Title: Mutations in Two Zinc Cluster Proteins Activate Alternative Respiratory and Gluconeogenic Pathways and Restore Senescence in Long-lived Respiratory Mutants of Podospora anserina

Running title: Alternative pathways and senescence in P. anserina

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ABSTRACT

In *P. anserina*, inactivation of the respiratory chain results in a spectacular lifespan extension. This inactivation is accompanied by the induction of the alternative oxidase. Although the functional value of this response is evident, the mechanism behind it is far from understood. By screening suppressors able to reduce the lifespan extension of cytochrome deficient mutants, we identified mutations in two Zinc cluster proteins RSE2 and RSE3, which are conserved in other ascomycetes. These mutations led to the overexpression of the genes encoding the alternative oxidase and the gluconeogenic enzymes, fructose-1, 6 biphosphatase and pyruvate carboxykinase. Both RSE2 and RSE3 are required for the expression of these genes. We also show that even in absence of a respiratory deficiency, the wild type RSE2 and RSE3 transcription factors are involved in lifespan control and that their inactivation retards aging. These data are discussed with respect to aging, the regulation of the alternative oxidase and carbon metabolism.

The filamentous fungus *Podospora anserina* is a model organism in which lifespan control has been extensively investigated. As in other organisms, it was clear from the beginning that lifespan is controlled by numerous external and genetic factors. Among these factors, mitochondrial activity seems to play a determinant role (reviewed in Lorin et al. 2006). But whereas mutations that compromise mitochondrial function in humans (reviewed in Wallace 2005) and mice (Kujoth et al. 2007, 2005, 2006; Trifunovic et al. 2005, 2004) lead to a variety of pathological lifespan-shortening diseases, in *P. anserina* they lead to a spectacular life extension. In this organism, all wild type cultures exhibit an unavoidable arrest of vegetative growth systematically associated with large rearrangements in the mitochondrial DNA (mtDNA). Inactivation of the respiratory complex III (mutant *cyc1-I*) (Sellem et al. 2007) or complex IV (mutant *cox5::ble*) (Dufour et al. 2000) results in an extreme increase of lifespan (more than 30-fold) associated with a reduction in ROS levels and an increased stability of the mtDNA. In *C. elegans*, a class of mutants (*Mit* mutants) with disruptions (either genetic or mediated by RNA interference) in genes essential for the mitochondrial electron transport chain (ETC) are also long-lived (reviewed in Rea 2005,
2007). How can loss of genes critical for mitochondrial activity lead to life extension in *P. anserina* and *C. elegans*? One characteristic shared by *C. elegans* Mit mutants and *P. anserina* respiratory mutants is the activation of compensatory metabolic pathways in an attempt to supplement deficits in ETC function. Such pathways could produce “less toxicity” e.g. by reducing mitochondrial ROS production or activating antioxidant mechanisms. In *P. anserina*, inactivation of genes essential for complex III or IV activity leads to the induction of an alternative oxidase (AOX) that catalyzes the transfer of electrons directly from the ubiquinol pool to oxygen and does not couple this transfer to proton translocation (Affoultit et al. 2002, Moore et al. 2002). Some phenotypic traits of the *cox5::ble* and *cyc1-1* mutants can be attributed to these characteristics: reduced growth rate, loss of fertility and reduced ROS production. In these mutants, only complex I is conserved as a site of proton gradient formation for ATP synthesis leading to a reduction of the energy yield associated with respiration. Furthermore the alternative oxidase is thought to have an antioxidant role, preventing over-reduction of the mitochondrial quinone pool known to favor superoxide production (Maxwell et al. 1999). The reasons for the spectacular long-lived phenotype of these mutants are more puzzling. One hypothesis proposed that the reduction of ROS and/or ATP production might be sufficient to account for lifespan extension. Another invoked a mitochondria to nucleus signaling pathway (Lorin et al. 2001). The AOX is encoded in the nucleus and imported into mitochondria. In fungi, AOX expression has been extensively studied in *Neurospora crassa* and *P. anserina*. The protein is not detectable under standard growth conditions. Its expression is strongly induced by mutations or chemicals that inhibit the ETC and significant regulation occurs at the level of transcription (Affoultit et al. 2002; Chae et al. 2007a, 2007b; Descheneau et al. 2005; Lambowitz et al. 1989; Li et al. 1996; Lorin et al. 2001; Tanton et al. 2003). In higher plants, AOX expression depends on developmental signals, stress conditions and inhibition of the respiratory chain (reviewed in Clifton et al. 2006). The AOX expression implies therefore the existence of one or more pathways for transducing signals from the mitochondria to the nucleus to control the expression of the gene. The mechanisms by which mitochondria communicate with the nucleus have been referred to as retrograde signaling (Butow and Avadhani 2004; Liu and Butow 2006; Rhoads and Subbaiah 2007). In *Saccharomyces cerevisiae*, one retrograde pathway (the RTG pathway) has been extensively studied and shown to be an important determinant of lifespan (Kirchman et al. 1999).

In an attempt to clarify the relationships between AOX expression, retrograde signaling and lifespan in *P. anserina*, we isolated fast-growing, short-lived revertants from the long-
lived respiratory \textit{cox5::ble} and \textit{cyc1-1} deficient mutants. We identified three mutations localized in two Zinc cluster transcription factors that control AOX expression both in \textit{P. anserina} (this work) and \textit{N. crassa} (CHAE \textit{et al.} 2007b). Interestingly, these two mutations activate the expression of the alternative oxidase and also of gluconeogenic genes.

**MATERIALS AND METHODS**

\textit{P. anserina} strains, growth conditions, transformation and genetic analysis: Except strain TS24 used for positional cloning, all the strains used in this study were derived from the s wild type strain (RIZET 1952). The \textit{gpd-aox} strain contains a transgenic copy of the \textit{aox} gene under the control of the strong constitutive \textit{P.anserina gpd} promotor associated with a hygromycin resistance cassette (LORIN \textit{et al.} 2001). The long-lived \textit{cox5::ble} and \textit{cyc1-1} strains have been described in DUFOUR \textit{et al.} (2000) and SELLEM \textit{et al.} (2007) respectively. The \textit{ΔPaKu70} strain inactivated for the \textit{KU70} mammalian ortholog, provides an efficient method to produce deletion mutants (EL-KHOURY \textit{et al.} 2008). The TS24 strain used for positional cloning was obtained from the progeny of crosses between the \textit{P. anserina S} (RIZET 1952) and the \textit{P. comata T} (ATCC 36713) strains. TS24 exhibits the wild type, fertile \textit{P. anserina} phenotype and retained, on chromosome III, at least 12 simple sequence repeat markers (SSR) characteristic of the \textit{P. comata} strain. The germination medium contains ground corn meal (50g/l), agar (12.5g/l) and ammonium acetate (4.4g/l). Minimal standard medium (M2) contains 1% dextrin as carbon source (ESSER 1974). When necessary, hygromycin, phleomycin, nourseothricin and antimycin A were added to the medium at 75 μg/ml, 10 μg/ml, 50 μg/ml and 10 μg/ml respectively. Transformation experiments were conducted as previously described (BERGES and BARREAU 1989) on protoplasts obtained by incubation with 40 mg/ml glucanex (Laffort). Genetic methods for \textit{P. anserina} have been described (ESSER 1974). For the construction of double mutant strains, the appropriate single mutants of opposite mating types were crossed. The \textit{Δrse2 Δrse3} strain carrying the two alleles inactivated by the same cassette conferring nourseothricin resistance and the double mutant strain \textit{rse2-1 rse3-1} carrying the 2 alleles that both confer the ability to grow without delay on a medium supplemented with antimycin A were identified by analysing the segregation of the cassette or the resistance to antimycin A in isolated asci.

Lifespan measurements: Lifespans were measured on M2 medium on three to five subcultures derived from two to five independent spores exhibiting a given genotype.
Cultures were grown in 30ml/30cm race tubes at 27°C in the dark. The lifespan of a strain was defined in centimeters as the mean length (given with standard errors) of growth of parallel cultures between the point of incubation of freshly germinated spores and the arrested edge of the dead culture. Survival curves, plotted as the percentage of surviving cultures in course of time, also defined the lifespan (in days) as the time at which 50% of the cultures are still alive.

**SSR markers and localisation of gene rse2:** Twelve single sequence repeats (SSR) markers overlapping 2 Mb on the long arm of chromosome III were found polymorphic between the *P. anserina* and *P. comata* isolates. Their characteristics markers are presented in Supplemental Material Table1. To position gene rse2, crosses between *(TS24) rse2+ (comata origin) and (s) rse2-1 (anserina origin)* parental strains provided us with a collection of monocaryotic spores for which linkage analysis was performed. The nature of the *rse2* allele was determined by growth on antimycin A, the nature of the twelve SSR markers was identified by PCR analysis. PCR amplifications were performed on rapid mini-preparations of DNA extracted from mycelium grown 24h after the germination of each spore.

**Nucleic acid and protein manipulation:** Southern blots were done using total DNA extracted by the minipreparation method (LECELLIER and SILAR 1994). Western blot analysis of the AOX protein was performed on isolated mitochondria as previously described (SELLEM *et al.* 2007). Immunochemistry was performed with an anti-AOX mouse monoclonal antibody generated against the AOX of *Sauromatum guttatum* (ELTHON *et al.* 1989). Additionally, blots were reprobed with an anti-βATPase rabbit antibody (a gift from J. Velours) as a standardization control. The bound antibodies were detected using an enhanced chemiluminescence detection system ( Pierce Supersignal West picochemiluminescent substrate). Quantifications of the signal intensity that reflects the amount of protein were performed using the ImageQuant program on at least three independent blots (Molecular Dynamics, Amersham Bioscience, Piscataway, NJ).

**Quantitative RT-PCR:** Total RNA from various strains grown for 48h on standard medium (1% dextrin) covered with cellophane disc, was extracted using the RNeasy plant kit (Qiagen) with RLT buffer and DNAase I according to manufacturer’s instructions except that mycelium was broken with glass beads in a Fastprep apparatus (40 sec, intensity 6.5). For qPCR analysis, 2 μg of RNA was reverse transcribed and random primed with oligo (dT)20 using the Supercript II reverse transcriptase (Invitrogen) according to the instructions of the supplier. Pairs of primers for PCR were developed for the *aox* (5’-GATGTCTGTCTCCCCATCGAC-3’/5’-GAGGAAATGTTGGCAGTGGT-3’), *gpd* (5’-
CACCGAGGACGAGATTGTCT-3'/5'-'TCAGGGAGATACCAGCCTTG-3'),  
*fbp* (5'-CACCAGTGACTTTACGCTCC-3'/5'-GGAGAATTGGAGGGCGTGGC-3') and  
*pck* (5'-ACAAACCATCCGACATGC-3'/5'-GGTCTTGTTTACTGTGTTGA-3') genes to give  
products of about 40-50bp in length. One primer of each set was designed across an  
exon/intron boundary to avoid amplification of any contaminating genomic DNA. The  
product of the first-strand cDNA reaction was diluted ten-fold before real-time PCR analysis.  
Amplifications were performed in duplicate in a LightCycler (Roche) using the LightCycler  
FastStart DNA MasterPLUS SYBR Green I kit (Roche) with no-reverse transcriptase controls  
to estimate the contribution of contaminating DNA. Amplification efficiencies were measured  
for each primer pair and every set of amplification reactions. For each strain, the levels of  
aox, *fbp* and *pck* transcripts were normalized using the *gpd* transcript level which was used as  
a standard because its expression remained stable in all the strains and conditions examined.  
At least three independent experiments were performed from one to three different RNA  
preparations. For a given strain and a given gene, results are expressed as the level of  
expression of this gene in this strain relative to the level of expression of this gene in the wild  
type strain.

**Cloning of rse2 and rse3 genes:** PCR amplifications of the *rse2* and *rse3* mutated genes  
were performed with pairs of primers 5'-GGCTCGAGGACGGGAACCGGGAACCAGGGAAG-  
3'/5'GGGGACTAGTCGAAGGGGCGGCATTGTG-3'and 5'CCCCCATGGCCGAGTAAATCTGGATTTTG-3'/5'-CCCAGATCTGCCGCGTGACCAGGACC-3' and cloned in the  
XhoI/Spe1 sites of the PBCHygro vector (SILAR 1995) or in the *NcoI/BglII* sites of the  
*pAPI508* vector (EL-KHOURY *et al.* 2008) respectively. Transformation of wild type  
protoplasts resulted in hygromycin or nourseothricin resistant strains purified through genetic  
crosses with the wild type.

**Inactivation of rse2 and rse3 genes:** Seven hundred nucleotides of the 5' and 200  
nucleotides of the 3' region of the *rse3* wild type gene were amplified with the primers 5'-  
GAAAGCGGCCGCGTGACCAGGACCAAG-3'/5'-GGGCCATGGCTCTATCTGGAGGGAGCTGGG-3'and  
5'-GGGAGATCTGGAGTGCAGTTATACTTGG-3'/5'-CACGCGGCCGCTTTTCGCCTCTTCTTTAAAC-3' respectively as described in EL-KHOURY *et al.* 2008 and  
cloned in the *BgIII/NcoI* sites of the pAPI508 vector containing the nourseothricin resistance  
cassette. Protoplasts of the Δ*KU70* strain were transformed and nourseothricin resistant  
transformants were isolated and purified through genetic cross with the wild type.  
Nourseothricin resistance co-segregated with antimycin sensitivity. Following the same  
strategy, *rse2* was inactivated using the primers 5'-AGGAAAAAACGGCGCCGCTTTGGGAAA
GGGGAGGAAG-3’ / 5’-GAAGATCGCAGTCGTTCGGCTTTGT-3’ for the 5’ region and
5’-AGGAAGCTTGGTGAGGCATCGACAAA-3’ / 5’-AGGAAAAAGCGGCCGCAAT
CCGCTCTCGGTCTT-3’ for the 3’ region and cloned in the HindIII/BglII sites of the
pAPI508 vector.

RESULTS

Mutations in genes rse2 and rse3 restore senescence in cox5::ble and cyc1-1 contexts:
Inactivation of complex III (cyc1-1) or complex IV (cox5::ble) leads to a spectacular increase
in lifespan associated with several phenotypic defects: alteration in germinating mycelium,
poorly coloured thin growing mycelium, reduction of the growth rate and female sterility
(DUFOR et al. 2000, SELLEM et al. 2007). To shed light on the parameters responsible for
these different characteristics, we isolated suppressor mutations able to improve the
phenotype of these mutants. Spontaneous revertants were obtained independently as sectors of
aerial fast growing mycelium from cox5::ble and cyc1-1 cultures. Most cultures grown in race
tubes led to such sectors. Two of them, sectors 2 and 3 were isolated from cox5::ble and cyc1-
1 cultures respectively. They were crossed with wild type in order to test the genetic basis of
the reversion and to obtain pure revertant strains (the sectors probably contain a mixture of
mutant and revertant nuclei). The presence of extragenic suppressors was revealed by
recovery in the progeny of these crosses of three types of ascospores: ascospores that
germinate to give sparse mycelium like the original mutant, ascospores that germinate
normally and ascospores displaying an intermediate germinating mycelium that gave rise to a
growing mycelium similar to that of the initial sectors. Genetic analysis of the two pure
revertant strains revealed that the two suppressor mutations named rse2-1 and rse3-1 were
unlinked to the original cox5::ble and cyc1-1 mutations. The characteristics of the revertants
and of the strains carrying the suppressor mutations dissociated from the initial respiratory
mutation are shown on Figure 1 and Table 1.

As shown in Table 1, the rse2-1 or rse3-1 mutations do not restore a wild type phenotype
to the cox5::ble and cyc1-1 mutants; however they considerably improve the germination of
the ascospores (germinating thalli of cox5::ble rse2-1 and cyc1-1 rse3-1 appeared more dense
and grew better than germinating thalli of cox5::ble and cyc1-1), they improve the aspect of
the growing mycelium that appears more aerial and coloured and they restore a growth rate of
0.48 ± 0.1 cm/day compared with 0.21 ± 0.01 cm/day for the mutants. In contrast they do not
restore female fertility to the cox5::ble and cyc1-1 mutants or normal ascospore pigmentation
to *cyc1-1*. As *P. anserina* crosses yield dicaryotic ascospores, the recovery of heterocaryotic *cox5::ble rse2-1/cox5::ble rse2* and *cyc1-1 rse3-1/cyc1-1 rse3* ascospores allowed us to test the dominance/recessivity of the suppressors and to conclude that they are dominant with respect to the improved phenotypes.

Interestingly, analysis of longevity of the *cox5::ble rse2-1* and *cyc1-1 rse3-1* strains revealed that the two suppressors also restored the senescence phenomenon (hence the name *rse* for Restorator of SEnescence). Longevity of the revertants was about 90 days (60 ± 10 cm) compared to about 17 days (11.3 ± 1.6 cm) for the wild type strain and more than 2 years (> 300 cm) for the *cox5::ble* and *cyc1-1* mutants. An analysis of the mtDNA content of the senescent revertant cultures revealed the presence of mtDNA rearrangements called senDNAs as in senescent wild-type cultures (ALBERT and SELLEM, 2002, BELCOUR et al. 1999). However in contrast to the wild-type strain in which senDNAα is systematically observed in large amount, the senDNAs in the revertants mainly originated from the γ region as previously shown in other mutants (LORIN et al. 2001 and Supplemental Figure 3).

Recombination between *cox5::ble* and *rse3-1* on one hand and between *cyc1-1* and *rse2-1* on the other hand revealed that either mutation, *rse2-1* or *rse3-1*, is able to suppress complex III and complex IV loss-of-function mutations.

**The mutations *rse2-1* and *rse3-1* are responsible for the constitutive expression of the alternative oxidase:** We previously showed that constitutive overexpression of the alternative oxidase in the *cox5::ble* and *cyc1-1* mutants improved mycelium aspect, growth rate and also restored the senescence process. This was demonstrated by expressing a fusion (*gpd-aox*) between the *gpd* promoter and the *aox* coding sequence in these mutants (LORIN et al. 2001; SELLEM et al. 2007). Because of the similarities between the effects of the *gpd-aox* transgene and the *rse2-1/ rse3-1* mutations, the level of expression of the alternative oxidase was examined in strains carrying these mutations. The *gpd-aox* strain was used as a control. The Western blot analysis (Figure 2) corroborated previous results (LORIN et al. 2001) showing that AOX is undetectable in the wild type grown under standard conditions whereas it is induced in long-lived respiratory mutants (*cyc1-1* for example in the Figure) and very strongly expressed in the *gpd-aox* strain. Our results show that it is also expressed in the strains carrying the *rse2-1* or *rse3-1* mutations. However, the AOX level is 2- to 3 fold lower in *rse2-1* and *rse3-1* strains than in strains carrying the *gpd-aox* transgene. These results were confirmed by (q)RT-PCR experiments shown in Figure 3. Expression levels of the *aox* gene were normalized to the *gpd* gene and the *aox* mRNA copy number was given a value of 1 in the wild type strain. Aox mRNA copy number increased about 20 fold in *rse3-1*, 40 fold in
rse2-1 and 60- to 80 fold in gpd-aox strains. In the cox5::ble (and cyc1-1) strain, aox transcript levels were increased about 20-fold compared to wild type. These levels were increased about 3-fold in the presence of rse2-1 or rse3-1 mutations and about 5-fold in the presence of the gpd-aox transgene. Altogether, these results are in accordance with data obtained by Western blot. The expression of the aox gene in rse2-1 and rse3-1 strains was also confirmed by testing the ability of these strains to grow on a medium containing antimycin A. Antimycin A is an inhibitor of complex III leading to induction of the alternative oxidase in wild type cells, allowing them to grow in presence of the drug after a delay necessary for the induction whereas the gpd-aox, rse2-1 and rse3-1 strains grow without delay on this medium because of the constitutive expression of AOX. Heterocaryotic strains rse2-1/rse2+ and rse3-1/rse3+ also grow without a delay on medium containing antimycin A, confirming the dominance of these mutations. The double mutant rse2-1 rse3-1 was constructed by genetic cross and it exhibited a phenotype very similar to that of each simple mutant indicating the absence of a synergistic effect between the two mutations.

The rse2 and rse3 genes encode two Zinc-cluster transcription factors: Since the strains carrying the wild type rse2+ and rse3+ or the mutated rse2-1 and rse3-1 alleles differ by their growth with or without delay on a medium containing antimycin A, the segregation of these alleles can be easily analyzed through crosses. Genetic analysis showed that rse2 and rse3 were localized on chromosomes III and IV respectively, near ura5 for rse2, near sir2 for rse3.

Taking advantage of the genome sequence of P. anserina (ESPAGNE et al. 2008) and of the characterisation on each of the seven chromosomes, of marker polymorphisms between the geographic strains P. anserina and P. comata, segregation analysis of gene rse2 with the markers linked to gene ura5 was undertaken. A total of 198 monocaryotic spores derived from the cross rse2-1 (anserina) X rse2+(comata) were generated. The rse2-1/rse2+ segregation was determined by growth on antimycin A and each spore was genotyped for 12 simple sequence repeat markers covering 2 Mb. All the polymorphic markers and the rse2 gene segregated in a 1:1 ratio. Of the 12 molecular markers, two always remained in parental association with the rse2 alleles. They covered about 250 Kb containing 67 predicted open reading frames (ORFs), 3 encoding putative transcription factors of which one (Pa_3_6340) is a zinc-cluster transcription factor containing the canonical motif CX2CX6CX5-12CX2CX6-8C. Genomic DNA was prepared from rse2-1 and wild type strains. PCR products that contained ORF Pa_3_6340 were generated and sequenced. The rse2-1 gene was shown to contain a single T to G substitution changing a Tyr into an Asp codon at position 300.
Sequence comparisons revealed that this protein is homologous to the recently reported AOD2 protein from *N. crassa*, which acts synergistically with another transcription factor of the zinc-cluster family, AOD5 (Chae et al. 2007b). This prompted us to search by BLAST the homolog of this protein in the *P. anserina* genome. The protein most related to AOD5 was Pa_4_8760 (54.7% identity) located on chromosome IV to which the *rse3-I* mutation was genetically assigned. Sequencing of the corresponding gene in wild type and *rse3-I* strains revealed a G to T substitution changing a Gly into a Val codon at position 642 in the *rse3-I* strain. The structure of the two genes and the position of the mutations are shown in Figure 4.

To confirm that the Y300D mutation in the *rse2-I* strain and the G642V mutation in the *rse3-I* strain are responsible for the constitutive expression of the AOX and the restoration of senescence in strains deficient for the III/IV respiratory complex, we took advantage of the dominance of the 2 suppressor mutations. The mutated genes were cloned in plasmids PBCHygro (Silar et al. 1995) and pAPI508 (El-Khoury et al. 2008) and introduced into a wild type strain by transformation. Hygromycin and nourseothricin resistant transformants resulting from a non-homologous integration were selected for each transformation. These *rse2+*(*rse2Y300D*) and *rse3+*(*rse3G642V*) transformants, which carried an endogenous wild type allele and an ectopic mutant allele, showed constitutive overexpression of the alternative oxidase (see *rse2+*(*rse2Y300D*) in Figure 3) revealing that that the introduction of the Y300D mutation in the *rse2* gene, or of the G642V mutation in the *rse3* gene, is sufficient to cause this phenotype. Furthermore strains with the *cox5::ble rse2+*(*rseY300D*) genotype obtained by genetic cross, exhibited the same growth phenotype and longevity as *cox5::ble rse2-I* (90 days compared to more than 300 cm for *cox5::ble rse2+*).

The *rse2* and *rse3* genes of eight other revertants derived from *cox5::ble* or *cyc1-I* cultures were sequenced. One revertant had a single base pair substitution changing a Glycine to Serine at position 303 in the *rse2* gene, only 3 amino acids from the *rse2Y300D* mutation (Figure 4). The seven other revertants carried wild type alleles for *rse2* and *rse3*.

The *rse2* and *rse3* gene products also activate the expression of gluconeogenic genes: The *rse2* and *rse3* gene products are conserved in several ascomycetes (see Supplemental Material Figures 1 and 2). RSE2 is homologous to AOD2 from *N. crassa* (53% identity), AcuM from *Aspergillus nidulans* (34% identity) and RDS2 from *S. cerevisiae* (28.8% identity). Recently it was shown that RDS2 in *S. cerevisiae* (Soontorngun et al. 2007), AcuM and AcuK in *A. nidulans* (Hynes et al. 2007) act as activators of the expression of genes encoding central enzymes in the gluconeogenic pathway, in particular phosphoenolpyruvate carboxykinase (PCK) and fructose-1, 6-biphosphatase (FBPase). PCK
catalyzes an early step in gluconeogenesis and converts oxaloacetate to phosphoenolpyruvate, FBPase catalyzes the final step in hexose monophosphate formation by dephosphorylating fructose-1,6-biphosphate to yield fructose-6-phosphate. This prompted us to examine the expression of the genes encoding these two enzymes in $rse2^{Y300D}$, $rse3^{G642V}$ and wild type strains by quantitative RT-PCR. Two CDS potentially encoding PCK (Pa_4_3160) and FBPase (Pa_4_9360) were identified by homology searches. RNA was isolated from the $rse2^{Y300D}$, $rse3^{G642V}$ and wild type strains grown on standard medium (containing 1% dextrin) and the abundance of transcript levels of the genes encoding Pa_4_9360 ($Papck$) and Pa_4_9360 ($Pafbp$) were examined in the three strains. The expression of the $aox$ gene was determined in parallel and the level of transcripts expressed from the constitutive $gpd$ gene was used as reference control. Figure 5 shows the expression level of each gene in a given strain compared to the expression level of the $gpd$ gene in the wild type strain. These experiments corroborate the results shown in Figure 3, an increase of $aox$ mRNAs levels of about ~40-fold in $rse2^{Y300D}$ and ~20-fold in $rse3^{G642V}$. They also reveal that the expression of $Papck$ and $Pafbp$ is significantly increased in the mutant stains: ~20-fold for $Papck$ in $rse2^{Y300D}$ and ~5-fold in $rse3^{G642V}$, ~5-fold for $Pafbp$ in $rse2^{Y300D}$ and ~2-fold in $rse3^{G642V}$.

To be sure that the activation of the $Papck$ and $Pafbp$ genes in the mutant strains does not result from a qualitative change of the properties of the mutated transcription factors, the expression level of the two genes and of the $aox$ gene was also tested in a wild type strain grown on antimycin A. As shown in Figure 5, the expression of the $aox$, $Papck$ and $Pafbp$ genes is increased 5-fold, 10-fold and 3-fold respectively in the wild type strain grown on antimycin A, indicating that $Papck$ and $Pafbp$ are indeed co-regulated with the $aox$ gene.

**Genes $rse2$ and $rse3$ are non-essential in respiratory competent strains grown in standard conditions but are both essential for induction of the alternative oxidase:** In order to determine the function of these transcription factors more clearly, strains deleted for the $rse2$ and $rse3$ genes were constructed by replacement with a cassette conferring resistance to nourseothricin (EL-KHOURY et al. 2008). The correct replacement was verified by Southern blot analysis (data not shown). Using $rse2::nat$ ($\Delta rse2$) and $rse3::nat$ ($\Delta rse3$) as parents in genetic crosses, we subsequently isolated the double-deleted strain $\Delta rse2 \Delta rse3$. All these strains were viable and displayed no impairment of growth, pigmentation or fertility on standard synthetic M2 medium (same mycelium aspect as the wild-type strain cf. Figure 1). These results demonstrate that $rse2$ and $rse3$ are non-essential genes in respiratory competent strains grown in standard conditions. However none of these strains, including the simple
Δrse2 and Δrse3 deletions, are able to induce the aox gene. This was demonstrated in two ways. First, none of the deleted strains is able to grow on a medium containing antimycin A even after the lag necessary for the wild type to begin growth. Second, the association by genetic cross of mutations cox5::ble and Δrse2 or cox5::ble and Δrse3 led to “lethal” spores that were unable to germinate. These results indicate that the inactivation of either of the two genes, rse2 and rse3, prevents the expression of the aox gene under inducing conditions and therefore that the two proteins RSE2 and RSE3 are both required for aox induction. Finally, we constructed the Δrse2 rse3<sup>G642V</sup> and rse2<sup>Y300D</sup> Δrse3 strains in which the deleted allele of one gene is associated with the mutated allele of the other. None of these strains was able to grow on a medium containing antimycin A, demonstrating that even when one of the two proteins is present in its mutant form, the other protein is still required to induce the alternative oxidase.

The RSE2 and RSE3 transcription factors are involved in lifespan control even in the absence of mitochondrial dysfunction: As stated above, the modification of the transcriptional pattern in the rse2<sup>Y300D</sup> and rse3<sup>G642V</sup> strains revealed that the suppressor mutations modify the expression or the activity of the RSE2 and RSE3 products. We examined the abundance of rse2 and rse3 transcript levels in the wild type, rse2<sup>Y300D</sup> and rse3<sup>G642V</sup> strains by quantitative RT-PCR experiments. Transcripts of both genes were virtually unchanged in the three strains (data not shown). This strongly supports the hypothesis of a modification of the activity and not of the abundance of the RSE2 and RSE3 products in the rse2<sup>Y300D</sup> and rse3<sup>G642V</sup> strains. In a respiratory deficient context, cox5::ble or cyc1-1, the rse2<sup>Y300D</sup> and rse3<sup>G642V</sup> mutations are responsible for severe lifespan reduction. We investigated whether in respiratory competent strains, the rse2<sup>Y300D</sup> and rse3<sup>G642V</sup> mutations and the resulting gene expression modifications also lead to a modified lifespan. As shown in Figure 6, the rse2<sup>Y300D</sup> and rse3<sup>G642V</sup> strains displayed a decreased lifespan compared to wild type (about 12 days versus 17 days) whereas deletion of either of the rse genes results in an increased lifespan (about 30 days for Δrse2 and 35 days for Δrse3). These results suggest that the RSE products contribute to shortening lifespan and that the gene expression modifications due to the rse2<sup>Y300D</sup> and rse3<sup>G642V</sup> mutations accentuate this effect. It is very unlikely that the decreased lifespan of the rse2<sup>Y300D</sup> and rse3<sup>G642V</sup> strains results from the overexpression of the AOX, since as previously shown (LORIN et al. 2001 and Figure 6), we confirmed that the gpd-aox strain carrying the gpd-aox transgene displays the same longevity as the wild type.
Characterization of mutations in two conserved Zinc-cluster proteins that control the expression of the alternative oxidase and gluconeogenic genes: We report in this study the characterization of mutations in two transcription factor genes, \textit{rse2} and \textit{rse3} (for Restorator of SEnsescence), each encoding a Zinc-cluster protein controlling the induction of the alternative oxidase and the expression of gluconeogenic genes. These mutations were selected in a screen for suppressors of the long-lived respiratory mutants \textit{cox5::ble} and \textit{cyc1-1}. They partially suppress the detrimental effects and restore the senescence process in these mutants. The three mutations reported here are located in two different genes, several other suppressors non allelic to \textit{rse2} and \textit{rse3} were obtained but have not yet been studied in detail. Thus, the screen was not exhaustive. The two gene products contain Zn(2) Cys(6) binuclear cluster DNA-binding domains. Database searches and recent published data reveal that these genes are present in other ascomycetes. \textit{RSE2} corresponds to \textit{AOD2} from \textit{N. crassa}, \textit{AcuM} from \textit{A. nidulans} and \textit{RDS2} from \textit{S. cerevisiae}. \textit{RSE3} corresponds to \textit{AOD5} from \textit{N. crassa} and \textit{AcuK} from \textit{A. nidulans}.

We show here that the mutations \textit{rse2}^{Y300D} and \textit{rse3}^{G642V} confer higher levels of mRNA of the alternative oxidase and gluconeogenic genes compared to the wild type strain and are dominant, strongly suggesting that they correspond to gain-of-function mutations. The mutations \textit{rse2}^{Y300D} and \textit{rse2}^{G303S} are not located in a conserved predicted functional domain (SCHJERLING and HOLMBERG 1996); however, their clustering pinpoints one region in \textit{RSE2} with potential significance for the function of this transcription factor and indicates that the integrity of this region is necessary to keep the transcription factor in a less active form. Both the affected residues Y300 and G303 are conserved in \textit{aod2} (\textit{N. crassa}), \textit{RDS2} (\textit{S. cerevisiae}) and \textit{acuK} (\textit{A. nidulans}) and lie in a region of the protein highly conserved between the four organisms. In the same way, the G642 of \textit{rse3} is conserved in \textit{aod5} (\textit{N. crassa}) and \textit{acuK} (\textit{A. nidulans}) and belongs to a short region that is conserved among the three organisms (see Supplemental Material, Figures 1 and 2). The question how these single amino acid substitutions modulate the activity of the RSE2 and RSE3 transcription factors is unresolved. Though the levels of \textit{rse2} and \textit{rse3} mRNA in the mutant strains are unchanged, hypotheses such as an increased protein stability conferred by the mutations cannot be excluded. However some other interesting possibilities can be proposed. For instance the \textit{rse3}^{G642V} mutation in the C-ter of RSE3 could make the transactivation domain more accessible or
increase its intrinsic activation properties (SCHJERLING and HOLMBERG 1996). The mutations \(rse2^{Y300D}\) and \(rse2^{G303S}\) in RSE2 could reveal a latent activation domain or could change an interaction with an inhibitory protein. The hypothesis in which conserved motifs in Zinc cluster proteins could have an inhibitory role is based on several studies in \(S.\ cerevisiae\) showing that deletion of these motifs renders these proteins constitutively active (MACPHERSON et al. 2006).

Regarding the relationships between RSE2 and RSE3, the observation that the \(\Delta rse2\ rse3^+\), \(\Delta rse3\ rse2^+\), \(\Delta rse2\ rse3^{G642V}\) and \(rse2^{Y300D}\ \Delta rse3\) strains are unable to induce the alternative oxidase, unambiguously demonstrate that both proteins are required for this induction even when one of them is present in a mutated form. This result agrees with observations reported in \(N.\ crassa\) in which neither the \(aod2\) nor \(aod5\) mutants are able to induce AOX (DESCHENEAU et al. 2005) and in \(A.\ nidulans\) in which neither the \(acuK\) nor \(acuM\) mutants are able to induce PCK (HYNES et al. 2007). In \(N.\ crassa\), electrophoretic mobility shift assays showed that AOD2 and AOD5 act synergistically to bind an alternative oxidase induction motif (AIM) present in the promoter of the \(aod-1\) gene which encodes the alternative oxidase. These data support the hypothesis that the two proteins interact with each other (CHAE et al. 2007b). The AIM motif consists of one pair of CGG repeats separated by 7bp and is essential for the inducible expression of the \(aod-1\) gene. It is present in the upstream sequence of the \(aox\) gene of \(P.\ anserina\) and other Sordariales (CHAE et al. 2007a), however, it is absent from the 900 bp upstream coding sequence of the \(Papck\) and \(Pafbp\) genes whose expression is also controlled by RSE2 and RSE3. Several explanations can be proposed for this observation. One possibility is that there is a cryptic motif that we have not spotted in the promoter of these genes. Another one is that activation of these genes requires other factors interacting with RSE2 and RSE3 and determining DNA-binding specificity. A third hypothesis is that RSE2 and RSE3 are indirect activators of the gluconeogenic genes by regulating the production of an inducing molecule.

**RSE2, RSE3 and the control of longevity in \(P.\ anserina\):** We have previously shown that in \(P.\ anserina\), inactivation of genes encoding components of the cytochrome pathway leads to the induction of the alternative oxidase and to a spectacular increase of lifespan (DUFOUR et al. 2000; SELLEM et al. 2007). We have also shown that the introduction of the \(gpd-aox\) transgene in the long-lived \(cox5::ble\) and \(cyc1-1\) leads to increased expression of the alternative oxidase in comparison with induced expression of this enzyme in the non-transgenic \(cox5::ble\) mutant and to the restoration of senescence associated with an improvement of the phenotype (LORIN et al. 2001; SELLEM et al. 2007). We show here that in
response to a block of the cytochrome pathway, there is induction of the \textit{aox} gene but also of the \textit{pek} and \textit{fbp} gluconeogenic genes and that this induction is under the control of the two Zinc cluster proteins RSE2 and RSE3. These results therefore question the reasons for the very great lifespan of the respiratory deficient mutants of \textit{P. anserina} and the role of the different pathways that are induced in the control of longevity. The observation that gain-of-function mutations of genes \textit{rse2} and \textit{rse3} lead to a decreased lifespan whereas deletion of these genes, in particular \textit{rse3}, results in an increased lifespan, strongly suggests that some (direct or indirect) targets of the RSE2 and RSE3 proteins contribute to shortening lifespan. Though the \textit{aox} gene is greatly induced in the \textit{rse2^{Y300D}} and \textit{rse3^{G642V}} mutants, it seems unlikely that the reduction of lifespan of these mutants results from this induction since the \textit{gpd-aox} transgenic strains show no reduction of lifespan. The \textit{aox} gene is therefore probably not involved in the control of lifespan in a respiratory competent context. In contrast, in a \textit{cox5::ble} or \textit{cyc1-1} context, we found a correlation between the mycelium phenotype, longevity and AOX levels. Increased \textit{aox} gene expression leads to a reduction in life extension and counters the detrimental phenotypic effects due to the \textit{cox5::ble} or \textit{cyc1-1} mutations. This positive correlation between the amount of AOX and the improvement of the phenotype of \textit{cox5::ble} and \textit{cyc1-1} mutants supports our proposed mechanism, that increasing the electron flow through the alternative pathway is accompanied by increased oxygen consumption and increased ATP formation at the first coupling site (LORIN \textit{et al.} 2001, 2006).

Many studies have demonstrated the central role for metabolic regulation in the aging process. While it is impossible to highlight all such studies, it is worth noting that a simple reduction of available glucose in the media results in life extension in \textit{P. anserina} (MAAS \textit{et al.} 2004) and yeast (LIN \textit{et al.} 2000). More relevant to our discussion is that in \textit{S. cerevisiae}, a metabolic shift from glucose metabolism and fermentation toward respiration plays a central part in this life extension (LIN \textit{et al.} 2002). In the same way, caloric restriction induces a metabolic reprogramming characterized by a transcriptional shift toward energy metabolism and up-regulation of gluconeogenesis in mouse skeletal muscle (LEE \textit{et al.} 1999). Recently, transcript profiling data from \textit{C. elegans} dauer larvae and long-lived \textit{daf-2} mutant adults revealed increased expression of genes encoding gluconeogenic enzymes (MCELWEE \textit{et al.} 2006). It is therefore possible that gluconeogenesis is a conserved pathway in the control of longevity in a wide spectrum of organisms.

A block of the respiratory cytochrome pathway is expected to lead to a wide spectrum of transcriptional changes in the cell. Our study shows that the two Zinc cluster proteins RSE2 and RSE3 are involved in this transcriptional response by activating the expression of the
genes encoding the alternative oxidase and major enzymes of gluconeogenesis. In order to identify the other genes whose expression is regulated by RSE2 and RSE3, transcriptome profiling of the strains carrying the different alleles (wild type, gain of function, deleted) of genes rse2 and rse3 will be conducted. This should allow us to gain insights into the physiological role of these Zinc cluster proteins and especially their role in the cellular response to a defect in respiratory function. We are convinced that these data will clarify the parameters involved in the control of lifespan in P. anserina.

Acknowledgments

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KUJOTh, G. C., C. LEEUWENBURGH, and T. A. PROLLA, 2006 Mitochondrial DNA mutations and apoptosis in


FIGURE LEGENDS

FIGURE 1. — Mycelium aspect of the wild type, \textit{cox5::ble} and \textit{cox5::ble rse2-1} strains. Petri plates of M2 medium were inoculated with an explant of each strain and incubated for 10 days at 27°C. The wild type strain exhibits a dense, aerial, colored mycelium and is fast-growing whereas the \textit{cox5::ble} mutant exhibits a thin, poorly colored mycelium and is slow growing. The \textit{cox5::ble rse2-1} revertant exhibits an intermediate phenotype.

FIGURE 2. — Western blot analysis of the AOX protein. Mitochondria (10 µg of mitochondrial protein) were extracted from the wild type (WT), \textit{cyc1-1}, \textit{rse3-1}, \textit{rse2-1}, \textit{gpd-aox}, \textit{cyc1-1 gpd-aox}, \textit{cyc1-1 rse3-1} strains and loaded on a 12% SDS-PAGE acrylamide gel. The AOX was revealed with a mouse antiserum against \textit{Sauromatum guttatum} AOX provided by T. Elthon. As an internal control, the blot was reprobed with a rabbit anti β-ATPase provided by J. Velours.

FIGURE 3. — Relative abundance of \textit{aox} transcripts. For each strain, the levels of \textit{aox} and \textit{gpd} transcripts were determined by quantitative RT-PCR performed on 1 to 3 different RNA preparations (1 to 3 replicates). For each experiment, the level of \textit{aox} transcripts was normalized using the level of \textit{gpd} transcripts as a reference. The diagram shows in each strain, the level of \textit{aox} transcripts relative to the level of \textit{aox} transcripts in the wild type. The error bars correspond to standard error. The \textit{rse2+(rse2Y300D)} strain corresponds to a strain in which an ectopic copy of the mutated \textit{rse2Y300D} has been integrated.

FIGURE 4. — Structure of the \textit{rse2} and \textit{rse3} genes. The amino acids mutated in the \textit{rse2} gene (upper part) and the \textit{rse3} gene (lower part), are indicated. Exons (E) are shown as solid boxes and introns as solid lines. The first and the last amino acids of each protein are indicated. Sites within exons that contain a motif identified as a possible zinc cluster are indicated by thin lines below the genes.

FIGURE 5. — Quantification of \textit{fbp}, \textit{pck} and \textit{aox} expression. Total RNA was extracted from cultures of the wild type strain grown under normal conditions (wt: black) or in the presence of antimycin A (10µg/mL) (wt-ant:white), and from cultures of the \textit{rse2Y300D} (light grey) and \textit{rse3G642V} (dark grey) mutants grown under normal conditions. qPCR reactions were performed on cDNA to quantify the level of \textit{fbp}, \textit{pck} and \textit{aox}, \textit{gpd} transcripts in each strain. Experiments were performed at least three times. As in Figure 3, in each experiment, the level of \textit{fbp}, \textit{pck} and \textit{aox} transcripts was normalized using the level of \textit{gpd} transcripts as reference. The diagram shows the level of \textit{fbp}, \textit{pck} and \textit{aox} transcripts in the different strains and culture conditions relative to the level of these transcripts in the wild type. The error bars correspond to standard error.
FIGURE 6. — Lifespan analyses. For each genotype at least 18 sub-cultures (representing the two mating types) were grown on M2 medium at 27°C in race tubes. Data were plotted as cumulative survival in time using Kaplan-Meier estimates. In parentheses is the mean longevity in centimeters ± standard error.
SUPPLEMENTAL MATERIAL:

Supplemental Figure 1. Alignment of RSE2 with homologs from *Neurospora crassa* (Nc), *Aspergillus nidulans* (An) and *Saccharomyces cerevisiae* (Sc). Black and grey highlights indicate identical and similar residues, respectively. Black circles indicate the zinc cluster domain. Stars indicate the position of the mutations Y300D and G303S.

Supplemental Figure 2. Alignment of RSE3 with homologs from *Neurospora crassa* (Nc), *Aspergillus nidulans* (An) and *Saccharomyces cerevisiae* (Sc). Same legend as in Figure 1A except that the star indicates the position of the mutation G642V.

Supplemental Figure 3. (A) *Hae*III restriction patterns of mtDNA extracted from independent senescent cultures of *cyc1-1 rse3-1* (1, 2, 3) and of wild type (w) strains whose lifespans were 35, 44, 37 and 9.6 cm respectively. (B) and (C) Southern hybridization with ³²P labeled probes corresponding to the α (B) or γ (C) region of the *P. anserina* mitochondrial chromosome (Albert and Sellem, 2002). Asterisks point out non-rearranged chromosomal *Hae*III fragments. Arrows point out fragments absent in juvenile wild-type cultures corresponding to junctions of circular senDNAs. Position of senDNAα is indicated by α. The senescent wild-type culture (w) only contained senDNAα whereas the 3 senescent cultures of *cyc1-1 rse3-1* only contained senDNAγ. The senDNAα detected on the blot hybridized with the γ probe (α) is the result of the repробin.
Supplemental Table 1. Characteristics of the 12 SSR markers used for the genetic linkage analysis of rse2
wild type

cox5::ble

cox5::ble rse2-1
**TABLE 1**  
Phenotypic properties of the *cox5::ble* and *cyc1-1* respiratory deficient mutants and of the *rse2-1* and *rse3-1* suppressors

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th><em>cox5::ble</em></th>
<th><em>cyc1-1</em></th>
<th><em>cox5::ble</em></th>
<th><em>cyc1-1</em></th>
<th><em>rse2-1</em></th>
<th><em>rse3-1</em></th>
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<tr>
<td><strong>Mycelium aspect</strong></td>
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<tr>
<td><strong>Germination rate</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.60 ± 0.03</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.48 ± 0.10</td>
<td>0.48 ± 0.11</td>
<td>0.50 ± 0.04</td>
<td>0.51 ± 0.04</td>
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<td><strong>Growth rate</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>(cm/day)</td>
<td></td>
<td>0.60 ± 0.03</td>
<td>0.21 ± 0.01</td>
<td>0.21 ± 0.01</td>
<td>0.48 ± 0.10</td>
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<td>0.50 ± 0.04</td>
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<td>sterile</td>
<td>sterile</td>
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<td>fertile</td>
</tr>
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</table>

<sup>a</sup> This phenotype exhibits a variable penetrance (SELLEM et al. 2007); <sup>b</sup> germination rate is given as +++ for wild type thalli, + for sparse and slow growing thalli and ++ for thalli of intermediate phenotype on germination medium; <sup>c</sup> growth rates are mean values ± standard deviation.