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A potential impact of DNA repair on ageing and lifespan in the ageing model organism *Podospora anserina*: Decrease in mitochondrial DNA repair activity during ageing

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Summary

The free radical theory of ageing states that ROS play a key role in age-related decrease in mitochondrial function via the damage of mitochondrial DNA (mtDNA), proteins and lipids. In the sexually reproducing ascomycete *Podospora anserina* ageing is, as in other eukaryotes, associated with mtDNA instability and mitochondrial dysfunction. Part of the mtDNA instabilities may arise due to accumulation of ROS induced mtDNA lesions, which, as previously suggested for mammals, may be caused by an age-related decrease in base excision repair (BER).

Alignments of known BER protein sequences with the *P. anserina* genome revealed high homology. We report for the first time the presence of BER activities in *P. anserina* mitochondrial extracts. DNA glycosylase activities decrease with age, suggesting that the increased mtDNA instability with age may be caused by decreased ability to repair mtDNA damage and hence contribute to ageing and lifespan control in this ageing model.

Additionally, we find low DNA glycosylase activities in the long-lived mutants grisea and ΔPaCox17::ble, which are characterized by among others low mitochondrial ROS generation.

Overall, our data identify a potential role of mtDNA repair in controlling ageing and life span in *P. anserina*, a mechanism possibly regulated in response to ROS levels.

Keywords

DNA repair; Mitochondria; Ageing; Base excision repair; DNA glycosylases; *Podospora anserina*

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1. Introduction

Reactive oxygen species (ROS) are generated in the electron transport chain and induce damage to cellular components, such as DNA, proteins and lipids and hereby alter their stability and function. According to the mitochondrial free radical theory of ageing, the accumulation of these alterations over time will contribute to deleterious cellular changes leading to dysfunction of mitochondria, of cells and ultimately of the organism, and thus the damage induced alterations contribute to the ageing process (Harman 1973; Harman 1991). A decrease in mitochondrial function and an increase in the number of defective mitochondrial DNA (mtDNA) molecules have been found to accompany ageing in many organisms (reviewed in (Wallace 1999; Osiewacz 2002a)). Due to its close proximity to the sites of ROS generation, mtDNA is subject to continuous oxidation and it has been proposed that mtDNA contain steady-state levels of oxidatively induced DNA lesions, which are several-fold higher than those found in nuclear DNA (Richter et al. 1988;Barja and Herrero 2000). The level of oxidatively induced mtDNA lesions increases with age (Ames et al. 1993), an increase which is more dramatic than the age-related increase of DNA lesions in the nucleus (de Souza-Pinto et al. 2001;Madia et al. 2007). As many of the oxidatively induced lesions are mutagenic, the oxidative attack by ROS is an important source of mtDNA mutations. More DNA mutations (including mtDNA mutations) are found in cells from old than young humans and mice, indicating that DNA lesions accumulate as mutations with age (Finette et al. 1994;Hamilton et al. 2001;Vermulst et al. 2007;Vermulst et al. 2008). For these reasons, accumulation of ROS induced oxidative lesions in mtDNA is considered a major cause of accumulation of mtDNA mutations, increased mtDNA instability and mitochondrial dysfunction with age (Shigenaga et al. 1994; Wallace 1999).

Several eukaryotic model organisms have been used for studying the impact of ROS, mtDNA instability and mitochondrial function on ageing and life span. The sexually reproducing filamentous ascomycete *Podospora anserina* (*P. anserina*) has a limited lifespan and has been the subject of many studies, revealing that the ageing process in this organism is influenced greatly by processes such as respiration, ROS generation and scavenging, mtDNA stability, mitochondrial protein import, mitochondrial fusion and fission and apoptosis (Osiewacz 2002b;Gredilla et al. 2006;Maas et al. 2007;Scheckhuber et al. 2007;Hamann et al. 2008). The ageing process in *P. anserina* thus displays many similarities to that seen in higher eukaryotes (for reviews see (Osiewacz and Hermanns 1992;Osiewacz and Borghouts 2000;Osiewacz 2002a; Osiewacz 2004;Osiewacz and Scheckhuber 2006;Scheckhuber and Osiewacz 2008)).

Advantageously, in *P. anserina* it is easier than in for example mice, to modify the rate of ageing by manipulating different cellular processes or by generating mutant organisms in order to modify ageing and lifespan and hereby investigate the mechanisms underlining these processes. Thus, *P. anserina* is an excellent ageing model for studying basic mechanisms involved in organism ageing.

In senescent *P. anserina* cultures a closed circular mtDNA structure (named plDNA or senα) accumulates and homologous recombination between short dispersed direct repeats and deletion of large parts of the mtDNA take place (Stahl et al. 1978;Cummings et al. 1979;Belcour et al. 1981;Kück et al. 1981;Osiewacz and Esser 1984). Similar age-related rearrangements have been reported in other organisms (reviewed in (Wallace 1999)), including mammals (Linnane et al. 1989;Cortopassi and Arnheim 1990;Katayama et al. 1991;Osiewacz and Hermanns 1992;Corral-Debrinski et al. 1992), indicating that the age-related decrease in mtDNA stability in *P. anserina* could be caused by a similar age-related accumulation of DNA lesions as observed in mammals.

The electron transport chain (ETC) of *P. anserina* resembles the ETC found in higher eukaryotes (Krause et al. 2004;Gredilla et al. 2006) and its composition is an important determinant for lifespan of the organism. Loss of function of the transcription factor GRISEA or of the copper chaperone PaCOX17 leads to increased lifespan as compared to wild type. The mean lifespan for the wild type strain 's' is approximately 25 days, while it for the grisea mutant and ΔPaCox17 knock out strain is 40 and 375 days, respectively (Prillinger and Esser 1977;Stumpferl et al. 2004). Activity of the Cytochrome c oxidase (complex IV) of the ETC depends on copper. In the mutants, the delivery of copper to Cytochrome c oxidase is affected; in grisea it is partially impaired, whereas in ΔPaCox17¨ble it is severely impaired. The reduced level of active Cytochrome c oxidase is, however, not lethal due to a shift from the copper/Cytochrome c oxidase dependent to an iron/alternative oxidase dependent electron transport chain (a shift which is partial for grisea). A characteristic of this shift in electron transport chain is increased mtDNA stability (Borghouts et al. 2001;Stumpferl et al. 2004).

It has been suggested that age-related accumulation of DNA lesions is caused by an age-related change in DNA repair mechanisms (Chen et al. 2002). Indeed, an age-related decrease in base excision repair (BER) has been observed in rats and mice (Chen et al. 2002;Imam et al. 2006;Gredilla et al. 2008). BER is the main pathway for repair of small DNA modifications caused by alkylation, deamination and oxidation, and it is the main DNA repair pathway found in mitochondria (reviewed in (Bohr 2002)). The BER pathway includes four distinct steps; recognition and removal of the modified base, cleavage of the DNA backbone, incorporation of nucleotide(s) into the gap, and finally ligation. DNA glycosylases recognize and remove the modified base, they are lesion specific and either mono- or bi-functional. The former remove the modified base rendering an abasic site, which is then processed by AP endonuclease (APE) via its lyase activity, while bi-functional glycosylases contain lyase activity in addition to their damage incision activity. The activities of a number of DNA glycosylases have been reported to decrease during ageing in mammals (Chen et al. 2002;Imam et al. 2006).

The subsequent part of the BER pathway follows one of two sub pathways: short- or long-patch BER. The short-patch BER involves the incorporation of a single nucleotide into the gap by a DNA polymerase followed by ligation. In long-patch BER, several nucleotides are incorporated, which is followed by cleavage of the resulting 5′-flap (by FEN1) and ligation (reviewed in (Bohr 2002)). That BER activity in the mitochondria is important, is evident in knock out mice for the repair activity of the mitochondrial specific DNA polymerase γ. These mice have decreased life span, show premature ageing (such as early onset of kyphosis and osteoporosis) and have increased levels of point mutations (Trifunovic et al. 2004). Knock out mice for any of the DNA glycosylases on the other hand display no apparent ageing phenotype (e.g. (Klungland et al. 1999;Nilsen et al. 2000)), probably due to backup mechanisms between the DNA glycosylases (Karahalil et al. 2003). The BER activities are conserved among eukaryotes (Taylor and Lehmann 1998), however, very little is known about DNA repair processes in *P. anserina.* An endo-exonuclease named p49 with catalytic properties similar to the mammalian Flap endonuclease 1 (FEN1) has been purified from *P. anserina* mitochondria (Bouex et al. 2002) and has been shown to be stimulated by recombinant human proliferating cell nuclear antigen (PCNA) (Laquel-Robert and Castroviejo 2003), possibly indicating DNA repair to take place in *P. anserina*.

Several DNA lesions arise from oxidative stress. 8-oxo-2′-deoxyguanosine (8oxodG), an oxidized guanine, is highly mutagenic (Grollman and Moriya 1993). 8oxodG has been found to accumulate in mtDNA with age in both mice, rats and humans (Richter et al. 1988;Mecocci et al. 1993;Takasawa et al. 1993; Lindahl 1993;Hayakawa et al. 1993;Short et al. 2005). Deamination is enhanced under oxidative conditions (Lindahl et al. 1993), and deoxy-Uracil (dUracil) is a deamination product of cytosine. If unrepaired, dUracil may result in a U:G mispair, and since uracil can pair with adenine it might give rise to mutagenic C→T transition

upon DNA replication. Moreover, transcriptional bypass of 8oxodG and dUracil by RNA polymerase can lead to altered transcription (Lindahl and Barnes 2000;Kuraoka et al. 2003). 5-hydroxy-deoxyuracil (5OHdUracil) is an oxidation product of cytosine and can form stable base pairs with all four bases, and is hence capable of causing both transition and transversion mutations (Thiviyanathan et al. 2005). Obviously, due to the mutagenic potential of these DNA lesions, it is important that cells can repair such lesions.

The fact that *P. anserina* shows decreased mtDNA stability with age makes it compelling to use this ageing model organism for studying the possible age-related change(s) in mtDNA repair and to elucidate the impact of this maintenance pathway in a model that is well suited for experimental manipulation at the organism level.

In the present study, we first tested *P. anserina* mitochondria for the presence of the activities of the first two steps of the BER pathway (recognition/removal of the damaged base and cleavage of the DNA backbone). We assayed for the recognition and removal of three different DNA lesions for which three different DNA glycosylases show preferential specificity; 8oxodG, dUracil and 5-OHdUracil, which in mammalian mitochondria are removed by 8-oxoguanine DNA glycosylase (OGG1), Uracil DNA glycosylase (UNG1) and endonuclease III homologue 1 (NTH1), respectively. When using a DNA substrate containing a dUracil lesion, the combined effects of UNG1 and APE are measured, since UNG1 is a monofunctional DNA glycosylase. Finally, we studied the repair of 5-OHdUracil in a transcription/replication DNA structure, since such a lesion may lead to single strand breaks during transcription/replication possible obscuring these processes (reviewed in (Hazra et al. 2007)). Such a DNA substrate has been described to be preferentially repaired by Nei endonuclease VIII-like homologue 1 (NEIL1) (Dou et al. 2003), which has been found to be present in mammalian mitochondria (Hu et al. 2005).

Here, we report for the first time the presence of DNA repair activity in *P. anserina*, *as we* find repair in its mitochondria. We investigated the DNA glycosylase activities in the mitochondria of both juvenile and senescent *P. anserina* and our investigation indicates that the mtDNA glycosylase activities decrease with age possibly contributing to the increased mtDNA instability with age, and supporting a causal link between mtDNA repair and ageing and lifespan control in *P. anserina*. In addition to studying the possible age-related changes in DNA repair, we also investigated the DNA glycosylase capacities in the mitochondria of the long-lived mutants grisea and ΔPaCox17::ble, which are characterized by high mtDNA stability. Our results show that compared to the wild type these mutants posses low mtDNA glycosylase activities.

2. Materials and Methods

2.1. Sequence alignments

The sequences for the *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Homo sapiens* DNA glycosylases and AP endonucleases from the NCBI database were aligned to the *P. anserina* genome using the http://podospora.igmors.u-psud.fr/blast_ol.html webpage. The protein vs. nucleotide (blastn), podospora anserina [v.2] blastn, tblastn, tbalstx criteria were used. The following protein sequences were used for the alignments: *Saccharomyces cerevisiae:* NP_013651.1 (Ogg1p), P12887 (Ung1p), NP_009387 (Ntg1p), P22936 (Apn1p) and NP_009534 (Apn2p), *Schizosaccharomyces pompe:* NP_587889 (ung1), NP_593210 (nth1), NP_588189 (apn1) and P87175 (apn2) and H*omo sapiens:* NP_058214.1 (OGG1), NP_003353.1 (UNG1), NP_002519 (NTH1), Q96FI4 (NEIL1), NP_542380.1 (APE1) and NP_055296 (APE2). For construction of Figure 1, the human and yeast Nth1/Ntg1 sequences were aligned using the www.expasy.ch/tools/sim-prot.html web page (default criteria).

2.2. Strains

In the present study the wild type '*s*' (Esser 1974), the mutant grisea (Esser and Keller 1976) and the mutant ΔPaCox17:ble (Stumpferl et al. 2004) of *P. anserina* were studied. Juvenile cultures were derived from a mononucleate ascospore. Culture conditions were as described by Gredilla et al (Gredilla et al. 2006). Senescent cultures were generated by growth of mycelia according to Hamann et al (Hamann et al. 2007). Mitochondria of the investigated long-lived mutants were in the juvenile state.

2.3. Purification of whole protein samples

5g of mycelium was placed under liquid nitrogen, homogenized using a mortar and pestle followed by resuspension in cold extraction buffer (1mM EDTA, 20mM HEPES (pH 7.4), 2mM DTT, 1:1000 Protease Inhibitor Cocktail (Calbiochem, San Diego)). The homogenized mycelium was subsequently centrifuged at 13,000g for 10min (at 4°C) and the supernantant was collected. Slowly NH_4SO_4 was added to the supernantant until reaching an end concentration of 5M. The mixture was stirred for 1 hour at 4°C. Finally, the supernantant was centrifuged at 7000g for 50min (at 4°C), the pellet was resuspended in 0.5-1ml resuspension buffer (1mM EDTA, 20mM HEPES (pH 7.4), 5mM DTT 10% glycerin, 1:1000 Protease Inhibitor Cocktail (Calbiochem, San Diego)) and the protein concentration was determined by Lowry (Lowry et al. 1951). The whole protein samples were stored at −80°C until use.

2.4. Purification of mitochondria

All steps were carried out at 4°C as previously described (Gredilla et al. 2006). Briefly, 5g of mycelium was homogenized in isolation buffer (10mM Tris (pH 7.5), 1mM EDTA, 0.33M sucrose, 0.2% BSA, 1mM PMSF, 5mM DTT, 1:1000 protease inhibitor cocktail (Calbiochem, San Diego) and two drops of antifoam (Sigma, Munich) with a glass-beater. The homogenized mycelium was filtered through a 60μm filter and spun down at 600g for 10 minutes (min) in order to remove intact mycelium and cell walls. The supernatant was subsequently filtered through glass-fiber in order to remove the remaining cell walls and the mitochondria were then pelleted by centrifugation at 15,000g for 20 min. The pellet was resuspended in isolation buffer (without BSA) and was put on a sucrose gradient (10mM Tris (pH7.5), 1mM EDTA, 1mM PMSF, 5mM DTT, 1:1000 protease inhibitor cocktail and 50%, 36% and 20% sucrose) and centrifuged at 25,000 rpm (Sorvall TH-641) for 1 hour in order to separate mitochondria and the vacuolar fraction. The mitochondrial band was collected from the gradient, diluted in isolation buffer and spun down for 20 min at 15,000g. Finally, the mitochondrial pellet was resuspended in mito buffer (20mM Hepes/KOH pH 7.4, 1mM EDTA, 2mM DTT and 5% glycerol), aliqouted and stored at -80°C until use. Protein concentrations were determined by the Lowry method (Lowry et al. 1951).

2.5. Western blotting

The absence of nuclear contamination in the mitochondrial samples was confirmed by Western blot analysis. 50μg whole protein samples and 50μg mitochondrial samples were separated on glycine gels (5% polyacrylamide stacking gel and 16% polyacrylamide resolving gel) for 1 hour at 100volt. Proteins were transferred to PVDF membranes (Millipore Immobilon-FL) using the semi-dry technique for 1 hour at 0.8mV/cm^2. Nuclear histone3s were detected by incubating the Western blots over night at 4°C in 1:2500 of a Histone 3 antibody (Abcam) in Odyssey blocking buffer (LI-COR Biosciences) with 0.1% Tween20. The Histone 3 antibody used for Western blotting is generated against a peptide (amino acid 100 to 136 of human histone 3) and does in *P. anserina* in addition to histone 3 recognize the histone H3-like centromeric protein CSE4. The Western blots were subsequently incubated (1h at room temp) in 1: 15000 of goat anti-rabbit IgG (Li-Cor IRDye 800CW (LI-COR Biosciences)) in Odyssey blocking with 0.1% Tween20 and 0.01% SDS and were developed using infrared imaging (Li-

Cor Odyssey system) according to the manufactures instructions. The presence of mitochondrial protein was confirmed using the same technique, except Western blots were reprobed with 1:5000 of a cytochrome c antibody (a kind gift from Prof. R. Lill (Philipps-Universität, Marburg, Germany)) in Odyssey blocking buffer (LI-COR Biosciences) with 0.1% Tween20.

2.6. Oligonucleotides

All oligonucleotides were purchased from DNA Technology (Denmark) and were 5′-end-labeled using T4 polynucleotide kinase (PNK) and ^{32}P-γ-ATP. Mixtures of 100ng oligonucleotide containing either the DNA lesion or an unmodified base, 20units of T4 PNK (Fermentas), PNK forward buffer A (Fermentas) and 333μCi ^{32}P-γ-ATP were incubated for 90min at 37°C, and then stopped with 0.05M EDTA. The unincorporated ^{32}P-γ-ATP was removed by G25 Micro spin columns (GE Healthcare). Annealing of the 5′-end-labeled oligonucleotide with its complementary strand was carried out by heating at 90°C followed by gradual cooling to room temperature.

2.7. Mitochondrial DNA glycosylase activities

The activity of the different DNA glycosylases in the mitochondrial extracts was determined *in vitro* by incision assays essentially as previously described (Souza-Pinto et al. 1999). After permeabilization in the presence of 0.05% Triton X-100 and 0.3M KCl, mitochondria were incubated at 27°C with 90fmol of the duplex ^{32}P-labeled oligonucleotide in a 20μl reaction containing 2% glycerol, 65mM KCL, 5mM DTT, 5mM EDTA, 20 mM Hepes and 0.1mg/ml BSA. In order to avoid unspecific cleavage of the DNA oligonucleotides, 15mM dNTPs were added to reactions with dUracil or 5-OHdUracil containing oligonucleotides, while 1.5mM dNTPs were added to reactions with 5-OHdUracil in a bubble or 8oxodG containing oligonucleotides. When investigating dUracil incision, 2.5μg of mitochondrial extract and 1 hour of incubation were used. For 5-OH-dUracil, 5-OHdUracil in a bubble and 8oxodG incision 3 hours of incubation and 2μg, 12μg and 25μg of mitochondrial extract were used, respectively. Reactions were stopped by addition of 0.4% SDS and 0.2μg/μl proteinase K, followed by 30min incubation at 55°C. Samples were mixed with 20μl of formamide loading buffer (80% formamide, 10mM EDTA, 1mg/ml xylene cyanol FF, and 1mg/ml Bromophenol Blue) heated at 90°C for 5 min and loaded on a denaturing 20% polyacrylamide gel. The radioactively labeled DNA was visualized using a Phosphor Image Screen (Amersham), Personal Molecular Imager™ (BioRad) and quantified using Quantity One Software (BioRad). Incision activity was calculated as the amount of radioactivity in the band corresponding to the damage-specific cleavage product divided by the radioactivity in the entire lane. This value was converted to fmol of incised DNA/10μg mitochondrial extract/hour of incubation.

2.8. Statistical analysis

All data are reported as mean ± standard error of the mean of minimum 3 cultures (having different genotypes) from each group (juvenile, senescent or mutant). The value for each culture is based on assays performed in triplicate. Comparisons between juvenile wild type and senescent wild type and between juvenile wild type and juvenile mutants were statistically analyzed with Student's t test. The minimum level of statistical significance was set at $p = 0.05$ for all analyses.

3. Results

3.1. Alignments of *S. cerevisiae, S. pombe* and *H. sapiens* mitochondrial BER protein sequences with the *P. anserina* genome

In order to explore the potential presence of BER protein encoding regions in the *P. anserina* genome, we initially conducted several alignments of known BER protein sequences with the *P. anserina* genome. Since *S. cerevisiae, S. pombe* and *P. anserina* all belong to the Ascomycota phylum, the sequences of Ogg1p, Ung1p/ung1, Ntg1p/nth1, Apn1/apn1 and Apn/apn2 were included in the alignments (the Apn1/apn1 and Apn/apn2 proteins are the yeast homologues of human APE1 and APE2, respectively). NEIL1 homologues have not been identified in *S. cerevisiae* and *S. pombe* and an OGG1 homologue has not been identified in *S. pompe*, consequently for these proteins less than three sequences were aligned with the *P. anserina* genome.

The results of these alignments are summarized in Table 1. As seen from the E-values, sequences with high homology to the sequences for the DNA glycosylases Ogg1p/OGG1, Ung1p/ung1/UNG1 and Ntg1p/nth1/NTH1 are localized on the *P. anserina* chromosomes number 1, 1 and 2, respectively. The predicted protein sequence for PaNth1 aligned to the Ntg1/nth1/NTH1 protein sequences is shown in Figure 1. As seen from Figure 1, the predicted PaNth1 sequence contains amino acids of presumed functional importance (such as the helix-hairpin-helix motif). The *P. anserina* genome does, however, not contain a sequence with high homology to the human NEIL1. In the case of Apn1/apn1/APE, a high degree of homology was found between the yeast sequences and sequences on the *P. anserina* chromosome number 1, while for Apn2/apn2/APE2 there was a high degree of homology between *S. pompe* apn2 and sequences on the *P. anserina* chromosome number 1.

These data suggest that proteins functioning in the first steps of BER possibly exist in *P. anserina*, and hence encouraged us to investigate the BER activity in this organism.

3.2. Mitochondria purified from *P. anserina*

In order to investigate the mitochondrial DNA repair capacity in *P. anserina*, mitochondria from wild-type strains were purified by differential centrifugation followed by ultracentrifugation. Mitochondrial fractions were essentially devoid of nuclear contamination, as determined by Western blotting (of whole protein and mitochondrial fractions) for the nuclear Histone H3-like centromeric protein CSE4. The presence of mitochondrial protein was confirmed by reprobing the Western blots for the mitochondrial protein cytochrome c (Figure 2).

3.3. BER activity in wild type juvenile *P. anserina*

The activities of DNA glycosylases in the purified mitochondrial extracts were analyzed by employing double-stranded DNA oligonucleotides containing specific base damages (Table 2). In the case of the dUracil DNA substrate the combined UNG1 and APE activity was measured, since UNG1 is a monofunctional enzyme. Initially, the assay conditions such as reaction buffer content and amount of mitochondrial extract were optimized in order to ensure steady-state catalytic rates. Representative autoradiograms of incisions assays with each of the four DNA lesion containing oligonucleotides and increasing amount of mitochondrial extract are shown in Figure 3. The incision activities were quantified and used for concentration-response curves (as shown for the 8oxodG-containing oligonucleotide in Figure 3C) and the amounts of extract ensuring steady-state catalytic rates were chosen. Consequently, 2μg, 2.5μg, 12μg, 25μg of mitochondrial extract were added when investigating removal of 5-OHdUracil, dUracil, 5-OHdUracil in a bubble and 8oxodG, respectively. The lesion specificity of the observed incision activities was confirmed by incubating lesion free oligonucleotides

under the same conditions as for lesion holding oligonucleotides. No incision activities were observed when incubating the mitochondrial extracts with the 51-mer control oligonucleotide (Figure 3A (left panel)) or the 30-mer control oligonucleotide (Figure 3B (left panel)).

For each of the different lesion containing oligonucleotides, the incision assay was repeated at least in triplicate for a minimum of four juvenile wild type cultures (having different genotypes). The quantifications for each lesion holding oligonucleotide are shown in Figure 4. Under our optimized assay conditions we observed that the incision activity of dUracil was the highest, while the incision activities of 8oxodG and 5-OHdUracil in a bubble were the lowest.

3.4. BER activity in wild type juvenile and senescent *P. anserina*

Since changes in BER capacity are known to occur during ageing in mammalian ageing models, changes may also be relevant for ageing in *P. anserina*. Therefore, once we had confirmed the presence of BER activities in *P. anserina*, we investigated the potential age-related change in the mitochondrial DNA repair capacity. Specifically, we analysed DNA glycosylase activities in the juvenile state and in the senescent state. Representative autoradiograms are shown in Figure 5A. The mitochondrial incision activities were quantified and as shown in figure 5B the incision activities of all four DNA lesions were found to be significantly lower in the senescent state than in the juvenile state ($p \leq 0.05$), that is, the mitochondrial DNA glycosylase activities decrease with age in *P. anserina*.

3.5. BER activity in juvenile wild type and juvenile long lived mutants

As mentioned above, the *P. anserina* mutants grisea and ΔPaCox17::ble are long-lived and characterized by a high level of mtDNA stability. One might expect that efficient DNA repair could contribute to the maintenance of mtDNA stability and consequently influence the lifespan of these mutants. Therefore, in addition to studying the possible age-related change in BER (juvenile *vs.* senescent), we investigated the DNA glycosylase activities in these long lived mutants. Representative autoradiograms and the quantification of the incision activities on all four lesion holding oligonucleotides are shown in Figure 6A and B. As seen in figure 6B the incision activities at all four DNA lesions were found to be significantly lower ($P \leq 0.05$) in the mutants as compared to wild type.

4. Discussion

In the current study we show for the first time the presence of mitochondrial BER activity in the fungal ageing model *P. anserina*. This activity declines with age possibly contributing to the age-related increase in mtDNA instability and consequently to mitochondrial dysfunction, ageing and lifespan. The two long-lived mutants grisea and ΔPaCox17::ble showed lower mitochondrial BER activity than wild type, indicating that the reason for lifespan extension in these mutants is not increased DNA repair but rather lower damage as a result of the lower ROS generation in these mutants ((Gredilla et al. 2006), R. Gredilla and H. D. Osiewacz, unpublished data), which respire via an alternative electron transport chain

By conducting several alignments we initially demonstrated that sequences in the *P. anserina* genome have high homology with the sequences of yeast and human DNA glycosylases and AP endonucleases. The finding that the sequences are well conserved among the organisms, points to the importance of the BER activities and supports the use of *P. anserina* for studying DNA repair and ageing and enable the comparison of results obtained in *P. anserina* to mammalian model organisms. Compared to higher order organisms the advantage of *P. anserina* as an ageing model organism is its short lifespan, the easy generation

of mutants and the fact that it is possible to study the exact same individual as both young and old (as opposed to studying several individuals living for different time spans).

However, hNEIL1 did not show high homology with the *P. anserina* genome, which unfortunately in the region of potential homology contains a number of undetermined nucleotides making the annotation in this region uncertain. A NEIL1 encoding sequence has been found in several mammals as well as in *Danio rerio* (zebra fish), yet not in yeast. When aligning the sequences for either *Escherichia coli* Nei endonuclease VIII or MutM (both have high homology with NEIL1) with the *P. anserina* genome, we did not find high homology either (data not shown). Nonetheless, we do observe lesion specific incision of the 5-OHdUracil in the bubble oligonucleotide substrate, demonstrating that a protein capable of incision at such a lesion must be present in *P. anserina* mitochondria.

In addition to the sequence alignments, we probed Western blots of *P. anserina* mitochondrial extracts using antibodies against mammalian DNA glycosylases or APEs. When using antibodies against OGG1 or UNG1, we observed a band at the expected position, yet additional bands were detected (data not shown), making these observations inconclusive. Laquel-Robert and Castroviejo have previously observed a single band when probing Western blots of *P. anserina* mitochondrial extract with an antibody against human PCNA (Laquel-Robert and Castroviejo 2003). Hence, it is possible that other antibodies against mammalian BER proteins could recognize BER proteins in *P. anserina* with a higher accuracy.

In any case, based on the alignments presented here, it is likely that BER proteins similar to those known from other eukaryotes exist in *P. anserina*.

Since the maintenance of genomic stability is of profound importance for cellular survival and for the ageing process, it is not surprising to find BER activities in *P. anserina*. The interrelationship between the levels of incision (of the four different DNA lesion holding oligonucleotides) observed, corresponds very well to that observed in mammalian cells (Karahalil et al. 2002), implying that the DNA glycosylase activities in *P. anserina* may be somehow similar to the mammalian activities. It was repeatedly reported that the senescent state of *P. anserina* holds instable mtDNA as compared to the juvenile state (Stahl et al. 1978;Cummings et al. 1979;Belcour et al. 1981;Kück et al. 1981;Osiewacz and Esser 1984). When investigating the mitochondrial DNA repair activity in the senescent state of *P. anserina*, we found a decrease in DNA glycosylase activities as compared to the juvenile state, indicating that the first steps of BER decline as *P. anserina* ages. Such an age-related decrease in BER activity could be expected to contribute to an age-dependent accumulation of mtDNA lesions and therefore to the age-related decrease in mtDNA stability, which would contribute to an age-related hampering of mitochondrial function and consequently to the ageing process. Hence, our results point to a causal link between mtDNA repair and ageing in *P. anserina*.

In addition, the ROS levels in the senescent mycelium tend to be higher than in the juvenile mycelium (R. Gredilla and H. D. Osiewacz, unpublished data). Such an increase could cause an increase in DNA lesion levels in the senescent state and in combination with a decrease in BER activity contribute to the ageing phenotype.

We can only reflect on what might cause the activities of the DNA glycosylases to decrease during ageing. It could be due to a reduction in the level of DNA glycosylases. We attempted to test this, yet the lack of suitable antibodies made it impossible to draw any conclusions. Accumulation of damaged protein has been reported to be associated with ageing (reviewed in (Hipkiss 2006)), thus the BER proteins themselves may become damaged during ageing possible reducing their efficiency. Recently, an age-related increase in oxidized subunits of the *P. anserina* mitochondrial ATP synthase was described (Groebe et al. 2007), indicating that age-related protein modifications take place in *P. anserina* mitochondria. In addition, the

spectrum of mitochondrial proteins has been reported to change in an age-related manner in *P. anserina* (reviewed in (Silar et al. 2001)), showing that *P. anserina* mitochondrial proteins are affected during ageing.

Finally, we investigated the BER activity in the mutants grisea and ΔPaCox17::ble displaying lifespan extension. For most organisms resistance to oxidative stress is considered to correlate positively with lifespan (Honda and Honda 2002;Vijg and Suh 2005; Murakami 2006) and DNA repair capacity has in *C. elegans* (Hyun et al. 2008), *D. melanogaster* (Whitehead and Grigliatti 1993) and possibly mammals (Foksinski et al. 2004) been reported to correlate positively with lifespan. Since the *P. anserina* mutants are long-lived and are known to contain lower levels of age-related mtDNA rearrangements than wild type (Borghouts et al. 1997;Stumpferl et al. 2004), it could be expected that these mutants display an efficient DNA repair system. Yet, we observed a lower mitochondrial BER activity in the mutants as compared to wild type. On first glance, these data may seem surprising. However, due to a switch from the standard Cytochrome c oxidase dependent to an alternative respiratory chain the mutants consume more oxygen and produce less ATP and less ROS ((Gredilla et al. 2006), R. Gredilla and H. D. Osiewacz, unpublished data).

Hence, in these mutants ROS induced molecular damage (including damage of mtDNA) can be expected to be lower than in the wild type. Indeed, as already mentioned, an age-related increase in damage to protein has been reported for *P. anserina* (Groebe et al. 2007). Reduced damage to mtDNA can possibly affect the stability of the mtDNA in a positive direction. Hence, it is possibly that less damage could allow the system to invest less into its repair capacity without negative consequences. An attractive, yet not proved control of such a system could be via the use of ROS as signals to control the expression of genes involved in repair functions. Hence, the observed decrease in BER in these mutants might be the result of such a mechanism. It has been reported that induction of oxidative stress can induce BER gene expression and/or increased BER protein levels (Grosch et al. 1998;Rusyn et al. 2000;Cabelof et al. 2002;Liu et al. 2008), however whether low oxidative stress can lead to reduced BER gene expression levels needs further exploration. Then again, it cannot be excluded that other DNA repair mechanisms than BER play an important role in these mutants.

In conclusion, for the first time we show the presence of BER activity in *P. anserina*, an activity which decreases with age, indicating that the decreased mtDNA stability observed with age may to some extent be caused by a decreased ability to repair oxidative lesions in mtDNA and that *P. anserina* may hold similar mechanisms of ageing as higher eukaryotes. Our alignment study indicates the existence of BER proteins in *P. anserina*. The reported initial analyses bear new perspectives to study the impact of DNA repair on ageing and lifespan control in a model organism which is easily manipulated experimentally. In particular, it will be interesting to investigate the effect of modulation of components of the repair pathways and to see how such modifications affect the network of pathways involved in ageing and lifespan control.

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Abbreviations

BER
: Base excision repair

mtDNA
: mitochondrial DNA

ROS
: reactive oxygen species

dUracil
: deoxy-Uracil

5OH-dUracil
: 5-hydroxy-deoxyuracil

8oxodG
: 8-oxo-2′-deoxyguanosine

OGG1
: 8-oxoguanine DNA glycosylase 1

UNG1
: Uracil DNA glycosylase 1

NTH1
: endonuclease III homologue 1

NEIL1
: Nei endonuclease VIII-like homologue 1

APE
: AP endonuclease

PCNA
: proliferating cell nuclear antigen

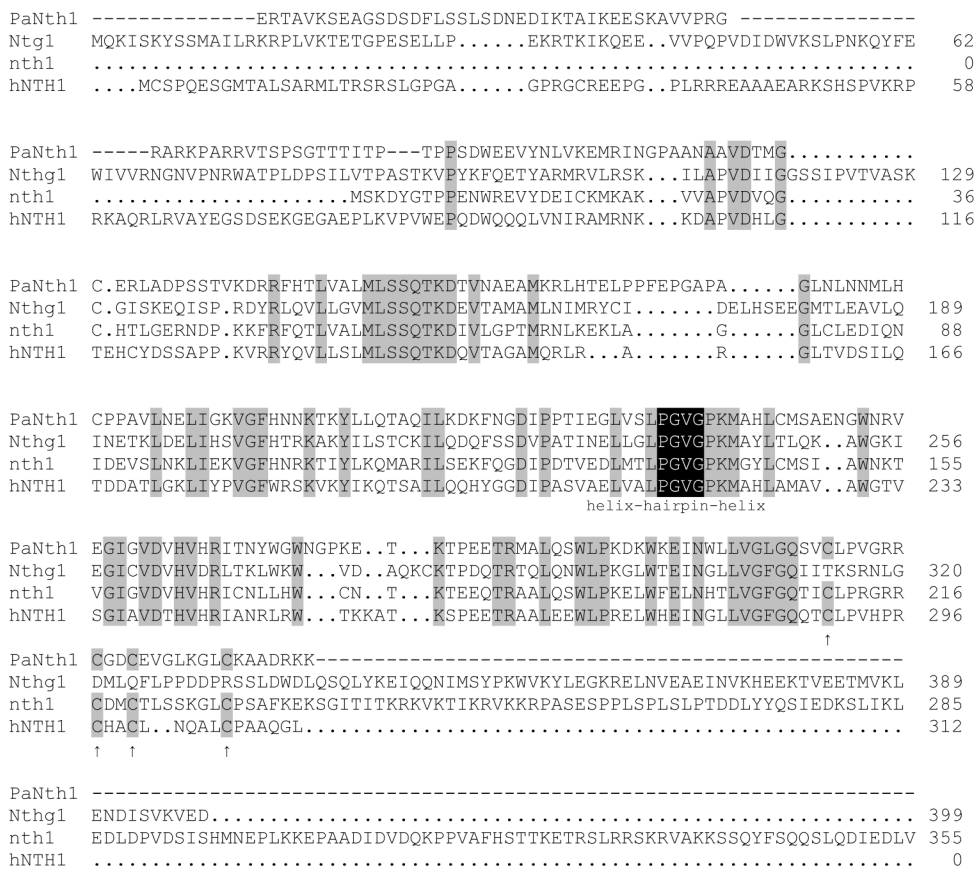

Figure 1. Alignment of the sequences for human and yeast Nht1 with the depicted sequence of PaNth1

The first 60 amino acids of the PaNth1 sequence is based on alignment to *Saccharomyces cerevisiae* Ntg1 sequence, while the rest of the PaNth1 sequence is based on the alignment to *Schizosaccharomyces pompe* Nth1 sequence. The helix-hairpin-helix motif, which is believed to interact with DNA, is indicated and the GVG hairpin is marked in black. The cysteine residues believed to be involved in binding a (4Fe-4S) cluster is indicated by arrows. Amino acids identical among all four species are shaded in grey.

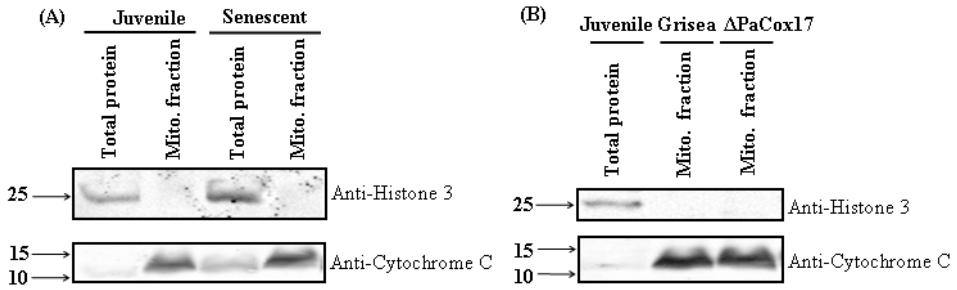

Figure 2. Western blot analysis of Histone H3-like centromeric protein CSE4 and Cytochrome C in whole protein and mitochondrial fractions

50μg of whole protein and 50μg of mitochondrial fractions of *P.anserina* wild-type strain (s) were loaded as indicated and resolved as described in Materials and Methods. Representative Western blot images are shown.

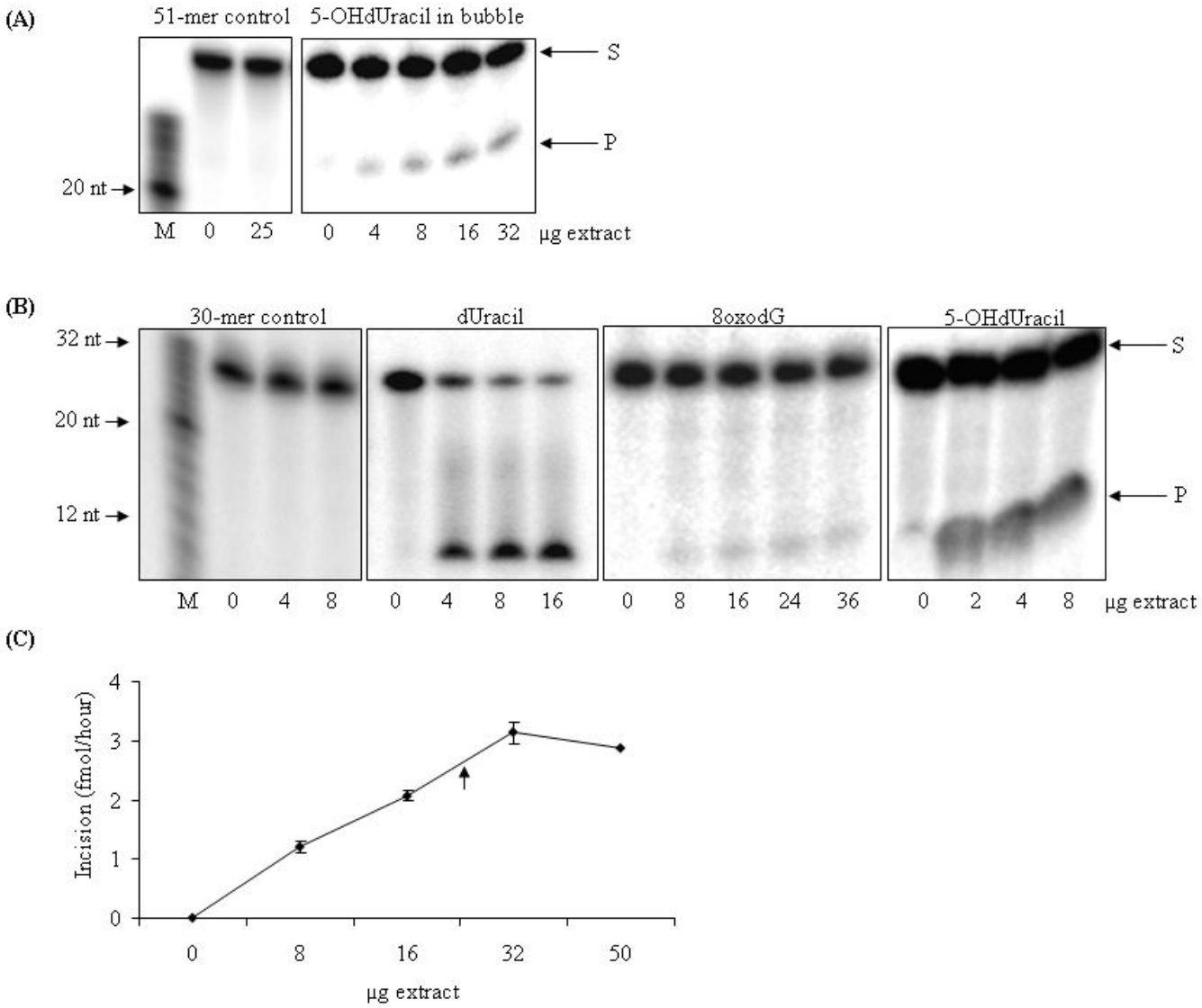

Figure 3. Representative autoradiograms showing BER activity in mitochondrial extract from juvenile wild type *P. anserina*

(A) Left panel: Incision of 51-mer undamaged control oligonucleotide after 3 hours of incubation. Right panel: Incision of the 5-OHdUracil in bubble substrate with increasing amount of mitochondrial extract after 3 hours of incubation. (B) Left panel: Incision of 30-mer undamaged control oligonucleotide after 3 hours of incubation. Right panels: Incision of dUracil, 8oxodG and 5-OHdUracil substrates with increasing amount of mitochondrial extract after 1 hour, 3 hours and 3 hours of incubation, respectively. (C) Quantification of incision of the 8oxodG holding oligonucleotide. Incision activities were calculated from the amount of radioactivity of the products relative to the radioactivity of the entire lane. Error bars represent standard error of the mean of the assay performed in triplicate. The arrow indicates the amount of extract chosen for incision assays with 8oxodG holding oligonucleotide (25µg).
S: substrate, P: product, M: marker, nt: nucleotides.

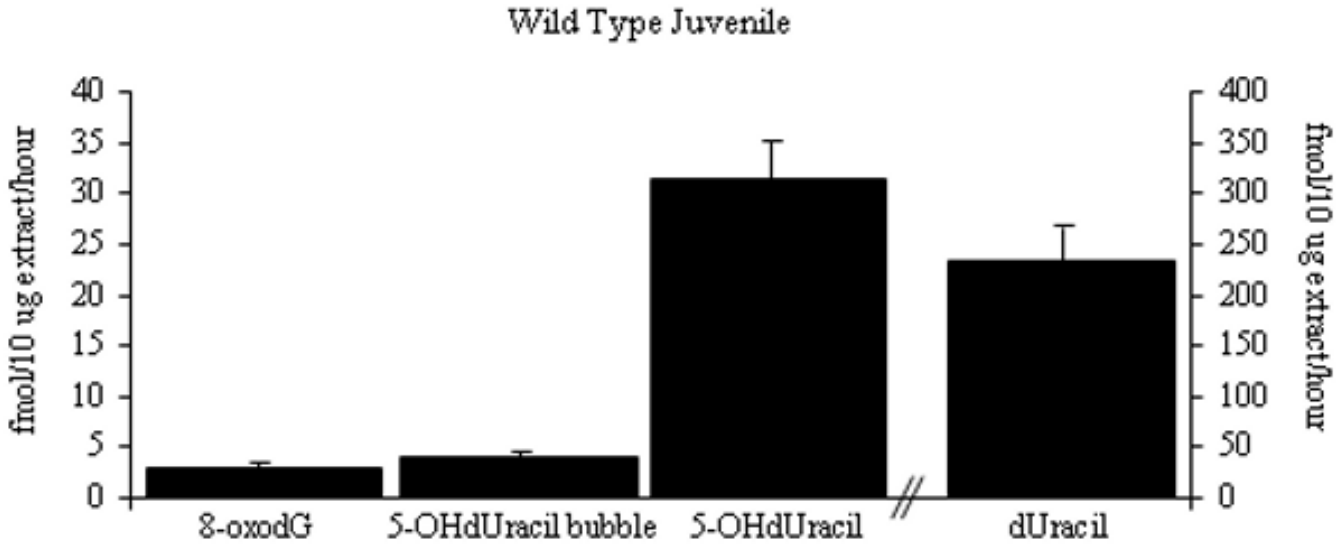

Figure 4. BER activity in mitochondrial extract from juvenile wild type *P. anserina*

Quantification of BER activity in mitochondria from juvenile wild type *P. anserina*. The graphs represent the averages of minimum 4 juvenile wild type cultures (having different genotypes) (n=5 for 8oxodG, n=7 for 5-OHdUracil in bubble, n= 4 for 5-OHdUracil and n=5 for dUracil). The error bars represent the standard error of the mean.

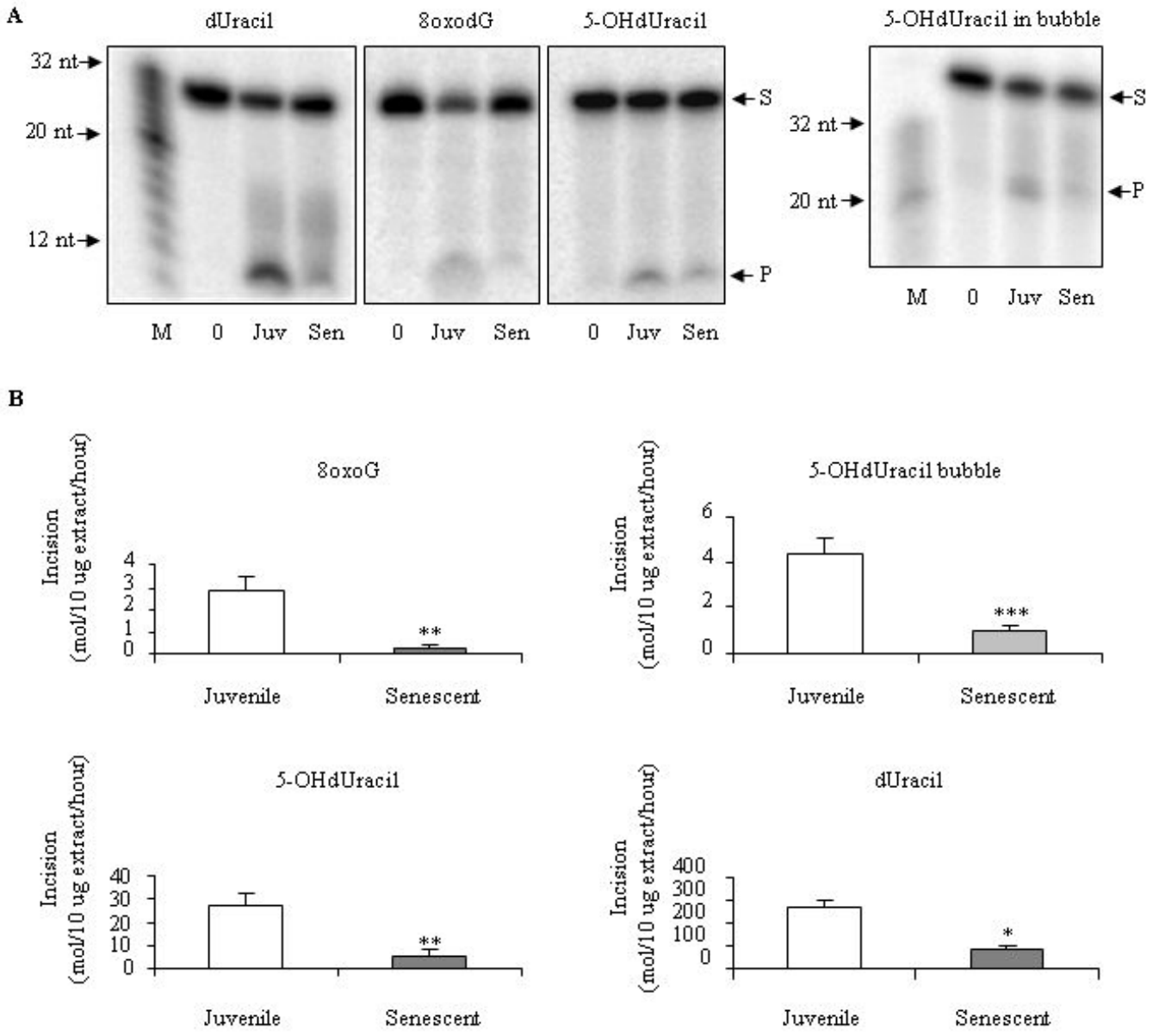

Figure 5. BER activity in mitochondrial extracts from juvenile and senescent wild type *P. anserina*

A) Incision of the dUracil, 8oxodG, 5-OHdUracil and 5-OHdUracil in bubble substrates with 2.5, 25, 2 and 12μg of mitochondrial extract and 1, 3, 3 and 3 hours of incubation time, respectively. Juv: juvenile, Sen: senescent, S: oligonucleotide substrate, P: oligonucleotide (incision) product, M: Marker, nt: nucleotides. B) Quantification of BER activity in mitochondria from juvenile and senescent wild type *P. anserina*. The graphs represent the averages of minimum 4 wild type cultures (having different genotypes) (8oxodG: n=4, 5-OHdUracil in bubble: n=5, 5-OHdUracil: n=4, dUracil: n=5). Error bars represent the standard error of the mean between cultures. *: significant difference in activity between juvenile and senescent cultures; * p=0.04; **p=0.03, ***p=0.02.

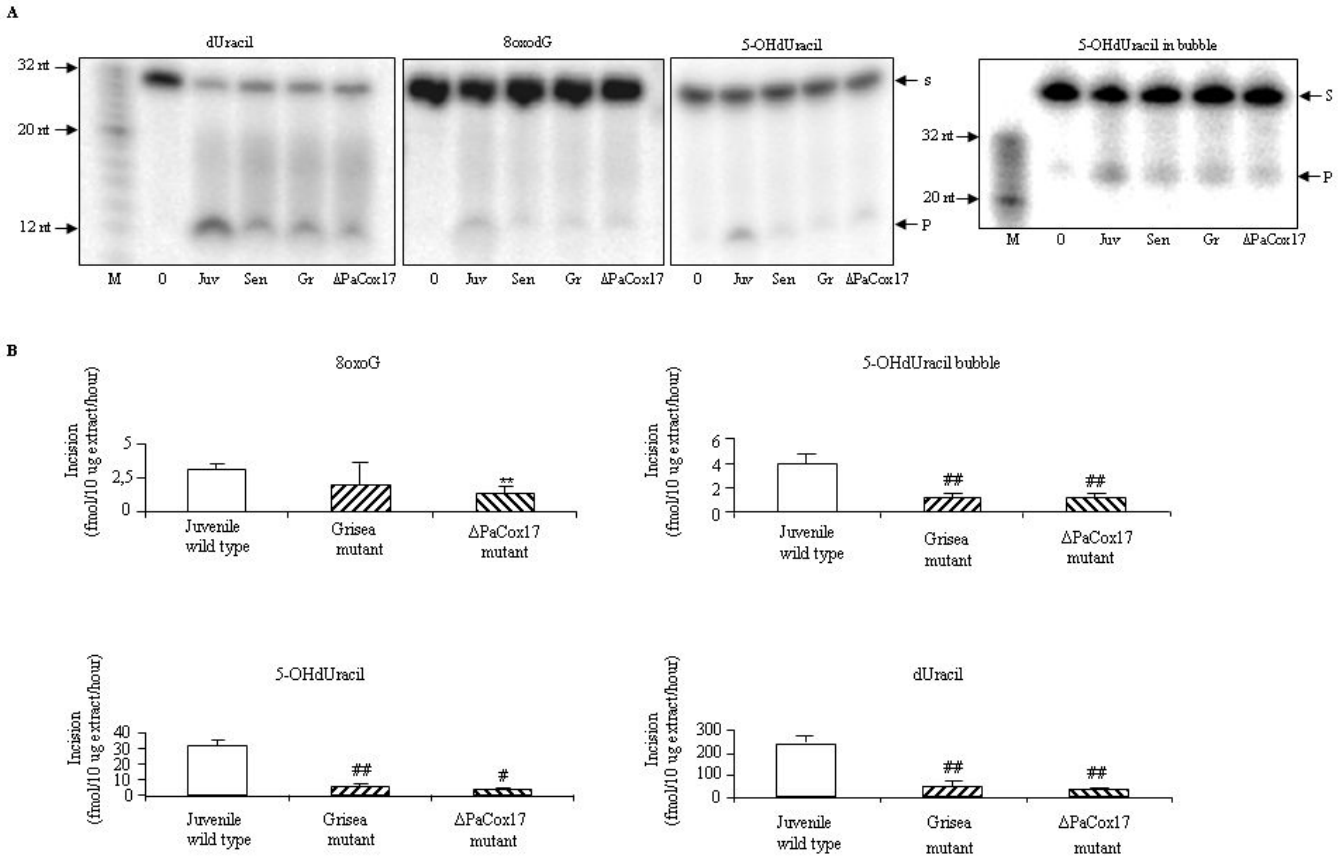

Figure 6. BER activity in mitochondrial extracts from juvenile and senescent wild type *P.anserina* and in juvenile grisea and ΔPaCox17::ble *P. anserina* mutants

A) Incision of the dUracil, 8oxodG, 5-OHdUracil and 5-OHdUracil in bubble substrates with 2.5, 25, 2 and 12μg of mitochondrial extract and 1, 3, 3 and 3 hours of incubation, respectively. Juv: juvenile, Sen: senescent, Gr: Grisea mutant, ΔPaCox17: ΔPaCox17::ble mutant, S: oligonucleotide substrate, P: oligonucleotide (incision) product, M: Marker, nt: nucleotides. B) Quantification of BER activity in mitochondria from juvenile wild type, juvenile Grisea and juvenile ΔPaCox17::ble *P. anserina*. The graphs represent the averages of minimum 3 wild type cultures (having different genotypes) (8oxodG: n=3, 5-OHdUracil in bubble: n=3, 5-OHdUracil: n=3, dUracil: n=3). Error bars represent the standard error of the mean between cultures. * and # indicate significant difference in activity between juvenile wild type and juvenile Grisea mutant cultures and between juvenile wild type and juvenile mutant cultures. **p=0.03, #p=0.004, ##p=0.003

Table 1

Alignments of *S. cerevisiae, S. pombe* and *H. sapiens* BER protein sequences with the *P. anserina* genome

"For each DNA glycosylase or AP endonuclease the order of listing (in left column) is: *S. cerevisiae* (top), *S. pombe* (middle) and *H. sapiens* (bottom). Identities: genomic sequence encodes identical amino acids, positives: genomic sequence encodes amino acid having similar properties.

	No. of amino acids in protein/No. of amino acids aligned	Identities (%) / Positives (%)	E-values	Chromosome no. in *P. anserina*
Ogg1p	376/296	34/47	4*10-38	1
hOGG1	424/318	39/55	2*10-56	1
Ung1p	395/208	51/62	2*10-51	1
ung1	322/192	54/69	1*10-57	1
hUNG1	304/169	54/68	2*10-44	1
Ntg1p	399/313	36/50	2* 10-41	2
nth1	355/245	51/63	1*10-60	2
hNTH1	303/239	47/59	4*10-51	2
hNEIL1	390/108	26/42	5*10-5	4
Apn1p	367/263	53/68	2*10-78	1
apn1	342/293	46/59	1*10-70	1
hAPE1	318/223	31/43	3*10-16	1
Apn2p	520/304	32/47	4*10-27	1
apn2	523/248	37/56	3*10-52	1
hAPE2	518/265	29/46	9*10-21	1

Table 2

The DNA oligonucleotides used in incision assays and the DNA glycosylases preferentially recognizing the DNA lesions

The modified bases are shown in bold. U: dUracil, 5: 5-OH-dUracil, 8: 8oxodG, A/T: undamaged control (30-mer), C: undamaged control (51-mer). Underlined bases indicate bubble area.

DNA lesion/substrate	Sequence	DNA glycosylase (preferentially recognizing DNA lesion)	Activity of DNA glycosylase
dUracil (dU)	5′-ATA TAC CGC G**U**C CGG CCG ATC AAG CTT ATT-3′ 3′-TAT ATG GCG CGG GCC GGC TAG TTC GAA TAA-5′	UNG1	Mono-functional
5-hydroxy dUracil (5-OHdU)	5′-ATA TAC CGC G**5**C CGG CCG ATC AAG CTT ATT-3′ 3′-TAT ATG GCG CGG GCC GGC TAG TTC GAA TAA-5′	NTH1	Bi-functional
8-oxo-2′-deoxy-guanosine (8oxodG)	5′-ATA TAC CGC G**8**C CGG CCG ATC AAG CTT ATT-3′ 3′-TAT ATG GCG CGG GCC GGC TAG TTC GAA TAA-5′	OGG1	Bi-functional
Undamaged control	5′-ATA TAC CGC G**A**C CGG CCG ATC AAG CTT ATT-3′ 3′-TAT ATG GCG C**T**G GCC GGC TAG TTC GAA TAA-5′	-	-
5-hydroxy dUracil in a 11 nt bubble (5-OHdU in bubble)	5′-GCT TAG CTT GGA ATC GTA TCA TGT A**5**A CTC GTG TGC CGT GTA GAC CGT GCC-3′ 3′-CGA ATC GAA CCT TAG CAT AGG CAC CCG ACA AAC ACG GCA CAT CTG GCA CGG-5′	NEIL1	Bi-functional
Undamaged control	5′-GCT TAG CTT GGA ATC GTA TCA TGT ACA CTC GTG TGC CGT GTA GAC CGT GCC-3′ 3′-CGA ATC GAA CCT TAG CAT AGG CAC CCG ACA AAC ACG GCA CAT CTG GCA CGG-5′	-	-