retrograde regulation because AOX is encoded by a nuclear gene that responds to signals produced from inefficiently functioning mitochondria. To identify genes required for AOX expression, we have screened the N. crassa gene knockout library for strains that are unable to grow in the presence of antimycin A, an inhibitor of complex III of the standard electron transport chain. From the 7800 strains containing knockouts of different genes, we identified 62 strains that have reduced levels of AOX when grown under conditions known to induce the enzyme. Some strains have virtually no AOX, whereas others have only a slight reduction of the protein. A broad range of seemingly unrelated functions are represented in the knockouts. For example, we identified transcription factors, kinases, the mitochondrial import receptor Tom70, three subunits of the COP9 signalosome, a monothiol glutaredoxin, and several hypothetical proteins as being required for wild-type levels of AOX production. Our results suggest that defects in many signaling or metabolic pathways have a negative effect on AOX expression and imply that complex systems control production of the enzyme.

KEYWORDS
alternative oxidase knockout library Neurospora crassa mitochondria

Although the proper formation of mitochondria requires the expression of genes found in both the nuclear and mitochondrial genomes, the vast majority of mitochondrial proteins are encoded in the nucleus. Thus, communication of mitochondrial status to the nucleus must occur to insure that the organelles meet the requirements for growth and development of the organism and to insure that they can properly respond to changing conditions and stress. Communication from the mitochondria to the nucleus that influences the expression of nuclear genes has been termed retrograde regulation (Jazwinski and Kriete 2012; Liu and Butow 2006; Woodson and Chory 2008).

The alternative oxidase (AOX) is a di-iron carboxylate protein that transfers electrons from ubiquinol to molecular oxygen and exists in the mitochondrial inner membrane (Albury et al. 2009; Andersson and Nordlund 1999; Berthold et al. 2000; Berthold and Stenmark 2003). It is found in a variety of organisms, including bacteria, protists, fungi, plants, and animals—but not mammals (McDonald 2008; McDonald and Vanlerberghe 2006). Depending on the organism, expression of AOX can be influenced by developmental signals, tissue specificity, and response to stress (Considine et al. 2001; Djajanegara et al. 2002; Finnean et al. 1997; Karpova et al. 2002; Nargang and Kennell 2010; Van Aken et al. 2009; Vanlerberghe and McIntosh 1997). In many organisms, AOX occurs at low-to-undetectable levels under normal growth conditions but becomes highly expressed when the standard, cytochrome-mediated, electron transport chain (sETC) is compromised. Thus, because AOX is encoded in the nucleus, it serves as a prime example of a gene that is controlled by retrograde regulation. However, the nature of the retrograde pathway(s) and the factors required to achieve regulation of AOX production are not well understood.

In fungi, a few genes are known that affect AOX production. In Candida albicans there is evidence for the involvement of a histidine
kinase in AOX regulation (Huh and Kang 2001). Early work with *Neurospora crassa* identified the structural gene for AOX as *aod-1* whereas another gene, *aod-2*, was found to be required for the expression of *aod-1* (Bertrand et al. 1983; Edwards et al. 1976; Lambowitz et al. 1989; Li et al. 1996). More recently, we used a reporter system and a traditional genetic screen to identify four additional genes, named *aod-4*, *aod-5*, *aod-6*, and *aod-7*, along with a new allele of *aod-2*, as required for AOX induction (Descheneau et al. 2005). Thus, these studies defined a minimum of five genes required for proper AOX production in *N. crassa*. We then demonstrated that *aod-2* and *aod-5* encode transcription factors (Chae et al. 2007b) of the Zn (II)$_2$Cys$_6$ binuclear cluster (zinc cluster) family (MacPherson et al. 2006). In *vitro* studies suggested that the proteins form a heterodimer, which binds a specific sequence within the *aod-1* promoter region to activate transcription under the appropriate inducing conditions (Chae et al. 2007a,b; Chae and Nargang 2009). Orthologs of AOD2 and AOD5 also are required for AOX production in *Podospora anserina* (Sellem et al. 2009) and *Aspergillus nidulans* (Suzuki et al. 2012).

Because the previously described mutant screen was not saturated, it seemed likely that additional genes might also be involved with AOX regulation. However, two factors led us to not simply repeat the screen. First, the screen was designed to detect mutations affecting transcriptional regulation of the *aod-1* gene. Additional factors affecting posttranscriptional processes that also may play a role in expression of AOX would not be detected. Second, a gene knockout library for *N. crassa* (Colot et al. 2006) has been created since our previous screen. Identification of strains affected in their ability to produce AOX in this library would allow direct identification of the genes without the need for mapping and rescue experiments. Here we describe 62 newly identified genes from the *N. crassa* knockout library that affect the production of AOX to varying extents.

**MATERIALS AND METHODS**

**Strains and growth of *N. crassa***

The *N. crassa* gene knockout library (Colot et al. 2006) was obtained from the Fungal Genetics Stock Center (FGSC) in a series of 96-well microtiter plates holding conidia from individual strains in each well (McCuskey et al. 2010). At the end point of the study described herein, plates 1 through 108 of the library had been examined. Strain 74A-OR23-1A (Nargang lab name NCN251) was used as a control for experiments with the knockout strains.

The tom20RIP sheltered heterokaryon, the methods used to manipulate the heterokaryon, and the control (strain HIV) for experiments with the heterokaryon have been described in detail previously (Harkness et al. 1994a). In summary, the tom20RIP sheltered heterokaryon contains two nutritionally complementing nuclei. One of the nuclei carries a functional tom20 gene, a mutation in the *pan-2* gene, and is sensitive to p-fluorophenylalanine (fpA). The other nucleus contains a nonfunctional tom20 allele that was destroyed by repeat-induced point mutation (RIP) mutagenesis. The latter nucleus also carries a mutant allele of the *mtr* gene that imparts resistance to fpA, as well as a mutation in the his-3 gene. The level of the Tom20 protein can be greatly reduced in the heterokaryon by growth in the presence of fpA plus histidine.

*N. crassa* cells were grown on solid or in liquid medium according to previously described methods (Davis and De Serres 1970) but using the modified Vogel’s salts developed by Metzenberg (Metzenberg 2004). Medium containing sorbose was used when colonies were desired whereas sucrose containing medium was used when filamentous growth was desired. When needed, inhibitors were added to media at the following final concentrations: antimycin A, 0.5 μg/mL (0.9 μM); chloramphenicol (Cm), 2 mg/mL (6 mM); fpA, 400 μM as described previously (Descheneau et al. 2005; Nargang et al. 1995). At the aforementioned concentration, antimycin A completely inhibits the sETC.

For screening the *N. crassa* knockout library, a 48-pin inoculation stamp was used that fit precisely into one half of the wells of the 96-well microtiter plates containing the library. Conidia from the library were stamped to Petri plates containing Vogel’s sorbose medium plus antimycin A and to Petri plates without the inhibitor as a control for growth under normal conditions. Petri plates containing antimycin A were examined 5–7 d after inoculation, and those without antimycin were examined 2–3 d after inoculation.

Growth rates in the presence or absence of antimycin A were measured in two ways. In some cases, filamentous growth was monitored by measuring the radius of growth from conidia inoculated in the center of a Petri dish containing Vogel’s sucrose medium at selected time points. In other cases the amount of growth of colonies produced on sorbose containing medium after 2 to 5 d of growth was observed.

**Mitochondrial protein import assays**

The procedures used to measure the import of mitochondrial precursor proteins have been described previously (Harkness et al. 1994b). In summary, radiolabeled precursors, synthesized by *in vitro* translation in the presence of [35S]-methionine, were incubated with mitochondria isolated from freshly grown mycelium. After import for specified time periods, proteinase K was added to remove unincorporated precursor proteins. Mitochondria were washed, and mitochondrial proteins were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). Proteins were transferred to nitrocellulose and exposed to X-ray film.

**Other techniques**

Standard procedures were used for gel electrophoresis of proteins (Lämmli 1970), western blot analysis (Good and Crosby 1989), and isolation of mitochondria (Nargang and Rapaport 2007). Measurement of the effect of inhibitors on oxygen consumption in growing cells using an oxygen electrode was as described (Tanton et al. 2003). Potassium cyanide (CN) and salicylhydroxamic acid were used to inhibit the sETC and AOX, respectively. In some figures, irrelevant lanes were removed electronically. Bands on mitochondrial import blots were quantified using Adobe photoshop.

**RESULTS**

**The basis of the screen—strains deficient in AOX are unable to grow in antimycin A**

The construction of a library of *N. crassa* strains containing individual gene knockouts (Colot et al. 2006) maintained on microtiter plates provided a resource to search for genes that might be involved in the production of AOX. When *N. crassa* is unable to respire through the sETC, it induces AOX, which allows continued growth via this alternative pathway of electron transport. However, if both pathways are blocked, cells are unable to grow. Thus, we reasoned that any of the knockout strains that were unable to grow in the presence of antimycin, which inhibits complex III of the sETC, should also be lacking AOX and the genes affected in such strains would in some way be required for AOX production or function. To test this principle, we set up a microtiter plate containing conidia from several previously described AOX nonproducing strains (Descheneau et al. 2005), including...
a mutant of the aod-1 gene, which encodes the AOX protein. As controls, we used the parental strain of the knockouts as well as two slow-growing mutants that affected the sETC, cya-5-34 (Nargang et al. 1978), and (mi-3) (Mitchell et al. 1953). A 48-pin stamp was used to transfer conidia from the microtiter plate to Petri dishes containing growth medium with and without antimycin A. Growth of the control and sETC mutant strains in the presence of antimycin A was obvious whereas AOX mutants failed to grow (Figure 1A). As a further test, we stamped out the section of the knockout library known to contain the two different mating type strains with a knock-out of the aod-5 gene (plate 1, co-ordinates C3 and C4), which we had previously identified as a transcription factor necessary for the expression of AOX (Chae et al. 2007b). Of the 47 strains that grew on the control plate without antimycin A, only the two containing the aod-5 knockout did not grow in the presence of antimycin A (Figure 1B).

Examining the knockout library for strains unable to grow on antimycin A

We then proceeded to test the complete library, which consisted of approximately 10,000 strains contained on 108 microtiter plates (96 wells each). Because many of the knocked-out genes are represented in duplicate as both A and a mating type strains, the actual number of knocked out genes screened was approximately 7800. In the initial screening we identified 218 colonies that grew very slowly, or not at all, on medium containing antimycin A while exhibiting obvious growth on medium lacking the drug. The strains represented by these colonies were then inoculated from the library plates into slants and subsequently restested for growth in Petri dishes containing medium with or without antimycin A. In this round of testing, several strains showed relatively good growth in the presence of the drug. The difference in results compared to the original growth observed in the stamped library might be due to low numbers of viable conidia in some wells of the library plates. Other strains were found to grow extremely slowly even on medium that lacked antimycin A. Strains in either of these two categories were not examined further leaving 126 strains with slow, or no growth, in the presence of antimycin A and reasonably robust growth in the absence of the drug.

To continue our screen, we wished to determine the level of AOX that could be produced in each of the remaining 126 mutants. Because the strains were selected based on inability to grow in the presence of antimycin A, we were unable to use the same drug as an AOX inducer for experiments requiring growing cells. Growth in the presence of Cm at 2 mg/mL results in severe but not total inhibition of synthesis of mitochondrionally encoded subunits of the respiratory chain and ATP synthase and results in AOX induction in wild-type cells of N. crassa (Li et al. 1996; Tanton et al. 2003). However, we have previously shown that cells unable to produce AOX grow at rates virtually indistinguishable from the wild type in the presence of Cm (Descheneau et al. 2005). We assume that the small amount of mitochondrial protein synthesis that occurs in the presence of Cm is sufficient to support growth of the cells. However, the effects on mitochondria are sufficient to elicit AOX induction.

After growth for 24 hr and 48 hr in the presence of Cm, mycelia were harvested and mitochondria were isolated. Western blot analysis of mitochondrial proteins revealed that 62 newly identified strains contained reduced levels of AOX at one or both of the time points (Figure 2). Because the NCU numbers of the predicted proteins at the Neurospora database can change on the basis of more defined annotations, the coordinates of the knockout library 96-well microtiter plates have been used as the primary name for the strains identified.

We have used the convention of: number of plate (1–108), followed by the letter of the row (A–I), followed by the number of the column (1–12). Based on a visual assessment of the amount of AOX present on Western blots, we placed each strain into one of three classes (Table 1). Class 1 strains produced virtually no AOX at either 24 hr or 48 hr, or at both time points. An example is strain 23H2 (Figure 2A). Table 1 shows 11 Class 1 strains, but three of these were previously described as AOX-deficient mutants (1C3, aod-5; 96H9, aod-1; 97B1, aod-2). Thus, there are eight new strains that have been identified and assigned to Class 1. The 20 strains in Class 2 exhibited a moderate reduction in the amount of AOX at 24 hr, 48 hr, or both time points. An example is strain 6A1 (Figure 2A). Class 3 contained 34 strains that exhibited some reduction in AOX at one or both of the time points. Strain 4B3 serves as an example (Figure 2E). The NCU number of the predicted proteins and the FGSC strain number of the knockout strain are also shown in Table 1. The BROAD genome sequence website was examined for each gene identified. Known gene functions or characteristic protein domains listed at that website are also included in Table 1. In addition, we have included information regarding the growth rate of the strains in the presence and absence of antimycin A as well as observations on the ability of the strains to form conidiasespores. For unknown reasons, despite the fact that we screened for mutants with poor growth in the presence of antimycin A, a few strains were ultimately found to have good growth in the presence of the drug.

Nature of genes defined by annotations and bioinformatics

The genes identified in the screen represent a broad range of functions. Among the 62 new genes, the two most highly represented groups were 16 hypothetical proteins (9E5, 23H2, 28B10, 41G6, 44H2,
Figure 2  AOX levels in knockout mutants identified in the screen. The indicated strains were grown for 24 and 48 hr in the presence of Cm. Mycelia were harvested, mitochondria were isolated, and mitochondrial proteins were subjected to SDS-PAGE and Western blot analysis using antibody to AOX, and to Tom70 as a loading control. Results are placed in rows A through H to facilitate reference to results for individual strains. The control (NCN251) was grown in similar fashion. A culture of the control without Cm also was used to demonstrate the lack of AOX without induction by Cm. AOX-deficient strains are shown with a control from the same Western blot. In some cases, irrelevant lanes were removed from the blots and are shown as white strips between lanes.
**Table 1** Summary and characterization of knockout strains identified as AOX deficient

| Knockout Library Grid Number | Mutant Class | Panel of Figure 2 Showing Western | NCU Number | FGSC Number | Known or Predicted Protein or Gene\(^a\) (Domain Identified) | Growth Defect\(^b\) | Growth on Antimycin A for 48 hr\(^c\) | Conidiation Defect\(^d\) |
|-----------------------------|-------------|----------------------------------|------------|-------------|-------------------------------------------------------------|------------------|---------------------------|------------------|
| 1C3\(^a\)                   | 1           | na                               | 03938.5    | 11227       | aod-5                                                       | 0                | 0                         |                  |
| 23H2                        | 1           | A                                | 08887.5    | 15957       | Hypothetical protein (major facilitator superfamily)        | 0                | 0                         |                  |
| 40E6\(^f\)                  | 1           | A                                | 05600.5    | 13805       | Ubiquinone biosynthesis protein (ABC1 family)               |                  |                           |                  |
| 41G6                        | 1           | A                                | 03589.5    | 13923       | Hypothetical protein                                         | Slight           | 0                         | Slight           |
| 47H10                       | 1           | A                                | 00778.5    | 16938       | sed5 vesicle protein (transmembrane adaptor Env26)          | Severe           | 0                         | Severe           |
| 52D8                        | 1           | B                                | 07281.5    | 14469       | Glucose-6-phosphate isomerase (phosphoglucone isomerase)     |                  |                           |                  |
| 83H3                        | 1           | A                                | 08365.5    | 18277       | RNA polymerase II mediator complex component Med8            | Slight           | 0                         | Slight           |
| 88H8                        | 1           | A                                | 01542.5    | 19221       | Hypothetical protein (HbrB like domain)                      |                  |                           |                  |
| 96H9\(^a\)                  | 1           | na                               | 07953.5    | 18947       | aod-1                                                       | 0                |                           |                  |
| 97B1\(^e\)                  | 1           | na                               | 03352.5    | 19465       | aod-2                                                       |                  |                           |                  |
| 100B5                       | 1           | A                                | 08158.5    | 19644       | Dual-specificity phosphatase                                 | Slight           | 0                         | Slight           |
| 1G9                         | 2           | D                                | 00157.5    | 11281       | COP 9 signalosome-1, csn-1                                  | Slight           | 0                         | Slight           |
| 1G11                        | 2           | D                                | 00467.5    | 11283       | csn-5                                                       | Slight           | 0                         | Slight           |
| 2A3                         | 2           | D                                | 08741.5    | 11299       | Striatin pro 11 (WD domain, G-beta repeat)                   | 0.7              | Severe                    |                  |
| 6A1                         | 2           | A                                | 06799.5    | 11001       | Fungal specific transcription factor, vad-5                  | 0                |                           |                  |
| 6F2                         | 2           | B                                | 09739.5    | 11062       | Hypothetical protein, ada-7 (Zn(2)-Cys(6))                   | 0.7              | Severe                    |                  |
| 7H4                         | 2           | B                                | 03875.5    | 11780       | Chromatin remodeling complex ATPase chain ISW1 (SLIDE, HAND, |                  |                           |                  |
| 12C8                        | 2           | D                                | 01266.5    | 12022       | Phosphoinositol-specific phospholipase C                     | 0.6              |                           |                  |
| 13G11                       | 2           | D                                | 04566.5    | 12420       | Protein kinase SNF1                                          | 0                | Slight                    |                  |
| 14A5                        | 2           | D                                | 03727.5    | 12091       | Hyphal anastomosis-2, ham-2                                  | 0.4              | Severe                    |                  |
| 28C5                        | 2           | C                                | 00007.5    | 16098       | pH response regulator palH, rim-21                          | 1.0              | Slight                    |                  |
| 28D10                       | 2           | C                                | 00593.5    | 16116       | COP9 signalosome-2, csn-2                                   | 0.2              | Slight                    |                  |
| 30C2                        | 2           | B                                | 06199.5    | 13193       | RNA binding protein Jsn1 (RNA recognition motif)            | 1.2              | Severe                    |                  |
| 44H2                        | 2           | B                                | 04826.5    | 16834       | Hypothetical protein                                         | 0                | Slight                    |                  |
| 45C3                        | 2           | D                                | 03035.5    | 13973       | Hypothetical protein                                         | 0.3              | Slight                    |                  |
| 50G8\(^f\)                  | 2           | A                                | 04607.5    | 14316       | Hypothetical protein                                         | 0.3              | Slight                    |                  |
| 81E9                        | 2           | E                                | 06084.5    | 20114       | Hypothetical protein (Vps51/Vps67)                          | 1.6              | Severe                    |                  |
| 83E1                        | 2           | B                                | 07881.5    | 18239       | Hypothetical protein                                         | 2.7              | Severe                    |                  |
| 85H8                        | 2           | E                                | 09803.5    | 18375       | Thioredoxin (glutaredoxin homolog)                           | Slight           | 0                         | Slight           |
| 96C10                       | 2           | B                                | 04245.5    | 18888       | Translocase of outer mitochondrial membrane 70, tom70        | 0                | Slight                    |                  |
| 96G8\(^b\)                  | 2           | E                                | 06727.5    | 18934       | Spermidine 3, spe-3 (Spermidine synthase)                    | 2.3              |                           |                  |
| 2C4                         | 3           | D                                | 03894.5    | 11324       | Serine/threonine protein kinase ste-20                       | 0.2              |                           |                  |
| 4B3                         | 3           | E                                | 04062.5    | 11473       | Peroxisome biogenesis factor 20                             | 1.1              |                           |                  |
| 4F11                        | 3           | G                                | 08480.5    | 11671       | Hsf-type DNA-binding domain-containing protein               | 0                | Slight                    |                  |
| 9E5                         | 3           | E                                | 08565.5    | 11945       | Hypothetical protein (NACHT domain)                         | 0.4              |                           |                  |
| 10B5                        | 3           | F                                | 09212.5    | 11545       | Serine/threonine protein kinase                              | 1.7              |                           |                  |
| 10B6                        | 3           | F                                | 06563.5    | 11546       | Serine/threonine protein phosphatase PP2A                    | Slight           | 0.5                       | Slight           |
| 10B8                        | 3           | H                                | 07489.5    | 11548       | Phosphatase-Z-like-1, ppz-1 (calcineurin-like phosphoesterase)| 0.4              | Severe                    |                  |
| 10C6                        | 3           | H                                | 03853.5    | 11559       | Peptidyl-prolyl cis-trans isomerase (tetraticopeptide repeat) | 2.7              | Slight                    |                  |

(continued)
45C8, 50G11, 51F9, 81E9, 82B6, 83E1, 88H8, 92B11, 93F6, 100F2, 108G10) and six kinases or phosphatases (2C4, 1085, 1086, 1088, 13G11, 100B5). If the previously identified aod-2 and aod-5 genes are included, there are also six predicted transcription factors (1C3, 6A1, 6F2, 97B1, 99C10, 100C8). There is no obvious relationship among the predicted functions of the eight new class 1 genes (Table 1) and in most cases it is not obvious how many of the predicted functions might compromise AOX production. One class 1 gene that might be imagined as influencing AOX expression is the dual specificity phosphatase deleted in 100B5, because kinases and phosphatases are well known as important regulatory enzymes. A complication with respect to class 1 strain 40E6 is that the region replaced in this knockout strain actually covers two genes. One of these (NCU05600.5) is listed as a ubiquinone biosynthesis protein with an ABC1 family domain, whereas the other (NCU12096.5) is an aspartyl aminopeptidase. We suspect that the absence of the gene encoding the putative ubiquinone synthesis protein would be the more likely of the two genes to affect AOX production because the reduced form of ubiquinone is the substrate for AOX. NCU05600.5 has recently been shown to contain an atypical serine-threonine kinase domain and the gene

| Knockout Library Grid Number | Mutant Class | Panel of Figure 2 Showing Western NCU Numbera | FGSC Number | Known or Predicted Protein or Geneb (Domain Identified) | Growth on Antimycin A for 48 hr | Conidiation Defectc |
|-----------------------------|-------------|-----------------------------------------------|-------------|----------------------------------------------------------|-------------------------------|---------------------|
| 13C11                       | 3           | F                                             | 06493.5     | Guanine nucleotide-binding protein alpha-1 (ADP ribosylation factor) | 3.3                           |                     |
| 28B10                       | 3           | G                                             | 08137.5     | Hypothetical protein                                       | 0                             | Slight              |
| 30F5                        | 3           | C                                             | 01471.5     | Nuclear protein SNF4 (CBS domain)                          | 0                             |                     |
| 32A11                       | 3           | C                                             | 01955.5     | Autophagocytosis protein Aut1                              | 1.9                           | Slight              |
| 42A2                        | 3           | D                                             | 01276.5     | N-acetyltransferase 5                                       | Slight                        | 0.5                 |
| 48E6                        | 3           | C                                             | 04771.5     | FR47-like protein                                           | 0.5                           |                     |
| 51F9                        | 3           | C                                             | 09530.5     | Hypothetical protein                                       | 1.4                           |                     |
| 52F6                        | 3           | C                                             | 08067.5     | Osmosensor protein (SH3 domain)                            | 3.5                           |                     |
| 63E6                        | 3           | H                                             | 03802.5     | Trimethyllysine dioxygenase (taurine catabolism dioxygenase) | 2.6                           |                     |
| 82B6                        | 3           | E                                             | 07112.5     | Hypothetical protein (pyridine nucleotide-disulphide oxidoreductase) | 1.5                           |                     |
| 83F11                       | 3           | F                                             | 01511.5     | Urease accessory protein ureG                               | Severe                         | 0.5                 |
| 92B11                       | 3           | G                                             | 07579.5     | Hypothetical protein (SCA7)                                 | 0.2                           | Severe              |
| 93F6                        | 3           | G                                             | 03708.5     | Hypothetical protein (putative methyl transferase)         | 1.8                           |                     |
| 94D6a                       | 3           | G                                             | 00712.5     | 3-hydroxy-3-methylglutaryl-coenzyme A dehydrogenase (sterol sensing domain, patched family) | 2.6                           |                     |
| 97D9                        | 3           | G                                             | 00360.5     | NAD-dependent epimerase/dehydratase                         | 0                             | Slight              |
| 98H11                       | 3           | G                                             | 09527.5     | Phosphoglucomutase 2                                       | 1.1                           |                     |
| 99C10                       | 3           | E                                             | 09686.5     | Clock-controlled gene-8, ccc-g-8                           | 0                             |                     |
|                            |             |                                               |             | (transcription factor opl1)                                 |                               |                     |
| 100C8                       | 3           | C                                             | 09208.5     | Transcription factor SPT8 (WD domain, G-beta repeat)       | 0                             |                     |
| 100F2                       | 3           | C                                             | 09527.5     | Hypothetical protein                                       | 2.0                           |                     |
| 101D3                       | 3           | F                                             | 09560.5     | Superoxide dismutase                                       | 0                             |                     |
| 103G4                       | 3           | F                                             | 09535.5     | 3-hydroxybutyl CoA dehydrogenase                           | 2.2                           |                     |
| 103G10g                     | 3           | H                                             | 08132.5     | Alpha-1,3-glucan synthase, Ags2                             | Slight                        | 0.5                 |
| 104D1                       | 3           | F                                             | 02973.5     | Mitochondrial carrier protein                               | 0.1                           |                     |
| 104E9                       | 3           | C                                             | 01533.5     | Para-hydroxybenzoate-polyprenyltransferase, Coq2           | 0                             |                     |
| 108C9                       | 3           | H                                             | 02979.5     | AMP deaminase                                               | 1.8                           | Slight              |
| 108G10g                     | 3           | H                                             | 08279.5     | Hypothetical protein                                       | 1.1                           |                     |

a Information from N. crassa database BROAD Institute (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html).
b Growth rates were measured by inoculating conidiospores at the center of Petri dishes (standard 100 mm × 15 mm size) containing Vogel’s medium. Plates were incubated at 30°. The radius of mycelial extension from the inoculation point was measured (in cm) and compared with the wild-type control strain that covers the surface of the plate (radius of growth from the inoculation point of 4.2 cm) in 48 hr. Strains with a “slight” growth defect were defined as having a growth radius of 1.5 to 4.1 cm in 48 hr. A “severe” growth defect was defined as having a growth radius of less than 1.5 cm in 48 hr. c Same as b, but the medium contained Antimycin A. Measurements of growth (in cm) were taken after 48 hr. The control strain grew 1.7 cm in 48 hr on average under these conditions.
d Formation of asexual conidiospores was considered slightly defective if conidiation on slants containing 3 mL of Vogel’s medium in a 1 cm × 10-cm test tube was similar to the control but took longer than the control strain. The defect was considered severe if very few conidia formed, even after extended times.
e The aod-1 (Li et al. 1996), aod-2, and aod-5 (Descheneau et al. 2005) genes were all identified during the screen and have been described in detail previously.
f Original predicted open reading frame was knocked out but is now known to be divided into two predicted genes.
g Strain maintained in the knockout library as a heterokaryon. This implies that the affected gene is essential for viability.
Table 2 Strains in which the gene knockout is predicted to encode a mitochondrially localized protein

| Strain | Mutant Class | NCU Number | Localization Criteriaa | Predicted Proteinb |
|--------|--------------|------------|------------------------|--------------------|
| 40E6   | 1            | 05600      | A2                     | Ubiquinone biosynthesis |
| 96H9   | 1            | 07953      | C1                     | Alternative oxidase (AOX) |
| 2A3    | 2            | 08741      | B1                     | Striatin pro 11 |
| 96C10  | 2            | 04245      | A1                     | Tom70 protein import receptor |
| 9G6    | 2            | 06727      | C1                     | Spermidine 3, spermidine synthase |
| 9E5    | 3            | 08565      | B1                     | Hypothetical protein |
| 13C11  | 3            | 06493      | D2                     | Guanine nucleotide binding protein alpha-1 |
| 2A3    | 3            | 08741      | B1                     | Hypothetical (BLAST yeast Mitochondrial ribosomal protein of the small subunit, has similarity to human mitochondrial ribosomal protein MRP-S36) |
| 82B6   | 3            | 07112      | A1                     | Hypothetical [HcaD domain conserved many organisms, NAD(FAD) oxidoreductase] |
| 101D3  | 3            | 09560      | A1                     | Superoxide dismutase |
| 103G4  | 3            | 09553      | A1                     | 3-hydroxybutyl-CoA dehydrogenase |
| 104D1  | 3            | 02973      | C1                     | Mitochondrial carrier protein |
| 104E9  | 3            | 01553      | C1                     | Para-hydroxybenzoate-polyprenyltransferase Coq2 |

a As defined previously (Keeping et al. 2011). Category A1 proteins were identified in their study from highly purified mitochondria using mass spectrometry. Category A2 proteins were identified by mass spectrometry in other studies or had other evidence of being located in mitochondria. B1 proteins were identified in an earlier study of the mitochondrial proteome (Schmitt et al. 2006) but no other evidence for or against their mitochondrial location exists. C1 proteins were predicted or demonstrated to be mitochondrial by conventional biochemistry. The D2 proteins are listed in MitoP2 Neurospora data, but other evidence that they are not mitochondrial also exists. This refers to a mitochondrial proteome database for several organisms, including Neurospora (http://www.mitop.de/).

b Prediction from the N. crassa database. Descriptions in brackets after the first description are taken from Keeping et al. (Keeping et al. 2011).

has been named stk-33 (Park et al. 2011). The role of the protein in ubiquinone synthesis is not entirely clear, and its closest S. cerevisiae homolog (YPL109c) has not yet been assigned a function. However, the proteins from both species are related to the S. cerevisiae YGL119W protein, also known as Coq8 (Do et al. 2001). The kinase domain of Coq8 is thought to regulate the activity of Coq3, a ubiquinone biosynthetic enzyme (Tausche et al. 2008). However, a coq8 deletion strain has also been shown to have a complex phenotype that includes underproduction of glutathione and sensitivity to oxidative stress (Gazdag et al. 2011). Members of the same family of proteins have also been linked to oxidative stress in Arabidopsis thaliana (Jasinski et al. 2008). It should be noted that a homolog of another ubiquinone synthesis protein, Coq2, was identified as a class 3 mutant (104E9).

Among the 20 class 2 knockouts, it is striking that three encode components of the COP9 signalosome (1G9, 1G11, and 28D10), whereas another two encode predicted transcription factors (6A1, 6F2). The latter two genes were previously identified as vad-5 and ada-7, respectively, in a study of Neurospora transcription factor knockouts (Colot et al. 2006). The class 3 genes represent a broad range of predicted function. Four class 3 genes are predicted to act as kinases or phosphatases.

It was of interest to determine how many of the genes identified in our screen encoded proteins that are known or predicted to be localized to mitochondria. An exhaustive compilation that lists proteins actually identified in purified N. crassa mitochondria by two-dimensional electrophoresis and mass spectrometry, as well as those predicted to be mitochondrial by various in silico analyses has recently been published (Keeping et al. 2011). Each of the NCU numbers identified in our study was compared to the compilation. The matches are shown in Table 2, along with the criteria used in assigning the protein as mitochondrial. Thirteen proteins were found to be known or possible mitochondrial proteins (Table 2).

Time-dependent AOX expression

When wild-type strains are grown for 24 and 48 hr in the presence of Cm, there is typically no difference in the amount of AOX present in their mitochondria (Figure 2, panel A, left and right blots; panel F, left blot). However, several of the strains identified from the KO library showed obvious differences in AOX abundance at the two different times of growth used in the screen. These observations suggest that different pathways of induction might be activated or used after different times of inhibition of the sETC. Although a few class 3 mutants showed this phenotype, it was most obvious in class 1 and 2, where mutants 1G9, 6A1, 7H4, 13G11, 30C2, 40E6, 41G6, 44H2, 50G11, and 52D8 contained more AOX after 24 hr growth in Cm than at 48 hr. In these cases a positive regulatory factor may become depleted or a negative factor may accumulate. Conversely, mutants 12C8, 14A5, 28C5, 81E9, 85H5, 100B5, and 101D5 contained more AOX after 48 hr than 24 hr. In these mutants a positive factor may accumulate or a negative factor might be depleted.

We chose one such class 1 strain (40E6) to determine whether the changes in AOX levels were gradual or sudden. As shown in Figure 3A, the level of AOX in the control strain mitochondria during growth in Cm was consistent over the course of the experiment from 12 to 36 hr. However, for 40E6 grown in Cm, the level of the enzyme was constant from 12 to 24 hr, but then it abruptly disappeared. Furthermore, there was direct correspondence between times that AOX could not be detected by western blot analysis with an absence of the CN- insensitive respiratory characteristic of AOX (Figure 3B). It should also be noted that even at the earlier time points, AOX levels are somewhat reduced in 40E6.

Tom70 acts as the major receptor for the AOX precursor protein

Among the genes identified as affecting AOX production, tom70 was somewhat unexpected (strain 96C10). Tom70 is a protein of the outer mitochondrial membrane TOM (translocase of the outer mitochondrial membrane) holo-complex (Ahting et al. 1999; Dekker et al. 1998). The TOM complex is responsible for the import of cytosolically synthesized mitochondrial preproteins into the organelle. Tom70, along with another protein of the complex, Tom20, are the major receptors for mitochondrial precursor proteins in the cytosol (Endo
reduced ef

proteins are still imported in mutants lacking Tom70, though with

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concentration over time. Arrows indicate the point at which the indicated

proteins once they reach the matrix. However, Tom20 also has a de-

laying an N-terminal targeting signal which is removed from precursor

Tom20 is best known for recognizing matrix-destined precursors car-

brane spanning domains such as AAC, the ATP/ADP carrier.

et al. (Brix et al. 1999; Steger et al. 1990). Because

nuclear properties of the strain in more detail. Although we saw

no growth of the strain on antamycin A containing medium after 48 hr

(Table 1), slow growth was detectable after four d (Figure 4A). We

then examined the steady-state levels of AOX protein in mitochondria

lacking Tom70 or depleted for Tom20. Because Tom20 is an

essential protein (Harkness et al. 1994a), we could not study a knock-

out strain. Therefore, we produced mitochondria with greatly re-

duced levels of Tom20 from a previously constructed tom20RIP

sheltered heterokaryon (Harkness et al. 1994a). Growth of the het-

erkaryon in the presence of fpa results in numerical superiority of

the nucleus that is devoid of a functional tom20 gene resulting in a

reduction of the Tom20 protein in the mitochondria of the culture

(See Methods). We isolated mitochondria from cultures of the

tom70 knockout strain (96C10), a Tom20-depleted strain, and appro-

priate controls. All strains were grown both in the presence and

absence of Cm. Mitochondrial proteins were subjected to SDS-

PAGE and analyzed on Western blots for the presence of various

mitochondrial proteins. Growth of the tom20RIP sheltered hetero-

karyon in the presence of fpa resulted in mitochondria virtually

devoid of Tom20, while Tom70 levels were unaffected (Figure 4B, Tom20
e xpt, tom20RIP lanes). Levels of AOX in these mitochondria

after growth in the presence of fpa and Cm were reduced compared

with AOX in tom20RIP cultures grown without fpa. However, the levels

of AOX in the tom20RIP cultures are virtually identical to the control

cells grown under the corresponding conditions (Figure 4B, Tom20
e xpt + fpa + Cm lanes for control and tom20RIP).

Thus, fpa appears to have an unknown effect in reducing AOX

levels, but the presence or absence of Tom20 does not result in

changes in AOX levels relative to the control. On the other hand, mito-

chondria isolated from the tom70 knockout strain have levels of

Tom20 similar to the control strain, but have much reduced AOX

levels compared with the relevant control (Figure 4B, Tom20
e xpt, + Cm lanes), consistent with the results in Figure 2B for 96C10. Res-

piration measurements on tom70 knockout cells grown in the pres-

ence of Cm showed that the reduced amount of AOX in the cells was

still sufficient to allow CN-insensitive respiration to occur (Figure 4C).

We assume that the reduced level of AOX in the mitochondria of

tom70 knockout cells grown in Cm is sufficient to allow slow growth

in the presence of antamycin A as seen in Figure 4A.

We also directly measured import of radiolabeled precursor

proteins into isolated mitochondria with reduced levels of Tom70

(Figure 4D). AOX import was obviously reduced in mitochondria

lacking Tom70, as was import of AAC a protein known to depend

on Tom70 for its import (Ryan et al. 1999; Söllner et al. 1990; Young

et al. 2003b). In contrast, import of the precursor of the β subunit of

the F1 portion of the mitochondrial ATP synthase (F1β), a protein that

contains a cleavable N-terminal targeting signal, occurred at a

slightly higher level in the mitochondria lacking Tom70. For two

twofold experiments, the percentage of import into the mitochon-

dria lacking Tom70 was 100% and 123% of the control for F1β; 39%

and 47% for AAC, and 35% and 48% for AOX. The simplest inter-

pretation of these data is that Tom70 is the major receptor involved in

the import of the AOX precursor. However, we cannot formally elim-

inate the possibility that another unknown factor that is decreased in

mitochondria lacking Tom70 may play a role in AOX import.

DISCUSSION

This study has identified many genes that are required to achieve full

expression of AOX under conditions that induce the enzyme in N.

crassa. Absence of many of the genes has only minor effects on AOX

(class 3), whereas loss of others has a more pronounced effect (classes

1 and 2). Many of the knockout strains identified in our screen exhibit

reduced growth rates and/or reduced ability to form conidia. Thus,

such genes are not likely to be specific for an AOX regulatory path-

way. Loss of these genes may affect several functions that include

AOX expression. More information on AOX expression at the tran-

scriptional and posttranscriptional levels, as well as the assembly of

the enzyme into the mitochondria inner membrane, will be required

to understand the effects in each case.
Although the AOX precursor protein contains a cleavable N-terminal targeting signal, its efficient import appears to be dependent on Tom70. Tom70 was previously shown to enhance the import of a few precursors containing N-terminal cleavable targeting signals (Hachiya et al. 1995; Hines et al. 1990; Hines and Schatz 1993; Schlossmann et al. 1994). A detailed investigation of precursors of this class revealed that their precursors were recognized by Tom20. However, the mature portions of these precursor proteins were prone to aggregation, which could be prevented by association with Tom70 (Yamamoto et al. 2009). It was suggested that the requirements for Tom70 is related to its known function as a docking site for cytosolic chaperone proteins (Fan et al. 2011; Young et al. 2003b), which would likely be associated with aggregation prone precursor proteins. Furthermore, a chaperone activity of Tom70 toward at least some precursors was also identified (Yamamoto et al. 2009). Thus, the AOX with its hydrophobic domains that interact with the inner mitochondrial membrane (Andersson and Nordlund 1999; Berthold et al. 2000; Berthold and Stemmark 2003) fits nicely into this model of Tom70 function.

Six genes were identified that are predicted to be transcription factors. Two of these, AOD2 and AOD5, were previously characterized as required for AOX production. However, it is now clear that these proteins are involved in the transcription of other genes as well. It has been shown in P. anserina that the orthologs of AOD2 and AOD5 (RSE-2 and RSE-3, respectively) are involved in transcription of AOX but also play a role in transcription of genes involved in gluconeogenesis (Sellem et al. 2009). Similarly, the A. nidulans orthologs (AcuM and AcuK, respectively) are involved in gluconeogenesis and AOX regulation (Hynes et al. 2007; Suzuki et al. 2012). Interestingly, the Aspergillus fumigatus orthologs also are involved in iron uptake (Liu et al. 2010). The other transcription factors identified here, may also have multiple roles, including possible contributions to AOX transcription.

We also identified six genes that encode putative kinases or phosphatases. Phosphorylation/dephosphorylation events are well known to be associated with effects on protein function and gene expression. Perhaps the most interesting gene in this category is the Snf1 kinase, which is disrupted in strain 13G11. The S. cerevisiae Snf1 kinase and its mammalian ortholog, AMPK, are known to play roles in sensing cellular energy levels (Hardie 2007; Turtuote et al. 2010; Young et al. 2003a). When activated by conditions resulting in reduced ATP levels, the kinase acts to phosphorylate transcription factors involved in expression of genes for processes such as gluconeogenesis and the glyoxylate cycle. In fact, the Snf1 kinase is involved in phosphorylation of Rds2 (Soontornrung et al. 2007), the Saccharomyces cerevisiae ortholog of N. crassa AOD2 (Chae et al. 2007b). Rds2 is involved in gluconeogenesis and increased Snf1-dependent phosphorylation of the protein is observed during growth in ethanol as compared to growth in glucose. Binding of Rds2 at the promoter for the FBP1 gene encoding fructose-1,6-bisphosphatase, was dependent on Snf1 activity (Soontornrung et al. 2007). Interestingly, the Snf4 gene (30F5) that encodes a homolog of a regulatory factor of the S. cerevisiae Snf1 kinase complex also was identified in our screen as a class 3 mutant. Thus, it will be of interest to determine if phosphorylation of AOD2 by N. crassa SNF1 and binding of malate may be involved in AOD2 activation.
Reactive oxygen species (ROS) are thought to play a role in mitochondrial biogenesis and in eliciting stress responses in many systems (Bae et al. 2011; Collins et al. 2012; Leister 2012; Rhoads and Vanlerberghe 2004; Yoboue and Devin 2012). Increased levels of ROS are known to be associated with increased levels of AOX in certain fungi such as C. albicans (Huh and Kang 2001), Magnaporthe grisea (Yukioka et al. 1998), and A. fumigatus (Magnani et al. 2007). On the other hand, this does not appear to be the case for A. nidulans (Pusztahegyi et al. 2011; Suzuki et al. 2012) or P. anserina (Borghouts et al. 2001). Our screen detected only two genes with an obvious relationship to cellular redox conditions. Superoxide dismutase, which converts superoxide to molecular oxygen and hydrogen peroxide, is inactivated in the class 3 strain 101D3, which has a minor AOX reduction after 48 hr growth in Cm. The specific protein (NCU09560.5) is predicted to encode a version of the enzyme that uses manganese as a cofactor, a hallmark of mitochondrial versions of the enzyme (Holley et al. 2010) and the protein was identified as a Neurospora mitochondrial protein in a proteomic study (Keeping et al. 2011). Because there is only a slight reduction of AOX in the strain after growth in Cm, it would appear that the signaling mechanism for AOX transcription is largely unaffected. Given that AOX is present in this strain during growth in Cm, it is not obvious why it does not grow in the presence of antimycin A (Table 1). One possibility is that the signaling mechanism for AOX production is different for growth in antimycin A vs. Cm. Another possibility is that the increased ROS that is likely produced upon antimycin A inhibition proves toxic to cells in the absence of the mitochondrial superoxide dismutase.

The gene removed in the class 2 mutant 85H5, is listed at the genome database as a thioredoxin that also has a glutaredoxin domain (NCU09803.5). BLAST searches against the S. cerevisiae proteome demonstrated that NCU09803.5 is most highly related to the monothiol glutaredoxins, Grx3 and Grx4 (supporting information, Table S1). These yeast proteins, as well as NCU09803.5, contain a thioredoxin-like domain at their N-terminus, but also the CGFS motif that defines monothiol glutaredoxins (Herrero and De La Torre-Ruiz 2007; Lillig et al. 2008; Meyer et al. 2009). The latter domain replaces the CXCC motif found in classical thioredoxins and dithiol glutaredoxins.

A search of the N. crassa database identified a GRX3 protein that represents a mitochondrial monothiol glutaredoxin and revealed that NCU09803.5 is the only non-mitochondrial form of the protein in the organism (see Table S1). This is similar to Schizosaccharomyces pombe, which contains only a single Grx3/Grx4 ortholog, called Grx4 (Chung et al. 2005). These proteins are known to play important roles in various aspects of iron metabolism, including iron sensing, influencing the localization and activity of iron responsive transcription factors, and general intracellular iron trafficking (Lill et al. 2012; Rouhier et al. 2010). With respect to our study, it is of interest to note that depletion of both Grx3 and Grx4 in S. cerevisiae has been shown to result in severe reduction in activity of di-iron enzymes in both the cytosol and mitochondria (Mühlenhoff et al. 2010). Thus, the defect in expression of AOX, a di-iron carboxylate enzyme, seems likely to be related to iron metabolism in the 85H5 strain. The observation that 85H5 does not grow in the presence of antimycin A even though substantial levels of AOX accumulate after 48 hr growth in Cm may reflect an accumulation of AOX protein without inserted iron.

Three class 2 mutants encode components of the COP9 signalosome (CSN). The CSN is a highly conserved complex in eukaryotes consisting of eight subunits in higher organisms, with some variation in the number of subunits in lower eukaryotes. The CSN is best known for its role in the regulation of protein degradation, but other activities, including possible direct roles in gene expression and regulation of transcription factors, have been associated with the complex or its subunits (reviewed in Chamovitz 2009, Wei and Deng 2003, and Wei et al. 2008). In N. crassa, study of a csn-2 disruption strain revealed a role for CSN in the regulation of the circadian clock (He et al. 2005). An in depth study of the N. crassa CSN complex revealed that it contains seven different subunits: CSN1 through CSN6 plus CSN7a (Wang et al. 2010). With the exception of CSN3, cells lacking any of the seven subunits had pleiotropic phenotypes, including defects in proteosome regulation, growth, conidiation, and circadian rhythms. Our screen identified knockouts of the csn-1, csn-2, and csn-5 genes as having effects on AOX production (Table 1). At the time we examined the library the csn-3 and csn-4 gene knockout among these yeast proteins, as well as NCU09803.5, contain a thioredoxin that also has a glutaredoxin domain

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