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MITOCHONDRIAL MECHANISMS OF AGEING

VISUALIZING THE AGEING PROCESS IN MITOCHONDRIA BY CONFOCAL LASER SCANNING MICROSCOPY

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ABSTRACT

The filamentous fungus *P. anserina* has proven to be a priceless model organism in ageing research and has established the mitochondria as main target of this research field. The present study aims to enhance the connections between the diverse integrative levels of ageing research, by directly observing the morphology of mitochondria during the ageing process. Mitochondrial morphology is visualized using confocal laser scanning microscopy of *P. anserina* samples expressing a GFP-tagged mitochondrial membrane protein ATG9. Comparisons are made between samples grown at normal conditions and at severe calorie restricted (SCR) conditions, and between wild-type strain s and in a strain carrying a deletion of mitophagy protein Pa*uth1*. At normal conditions, the elongated tubular networks of mitochondria observed at young age show increased fragmentation during the process of ageing. Furthermore, peculiar ring-shaped mitochondria appear to develop upon ageing. SCR counteracts these morphological changes and thus keeps mitochondria 'young', while Pa*uth1*-deletion accelerates the development of such modifications. Pa*uth1*-deletion further appears to be involved in lipofuscin production and disruption of *P. anserina*'s macroscopic and microscopic growth pattern. A hypothesis is proposed explaining all these observations by an imbalance of mitochondrial fusion and fission proteins, possibly in combination with other cellular damage, and impaired mitophagy. A second, theoretical, hypothesis is put forward linking fission events with mitochondrial fitness, and consequently, longevity.

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INTRODUCTION

People have always been fascinated by the cause and reason of ageing, and more specific, the possible deceleration or even prevention of this process. Additionally, age-related diseases and the apparent overlap in research field with cancer, gives ageing research also a significant medical importance. Understanding these ageing-correlated issues relies greatly on the knowledge of the underlying molecular and cellular mechanisms, and many years of research have been dedicated to elucidate these (see e.g. Harman, 1981; Partridge & Gems, 2002; Kirkwood, 2002).

Ageing can be defined as "the progressive and irreversible loss of physiological functionality with a concomitant increase in both morbidity and mortality of the organism" (Scheckhuber, Rödel & Wüstehube, 2008), and ageing research encompasses all research fields that (partly) address this. Lower eukaryotes have been used for ageing research mostly, due to their relative easy cultivation and short lifespan. One organism in specific, namely the filamentous fungus *Podospora anserina*, has been utilized to uncover ageing mechanisms already since its ageing process, termed "senescence", was described by Rizet (1953) more than fifty years ago. *P. anserina* proved to be a very valuable model for ageing research, because of its rapid ageing, i.e. within two to three weeks, and because this organism is at the borderline between mono- and multi-cellular organisms (Scheckhuber & Osiewacz, 2008). It is the species in which the principal role of mitochondria in ageing was established first (reviewed in Silar *et al.*, 2001), after which the same was found to be true for a wide range of other organisms (see Lorin *et al.*, 2006).

Mitochondria are dynamic organelles that undergo frequent fusion and fission events, regulating their quantity and morphology, and consequently also function (Bereiter-Hahn & Vöth, 1994). They perform the vital ATP production in eukaryotic cells and are for this reason also highly involved in processes of apoptosis and ageing. Harman (1956) proposed in his free radical theory of ageing, that mitochondrial derived reactive oxygen species (ROS) instigate molecular damage, leading to ageing. Besides being the main source of ROS, in particular respiratory chain complexes I and III, mitochondria are also the

primary target of the effects of ROS, causing damage in mitochondrial proteins, DNA and membranes. This was proposed first by Miquel *et al.* (1980; updated in Miquel, 1992) in their mitochondrial theory of ageing, and subsequently became a widely supported theory when empirical evidence was obtained in favour.

The involvement of mitochondria in the ageing process also becomes apparent when considering cellular repair systems counteracting e.g. ROS derived mutations and modifications. These systems actively remove and replace damaged molecules and organelles, and may even induce apoptosis when cellular damage is too severe (Scheckhuber & Osiewacz, 2008). The connection of such repair systems and ageing has primarily been established due to studies in mutants in which life span is affected, like autophagy mutants, or more specifically mitophagy mutants (Lemasters, 2005). However, the exact underlying mechanisms are still unclear.

Additionally, calorie restriction is found to extend life span in many organisms (reviewed in Lorin *et al.*, 2006), further confirming the key role of the respiratory function of mitochondria in ageing. Moreover, respiratory mutants that have a reduced metabolic rate are also found to be affected in their life span (Lorin *et al.*, 2006; Groebe *et al.*, 2007; Eisenberg *et al.*, 2007). Rate of metabolism, either through dietary restriction or mutation, is clearly linked with longevity, which has led to the "metabolic theory" (Hekimi *et al.*, 1998; Lakowski *et al.*, 1998). Yet again, the characteristics of this relation need further elucidation.

The important role of mitochondria in the ageing process is thus firmly established, supported by a huge body of biochemical, genetic, cell-biological and molecular evidence. However, connections between these different integrative levels of research remain limited, which blurs the overview of ageing pathways. Additionally, many of the involved cellular mechanisms are unclear. Morphological investigation of mitochondria during the ageing process may yield a vital link between these diverse fields of research, and forms the basis of the present study. Previous studies suggest an association between mitochondrial morphology and mitochondrial function (Bereiter-Hahn & Vöth,

1994), including their function in apoptosis and ageing processes (Scheckhuber, Rödel & Wüstehube, 2008; reviewed in Osiewacz, 2002a; Karbowski & Youle, 2003), yet hardly scrutinize the exact observed morphology. In this study, the morphology of mitochondria during ageing is investigated, and the effect of severe calorie restriction (SCR) and mitophagy impairment is also included, using *P. anserina* as model organism.

MATERIAL & METHODS

P. anserina strains

P. anserina strains used in this study were derived from the wild-type strain s as first described by Rizet (1953). For visualizing mitochondrial morphology, a mating-type minus (mat^-) strain was used that expresses a green fluorescent protein (GFP)-tagged ATP synthase subunit 9 (ATP9) (Sellem *et al.,* 2007), further referred to as the ATP9*-strain. The gene encoding ATP synthase subunit 9 is transcribed in the nucleus, but its product is localized in the mitochondrial membrane. Additionally, the ATP9*-strain carries the *E. coli aph* gene, providing resistance to hygromycin (Sellem *et al.*, 2007).

For the morphological study of mitophagy aberrations, a strain was used that carries a Pa*uth1* deletion, a gene that appears to code for a SUN super-family member mitochondrial outer membrane protein. In yeast, a homologous protein, UTH1, exists that has been identified to act as determinant of autophagic degradation of mitochondria (reviewed in Camougrand *et al.*, 2004), i.e. mitophagy. For this reason, it is thought that this Pa*uth1*-knockout *P. anserina* strain is unable to perform mitophagy. For visualization, this strain (mat^+) was crossed with the ATP9* strain and single mating-type ascospores were collected by visual examination. Due to nourseothricin resistance in the Pa*uth1*-knockout strain, single mating-type ascospores carrying both the Pa*uth1*-deletion and the GFP-tagged ATP9, could be selected by germination on plates containing 100 µg/ml hygromycin and 50 µg/ml nourseothricin. The novel strain derived from this was used for examining mitochondrial morphology and will hereafter be designated as ΔUTH1*.

P. anserina cultivation

Samples were grown on *P. anserina* synthetic medium (PASM), containing per litre: 0.5 g KH_2PO_4, 0.6 g $K_2HPO_4.3H_2O$, 0.5 g $MgSO_4.7H_2O$, 1 g urea, 20 g agar, 1 ml of a 10 mg per 100 ml biotin/thiamine solution, 1 ml of a trace-elements solution containing per 100 ml: 500 mg citric acid.H_2O, 500 mg $ZnSO_4.7H_2O$, 100 mg $Fe(NH_4)_2(SO_4)_2.6H_2O$, 25 mg $CuSO_4.5H_2O$ (of 16 mg $CuSO_4$), 5 mg $MnSO_4$, 5 mg H_3BO_3, 5 mg $Na_2MoO_4.2H_2O$, and a percentage of glucose depending on diet. For wet mount microscope visualization, liquid medium was prepared which lacked agar and was made isotonic with saccharose, as determined by a cryoscopic osmometer (Gonotec, Germany). For samples grown on normal diet, indicated as "N", 2% w/v glucose was added, while for samples grown at SCR conditions, indicated as "SCR", 0.02% w/v glucose was added to the medium. The pH of the final medium was made 5.8 and incubations were always done at 27°C in the dark, unless indicated otherwise. For germination, PASM containing 2% glucose was supplemented with 60 mM ammonium acetate and incubated for two days. Ammonium acetate is added to emulate the low pH of the gastro-intestinal track of herbivores through which ascospores naturally pass. From the freshly germinated ascospores, a small piece cut from the mycelium was transferred to the edge of 14 cm Petri-dishes containing PASM medium, supplemented with 2% (for N) or 0.02% (for SCR) glucose. Samples were allowed to grow until those grown at normal diet (N) reached a state of senescence, typically 14 to 16 days (own observations). Growth was checked at least three times a week and samples were considered senescent, i.e. dead by ageing, when no growth was observed since the previous check. Samples grown at SCR conditions showed no senescence after 16 days and have been observed not to do so for sometimes even over 3.5 years thus far (van Diepeningen, personal communication).

Confocal Laser Scanning Microscopy

PASM plates covered with a thin cellophane sheet were inoculated with samples from the above mentioned 14 cm Petri-dishes, from positions of about 1 day and of about 13 days old, and were allowed to grow for 24 hours at 27°C in the dark. It has been reported that age in *P. anserina* mycelium is determined by its growth in distance and remains effectively unaffected by time (Silar *et al.*, 2001; Osiewacz, 2002b).

After incubation, pieces of cellophane covered by mycelium were cut out, prepared as a wet mount with isotonic liquid medium and used or direct visualization. Visualization was performed using an inverted confocal laser scanning microscope (LSM510, Zeiss, Germany), equipped with a Plan-Apochromat 63x 1.4 NA oil immersion objective. Growth tips of the samples were examined at an illumination wave length of 488 nm from an argon laser using a main dichroic beam splitter of 488 nm. GFP-signal was detected through a 98 µm pinhole and emission band pass filter of 505-550 nm.

Only growing hyphal tips were used for analysis, because functional mitochondria are in clear demand here (Osiewacz, 2002b). Energy requirements in these cells can, consequently, be considered about equal, which improves comparability between samples. Furthermore, growth tips are located in regions of significantly less pigmentation and less dense hyphae, which improves visualization.

Of each sample, three-dimensional images were made using z-stacks consisting out of at least 60 optical slices with a step-size of 0.14 µm. In this way, the entire growth tip is scanned and the morphology and connection between mitochondria, having a typical diameter of 0.5 µm, can be easily resolved. Mitochondrial morphology was subsequently examined using the Zeiss LSM Image Browser v4.2.0.121.

RESULTS

ATP9*-strain

Visualization of the mitochondrial morphology in the ATP9* strain, grown under normal glucose levels (N) and SCR conditions (SCR), for two days (young) and 14 days (senescent under N conditions), yielded some interesting results. Elongated tubular networks of mitochondria are visible in samples from each condition (see Figure 1). In 14 day old samples grown at normal glucose conditions, however, small punctiform fragments of mitochondria are visible which are not connected to a mitochondrial network. Such fragments were solely observed in these virtually senescent samples when grown at N conditions, with the exception of one observation of fragmented mitochondria in a young N sample. Strikingly, also ring-shaped mitochondria were visible in half of the images taken of 14 days grown samples at N conditions. Furthermore, samples grown at SCR seem to contain a higher amount of mitochondria and this observation was confirmed statistically (van Diepeningen *et al.*, in press).

A mitochondrion is defined as "fragment" when it is not attached to a mitochondrial network, and its individual length is not longer than 1 µm (mitochondria have a diameter of about 0.5 µm). Fragments have a very clear distinction, because mitochondrial networks in this model are usually not shorter than 10 µm and networks with a length of more than 50 µm have been observed (own observations). Ring-shaped mitochondria are only defined as such when they are detached from a mitochondrial network. These ring-shaped mitochondria appear to have an identical diameter as mitochondrial networks have, but the tips seem fused to each other.

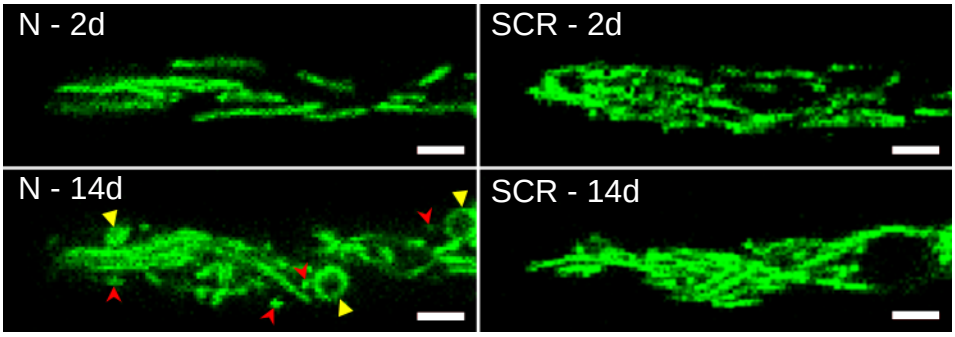

Figure 1 - Fluorescence images of mitochondria from living growth tips of a strain s of *P. anserina* during aging on different diets. The white bar in the right bottom corner of each image equals a length of 2µm. N indicates that the samples are grown on normal glucose concentration (i.e. 2%), while SCR indicates growth conditions under SCR, i.e. medium containing 0.02% glucose. From both conditions, samples are taken at two days of growth (2d) and at 14 days of growth (14d).

ΔUTH1*-strain

ΔUTH1* appears to grow in phases and, interestingly, also forms a lipofuscin-front, after 18 or 19 days of growth, when grown at SCR conditions (see Figure 2). It must be said, however, that ΔUTH1* was only grown on 14 cm Petri-dishes in two occasions and thus the observations mentioned may be incidental. Therefore, this experiment should be repeated. Additionally, these samples were transferred from 27°C to 4°C for storage at 17 days of growth. Consequently, the lipofuscin formed during the limited growth witnessed during storage. Still, this kind of phenotype was not observed in any of the wild-type strains at SCR conditions. This is true for both the seemingly pulsed growth as well as the lipofuscin formation, so it is very well possible that the Pa*uth1*-disruption also has some causal influence in these peculiar findings.

At microscopy level, ΔUTH1* appears to have a very erratic manner of growth as well. Typically, the mycelia of this mutant show a varying diameter, indicating pulsed hyphal elongation, strikingly different from the rather smooth growth pattern of the s-strain wild-type (see Figure 3). Additionally, hyphae appear to split more often than the wild-type and apical hyphae regularly grow further than its parental hyphe. Such phenotype has never been reported in the wild-type. The ΔUTH1*-mutant also shows bursting hyphal tips, even at young

age (Figure 4), identical to bursting hyphal tips seen in highly senescent wild-type samples.

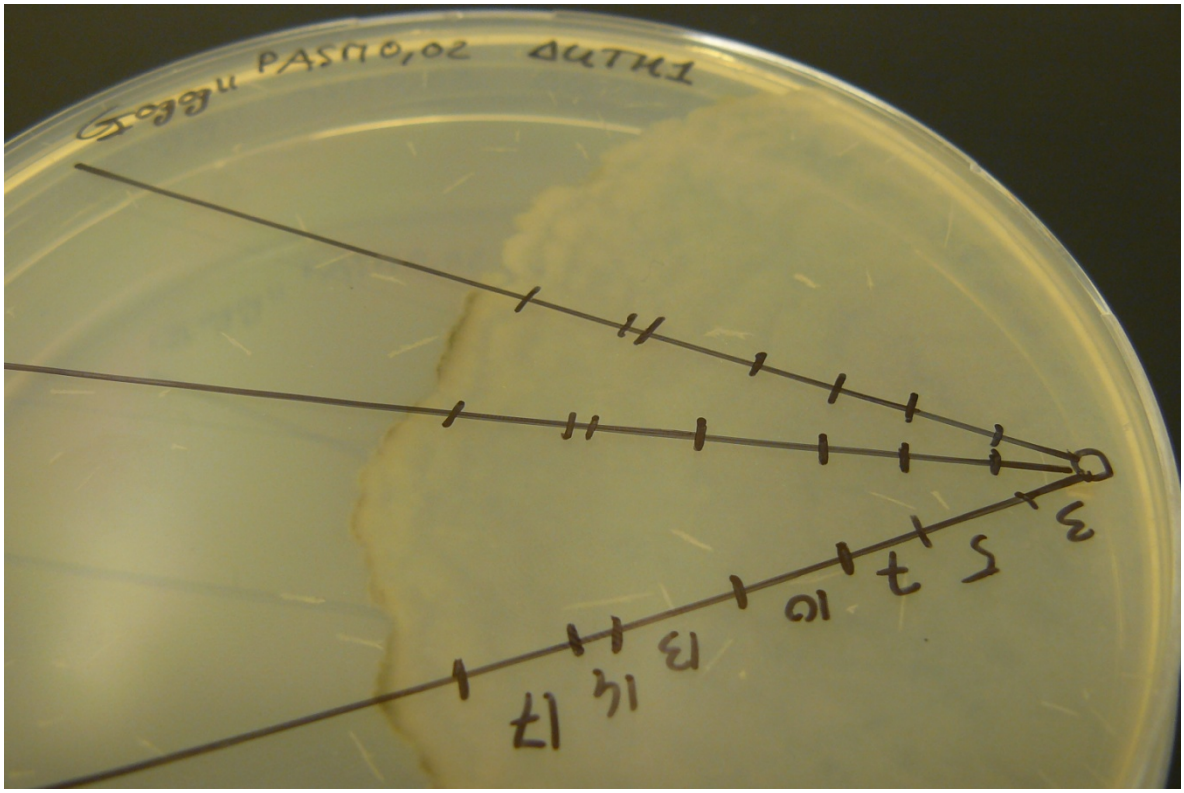

Figure 2 - Morphology of the ΔUTH1 mutant of *P. anserina* when grown under SCR conditions. A phased manner of growth and a lipofuscin front are visible.

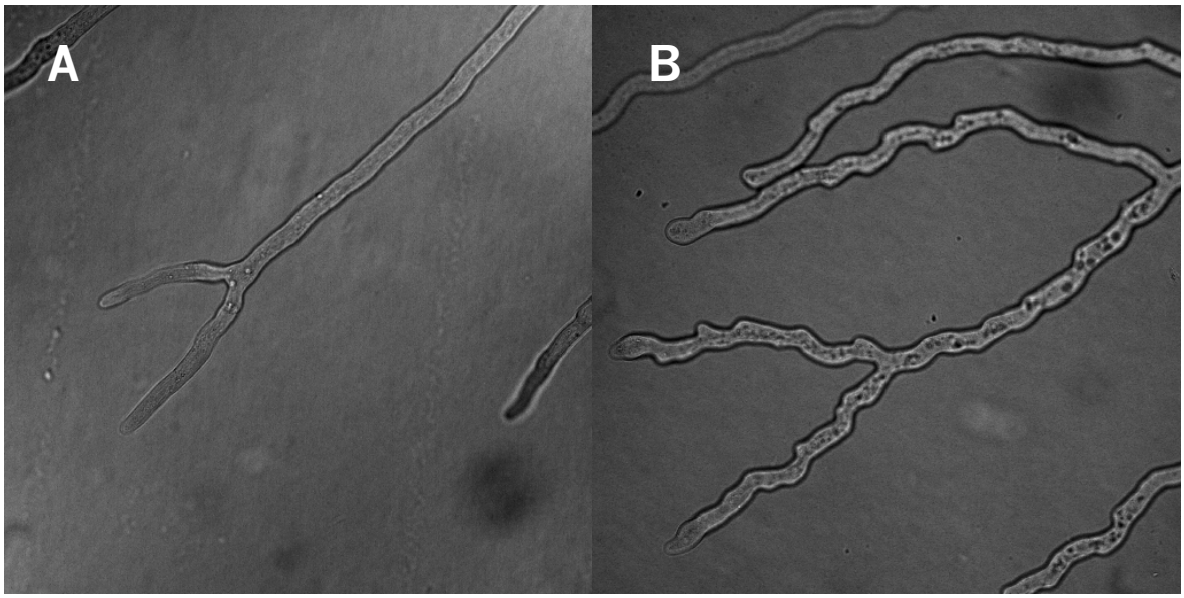

Figure 3 - Microscopic images of hyphae of *P. anserina* wild-type strain s (A) and ΔUTH1 mutant (B) at young age, i.e. two days after germination.

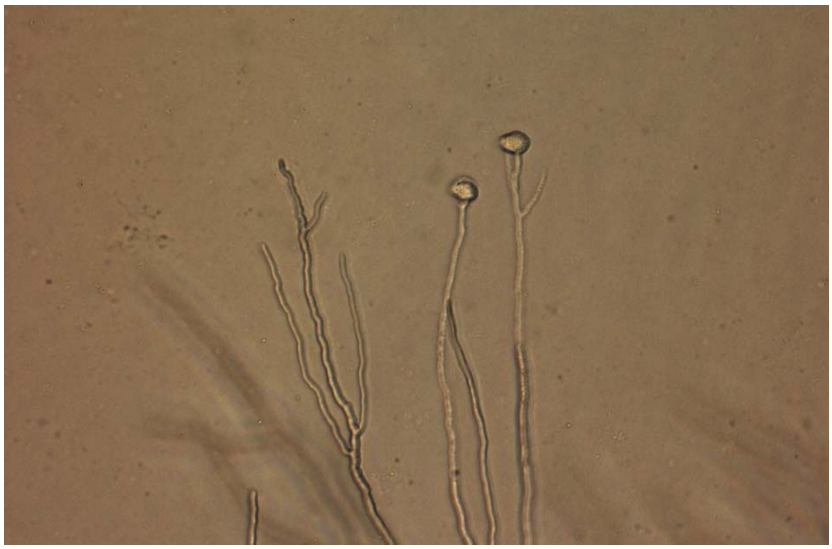

Figure 4 - Bursting of hyphal tips in a ΔUTH1-strain.

When visualizing mitochondrial morphology using the confocal laser scanning microscope, mitochondria of ΔUTH1* look remarkably different from the wild-type too (see Figure 5). The normally observed filamentous mitochondrial networks are absent in the ΔUTH1* strain. Instead, thin fragments of mitochondria are scattered among the cell and occasionally a big mitochondrion or an aggregate of mitochondria is visible, even in young samples visualized two days after sporulation. In contrast to the fragments found in virtually senescent wild-type samples grown at N conditions, the fragmented mitochondria in ΔUTH1* have a smaller diameter than the standard diameter of mitochondrial networks, i.e. less than 0.5 µm (compare Figure 1: N - 14d, with Figure 5B).

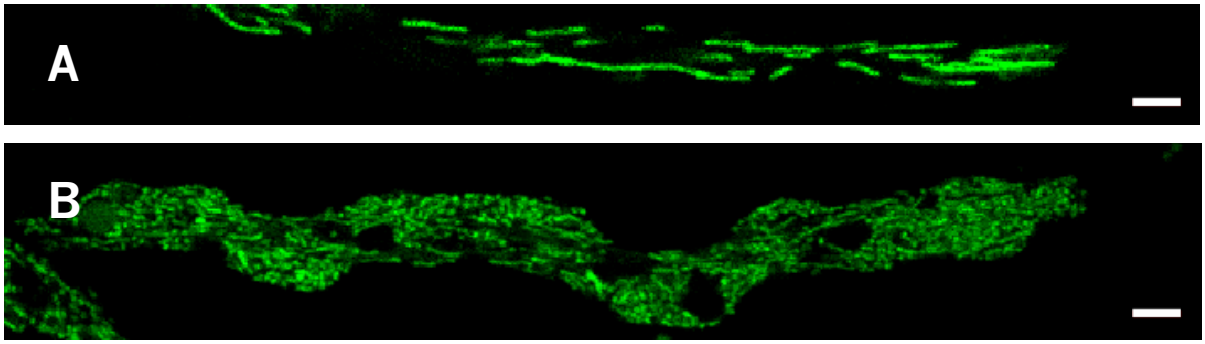

Figure 5 - Fluorescence images of mitochondria from living growth tips of *P. anserina* wild-type strain s (A) and ΔUTH1 mutant (B) at young age, i.e. two days after germination. The white bar in the right bottom corner of each image equals a length of 2 µm.

DISCUSSION

ATP9*-strain

In the ATP9*-strain, which can be considered wild-type, mitochondria of young samples form elongated, tubular networks that have been described in *P. anserina* before (e.g. Scheckhuber, Rödel & Wüstehube, 2008), but also in non-senescent and metabolically active cells from many other organisms (Ishihara *et al.*, 2003; Messerschmitt *et al.*, 2003; Jendrach *et al.*, 2005; reviewed in Karbowski & Youle, 2003; Westermann, 2008). Interestingly, mitochondrial morphology does not appear to change under SCR conditions over the observed time span. Under SCR conditions, mitochondria thus seem to remain in their 'young' morphology, i.e. filamentous networks. So, SCR appears to keep the *P. anserina* culture young at mitochondrial level.

In contrast, when grown at normal glucose concentrations, mitochondrial morphology changes markedly when cells become senescent. Fragmentation of the mitochondrial networks is observed, which is in accordance with previous observations of senescent *P. anserina* cultures (e.g. Scheckhuber *et al.*, 2007). Evidently, fragmentation of mitochondria increases with age at N conditions. It is not clear, however, whether such mitochondrial fragmentation is merely a side effect of age-related damage or that it plays an active role in senescence. Mitochondrial fragmentation has been associated with apoptotic events (Karbowski & Youle, 2003), but it remains to be elucidated if fragmentation of mitochondria is involved in e.g. apoptotic signal transduction or even induction of apoptosis.

Another observation in senescent samples at N conditions was ring-shaped mitochondria. Ring-shaped mitochondria, also referred to as cup-shaped or hooded mitochondria, have been described before in several tissues of a number of organisms (Christensen & Chapman, 1959; Stephens & Bils, 1965; Senger & Saacke, 1970; Skinnider & Ghadially, 1976; Grodums, 1977; Setoguti, 1977; Krech *et al.*, 1981; Lauber, 1982; Tavares & Paula-Barbosa, 1983; Kataoka *et al.*, 1991; Messerschmitt *et al.*, 2003; Almsherqi *et al.*, 2007; Scheckhuber, Rödel & Wüstehube, 2008). In all of these cases, pathology, irradiation, increased metabolic activity and/or a disrupted expression of

fusion or fission proteins are associated to the ring-shaped morphology. To my knowledge, the results of the present study link ring-shaped mitochondria to ageing for the first time. Considering all these findings, one could speculate that ring-shaped mitochondria develop due to cellular damage. In cases of increased metabolic activity, a consequential increased production of reactive oxygen species (ROS) could lead to damaging effects (see e.g. Sellem *et al.*, 2007). Furthermore, when young samples are irradiated by a high intensity laser light, all mitochondrial networks appear to rearrange to ring-shaped mitochondria within half an hour (own unpublished results). It thus seems that the formation of ring-shaped mitochondria is indeed damage induced.

Studies in which alterations of mitochondrial protein levels have been shown accountable for the eventual formation of ring-shaped mitochondria, seem to have contradictory results. Skinnider & Ghadially (1976) observed ring-shaped mitochondria after drug-induced inhibition of mitochondrial protein synthesis, while a study of Almsherqi *et al.* (2007) describes ring-shaped mitochondria after inducing an increased mitochondrial protein synthesis. Correspondingly, a lack of mitochondrial inner membrane protein Mdm33 (Messerschmitt *et al.*, 2003) or an over-expression of mitochondrial fission protein Pa*Mdv1* (Scheckhuber, Rödel & Wüstehube, 2008) also leads to a ring-shaped morphology of mitochondria. Possibly, a disturbance in the mitochondrial protein spectrum may explain these seemingly conflicting results. These findings support the aforementioned hypothesis that cellular damage, in this case represented as alterations of mitochondrial protein levels, induce ring-shaped mitochondria. It is conceivable that an imbalance of counteracting fusion and fission proteins may trigger such abnormal mitochondrial morphologies, but this needs further investigation.

ΔUTH1*-strain

The observation of lipofuscin in ΔUTH1* samples grown at SCR conditions is remarkable. Lipofuscin, also known as the 'age pigment' or 'wear-and-tear pigment', is a brown-yellow material and is widely considered to be a biomarker for ageing (see e.g. Terman & Brunk, 2004). This age pigment is shown to be a polymeric material consisting mostly of lipid and protein degradation residues, but also metals and carbohydrates may be included. Lipofuscin is undegradable and can not, or hardly, be exocytosed, causing it to accumulate in post-mitotic cells (Terman & Brunk, 2004; Terman *et al.*, 2007). Its production is commonly observed in wild-type strains (s and Wa32) when grown on 2% and 0.2% glucose, i.e. N and calorie restricted conditions, respectively, prior to growth arrest and cell death. To my knowledge, lipofuscin production has not been reported in samples grown under SCR conditions at 4°C before. The reason that it was now observed in ΔUTH1* on 0.02% glucose could possibly be explained by accumulation of mitochondrial damage, because of inhibited mitophagy in these mutants. So, damaging of mitochondria does seem to occur at SCR, yet slower than when a higher concentration of glucose is present in the medium.

Indeed, mitochondrial morphology suggests that mitophagy in ΔUTH1* is hampered. The observed thin fragments can be explained by absent or ineffective disposal of defective mitochondria. Damaged mitochondria are unable to fuse with each other due to their low membrane potential, since inner membrane potential is essential for fusion (Legros *et al.*, 2002; reviewed in Westermann, 2008), and consequently, mitochondria do not form networks. Simultaneously, fragments of mitochondria are not broken down and accumulate, resulting in the images observed and, apparently, lipofuscin accumulation.

The P*auth1* deletion in ΔUTH1* also plays a role in growth pattern. Both at macroscopic and microscopic level, the ΔUTH1* mutant seems to grow in a pulsating manner. Together with the findings of bursting hyphal tips, this may indicate a stress response to failing internal processes.

Conclusion

To explain all the observed morphologies of mitochondria, we should take mitochondrial fusion and fission activity in consideration. For what reason do mitochondria undergo fusion and fission?

Fusion leads to the formation of mitochondrial networks, which facilitates a joint membrane potential and therefore a more evenly distributed energy production throughout the cell. More importantly, fusion plays a vital role in the distribution and maintenance of mitochondrial components (Scheckhuber, Rödel & Wüstehube, 2008). Complementation of damaged or missing mtDNA, RNAs, respiratory chain subunits and membrane proteins is the most important feature of this mitochondrial maintenance (Legros *et al.*, 2002) and serves as a rescue mechanism for defective mitochondria, as proposed in the 'rescue hypothesis' (Jendrach *et al.*, 2005). Because of this, fusion is seen as a defence mechanism against the accumulation of somatic mutations and thus can be considered an activity that delays cellular ageing (Westermann, 2008). Indeed, the impairment of fusion results in loss of mtDNA, malfunctioning respiration and an increased ROS production (Bereiter-Hahn & Vöth, 1994), underlining the importance of fusion. In evolutionary terms, forming long networks of mitochondria is thus beneficial to the fitness of the mitochondrial population and the cell in a whole.

On the other side, fission is important for reproduction of mitochondria, since this is achieved only through the binary division of existing mitochondria (Lemasters, 2005). In case no division of mitochondria would take place, it would be impossible for dividing cells to transmit their mitochondria to progeny cells. Furthermore, fission is also associated with apoptosis through fragmentation and possibly with the release of pro-apoptotic compounds (see Scheckhuber, Rödel & Wüstehube, 2008; Westermann, 2008). All of this, however, does not explain the high frequency in which fission occurs. Apparently, fission serves an additional function which requires it to occur continuously. A clue of what this function might be can be found in the literature. Lemasters (2005) mentions that degradation of mitochondria, i.e. mitophagy, is an ongoing process and proceeds without any form of external

induction, while Terman *et al.* (2007) review that smaller mitochondria, i.e. fragments, are degraded more rapidly than large ones. When taking this together with the finding that mitochondrial fusion is highly dependent on membrane potential, i.e. fusion only occurs between mitochondria that have a high inner membrane potential (Legros *et al.*, 2002), one could speculate that fission serves a purpose in removing damaged components from mitochondrial networks.

By randomly forcing out parts of the mitochondrial network, the performance of this fragment can be 'assessed' independently. In case the isolated fragment is able to uphold a high membrane potential separate from the network, it is able to fuse back with a mitochondrial network. However, when this mitochondrial fragment is unable to maintain a high inner-membrane potential as fragment, it will not fuse back, thus remain a fragment and consequently undergo mitophagy more rapidly. Thus, according to the 'fragment assessment' hypothesis proposed here, defective parts that decrease the fitness of the mitochondrial network, and again, the entire cell, are effectively removed. The finding that fission is increased when mitochondrial function is impaired (Scheckhuber, Rödel & Wüstehube, 2008) partly supports this idea, because in this case there is a higher pressure on removing defective parts in the mitochondria population in order to restore a fully functional mitochondrial network.

Still, because of what do fragments accumulate, i.e. do not get removed by mitophagy? According to the mitochondrial theory of ageing, accumulation of damaged mitochondria is on the basis of the ageing process (Linnane *et al.*, 1989). Jendrach *et al.* (2005) have summarized three possible theories of how defective mitochondria can achieve majority over intact mitochondria. They point out that for two of these theories, fusion of mitochondria is necessary, which is not in line with the finding of mitochondrial fragmentation in the present study. However, in the third discussed theory, the 'SOS hypothesis' (de Grey, 1997), a lack of fusion, and thus fragmentation, is required. This necessity of impaired fusion is consistent with the findings of Scheckhuber, Rödel & Wüstehube (2008) that *P. anserina* strains in which the fusion protein

PaFzo1 is over-expressed, no mitochondrial fragmentation is observed during senescence. The 'survival of the slowest' (SOS) hypothesis further postulates that mitochondria which are degraded at the lowest rate will out-compete the others. The finding that defective mitochondria inflict less damage to their membranes due to self-produced ROS (reviewed in Kowald, 1999), supports this theory, since mitochondrial degradation rates are associated with the membrane damage of the mitochondria. The higher the amount of damage, the more rapid their degradation will be. Furthermore, it is possible that fragments can accumulate due to damaged effectors (i.e. proteins) of mitophagy, in extreme cases, a full deletion of a mitophagic protein as in ΔUTH1*. A combination of the SOS-hypothesis and impaired mitophagy is very plausible as well, and further studies are needed to ascertain the exact mechanisms that initiate fragmentation at senescence.

It seems apparent that accumulation of cellular damage, or more specifically protein damage, plays an important role in the formation of mitochondrial fragments and ring-shapes. Whether this damage is a consequence of defective mitochondria and their products, e.g. ROS, or the other way around, is still unclear.

It would be interesting to investigate at what point in the lifespan of *P. anserina* accumulation of fragments starts and what the performance, i.e. energy producing capacity, of mitochondrial networks and fragments is at that point. This information will indicate whether accumulation of mitochondrial damage occurs prior to or following fragmentation, or due to a combination. Furthermore, it could give more information about the energy producing capacities of ring-shaped mitochondria, since it has been suggested that ring-shaped mitochondria are more efficient than regular shaped mitochondria (Senger & Saacke, 1970). 'Mitochondrial performance' could be examined by analyzing mitochondrial membrane potential at several time intervals and using 2-(4-(dimethylamino)styryl)-1-methylpyridinium iodide (DASPMI)-staining (Ramadass & Bereiter-Hahn, 2008). DASPMI uptake in mitochondria is dependent on mitochondrial membrane potential and DASPMI is additionally released immediately when membrane potential decreases (Bereiter-Hahn,

personal communication). However, since DASPMI and GFP optical excitation spectra overlap, another fluorescent dye or tag should be used to visualize mitochondrial morphology during dual imaging. Additionally, it would be interesting to study the dynamics of mitochondrial performance in correlation with mitochondrial morphology and age of ΔUTH1* and other autophagy mutants, e.g. ΔPa*atg1* and ΔPa*atg8*, and strains carrying pAL2-1 plasmid or homologues.

Concluding, what do these findings contribute to our knowledge of mitochondrial mechanisms in the ageing process? The observed fragmentation is caused by impaired fusion and/or lack of autophagy of fragmented, damaged mitochondria. This will instigate a decrease in performance of the mitochondrial population and an accumulation of damaged mitochondria which result in cellular dysfunction. Both will lead to a deterioration of mitochondria and the cell in a whole, finally resulting in senescence. It is difficult to determine what causes this hampered fusion and mitophagy and it might very well be caused by a combination of multiple complex pathways. What is clear is that somewhere in these pathways, SCR conditions are able to prevent fragmentation or its initiator, and consequently prevent or severely delay ageing in a whole. Conversely, loss of mitophagic activity, due to loss of Pa*uth1*, is able to restore and aggravate fragmentation again. Diet and autophagy of defective mitochondria, preceded by fission, are thus very important aspects in prolonging lifespan of *P. anserina*. But to what extend can these findings be translated to other organisms? Although some pathways in ageing research are specified to only a small group of mitochondria, many molecular pathways that are found in model organisms appear to be conserved (Camougrand *et al.*, 2004; Lorin *et al.*, 2006; Groebe *et al.*, 2007; Westermann, 2008). Strong suggestions have been made that fusion and fission machinery and mitochondrial morphology during ageing may be identical among organisms (reviewed in Osiewacz, 2002a; Westermann, 2008). The lifespan prolonging properties of calorie restriction has also been widely established (see e.g. Harman, 1981; Partridge & Gems, 2002; Lorin *et al.*, 2006 for reviews). Extrapolation of mechanisms of such basal aspects as diet and

mitophagy to other organisms thus seem justified. The visual approach of the present study has lead to some new and important findings and has further confirmed the tight link of mitochondria with ageing. However, many questions remain unanswered, so, clearly more research combining visual cell biology with molecular biology is necessary to further develop our knowledge in the intriguing field of ageing.

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