Chapter 3
The Role of Mitochondria in the Aging Processes of Yeast

Michael Breitenbach, Peter Laun, J. Richard Dickinson, Andrea Klocker, Mark Rinnerthaler, Ian W. Dawes, May T. Aung-Htut, Lore Breitenbach-Koller, Antonio Caballero, Thomas Nyström, Sabrina Büttner, Tobias Eisenberg, Frank Madeo, and Markus Ralser

Abstract This chapter reviews the role of mitochondria and of mitochondrial metabolism in the aging processes of yeast and the existing evidence for the "mitochondrial theory of aging". Mitochondria are the major source of ATP in the eukaryotic cell but are also a major source of reactive oxygen species (ROS) and play an important role in the process of apoptosis and aging. We are discussing the mitochondrial theory of aging (TOA), its origin, similarity with other TOAs, and its ramifications which developed in recent decades. The emphasis is on mother cell-specific aging and the RLS (replicative lifespan) with only a short treatment of CLS (chronological lifespan). Both of these aging processes may be relevant to understand also the aging of higher organisms, but they are biochemically very different, as shown by the fact the replicative aging occurs on rich media and is a defect in the replicative capacity of mother cells, while chronological aging occurs in postmitotic cells that are under starvation conditions in stationary phase leading to loss of viability, as discussed elsewhere in this book. In so doing we also give an overview of the similarities and dissimilarities of the various aging processes of the most often used model organisms for aging research with respect to the mitochondrial theory of aging.

Keywords Mitochondria · Mutation · DNA repair · Somatic mutation theory · Hypoxia

Introduction

Mitochondria came into existence at the base of eukaryotic evolution and, arguably, are a prerequisite for multicellular development due to the improved energy metabolism of cells that contain mitochondria (Lane and Martin 2010). Mother Nature has "played" with mitochondria like with any other invention she has made

M. Breitenbach (✉)
Division of Genetics, Department of Cell Biology, University of Salzburg, Salzburg, Austria
e-mail: michael.breitenbach@sbg.ac.at

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in the biosphere. This has led to additional functions of mitochondria besides ATP production, like the function in programmed cell death with its close relationship with aging (see below) and the development of the capacity to exchange signals with the nucleo-cytoplasmic system of protein synthesis and the capacity of the cell to degrade mitochondria in case of severe loss of function. Fortunately, nearly all of these mitochondrial functions have been conserved during eukaryotic evolution. Yeast is well suited to study the functions of mitochondria in aging due to its rapid growth and cell division cycle, its highly developed genetics, the fact that respiration-deficient mutants are viable and can be easily obtained, and, in particular, the availability of methods for introducing nearly every genetic change at will in the chromosome as well as in the mitochondrial genome.

But what can yeast cells really tell us? As has been argued above, because of the conservation of mitochondrial functions in evolution, there is reason to believe that the results obtained with yeast genetics can be relevant for answering the one question that is behind all other questions: Why do we humans age? Can we manipulate, slow down or perhaps stop the intrinsic aging process that appears to be operative in nearly all living things and, of course also in humans?

To get started, we will review here the mitochondrial theory of aging (TOA) in its historical context. But also this theory does not supply us with a single or single most important cause of aging. Mitochondrial defects that occur naturally during aging lead to an increase in internal oxidative stress or an imbalance of redox homeostasis. But the genetic and pharmacological interventions that were applied to cells and organisms to influence their redox environment, in most cases led to only moderate changes in the lifespan.

A Short History of the Mitochondrial Theory of Aging

High energy ionizing radiation (X-rays, gamma rays) produces radicals in aqueous solutions and in living cells. The combination of ionizing radiation and oxygen is much more efficient in radical production, cell killing, and mutagenesis than either treatment alone, leading to the concept of oxygen toxicity in biology. These are typical discoveries of the decade from 1950 to 1960, when "radiation biology" was in its heyday and nuclear reactors were built. Irradiation of living cells, particularly in the presence oxygen, was shown to lead to defects phenotypically similar to those of aging cells. It is important to note that, contrary to the early beliefs, this does not mean that the very low level of natural high energy background radiation is the cause of natural aging, but it was still an important stimulus for the development of aging theories and for directing the research of subsequent decades.

Gerschman (1954) showed that oxygen radicals actually can be formed in living cells. A list of the most important reactive oxygen species (ROS) formed in living cells following the primary production of superoxide radical anion and of the enzymes converting and metabolizing them, as well as the pathophysiology caused by these ROS is given in another chapter (Aung-Htut et al. Chapter 2, this volume). It is now appropriate to broaden the concept of ROS and to speak of "RONS" (reactive

oxygen and nitrogen species) because of the importance of nitric oxide (NO, which is a radical) and peroxynitrite, a highly reactive non-radical species (for review: Halliwell et al. 1987; Halliwell and Gutteridge 1984, 1988).

The "oxygen radical" theory of aging (TOA) (Harman 1956) and the "somatic mutation" TOA (Szilard 1959; Orgel 1963) are closely related because oxygen radicals are mutagenic and the two theories were created at about the same time based on the experimental findings of the 1950s. In 1956, Denham Harman proposed that aging, which is a gradual loss of function in all constituents of living cells, might be caused by the chemical damage due to oxygen radicals, and that the molecular damage which is formed downstream of the short-lived primary radical species might accumulate during the lifetime of a cell and of an organism. All of these theories are interconnected and all of them had to wait for decades before the predictions contained in them could be tested by precisely aimed experiments.

Around 1965–1970, the location, metabolic importance and molecular details of the respiratory chain of the inner mitochondrial membrane gradually were elucidated (Racker 1977; Mitchell 1961). Later, mainly through the work of Britton Chance (for review see: Chance et al. 1979) it was shown that mitochondria not only transform oxygen directly to water (in complex IV of the respiratory chain) but also transform oxygen by an "unwanted" one electron process into the superoxide anion radical. Superoxide is a comparatively long-lived and slow-reacting radical species (but still more reactive and short-lived than most non-radical molecules). An important step in understanding oxygen radical biology was the discovery of superoxide dismutase (SOD) by Fridovich and his students (McCord and Fridovich 1969) at about the same time. Later it was shown that eukaryotic cells produce relatively large amounts of two different superoxide dismutases underscoring the physiological importance of this part of oxygen radical metabolism. The manganese SOD (Sod2) is present in the mitochondrial matrix while the copper/zinc SOD (Sod1) is located mainly in the cytoplasm but some is also found in the mitochondrial intermembrane space.

Based on the finding that most oxygen radicals derive from mitochondria, Harman in 1972 developed a new TOA, now called the "mitochondrial theory of aging" (Harman 1972). Subsequently, the major points of "leakage" of electrons from the respiratory chain were found to be complex III, and complex I. Therefore in yeast, which lacks complex I, complex III is the only physiological point of superoxide formation in the respiratory chain. However, in yeast, in place of complex I, NADH reducing equivalents can be fed into the respiratory chain by Nde1 or Nde2 (external NADH) or Ndi1 (internal NADH) without proton pumping, and these proteins were also found to be capable of producing superoxide (Luttik et al. 1998; Marres et al. 1991; Li et al. 2006). It is controversial which percentage of the electron flow ends up in superoxide, but a rough estimate is 1% under in vivo conditions (Cadenas and Davies 2000).

The present view of the role of ROS in aging includes a signalling function of the ROS and the importance of non-mitochondrial sources of superoxide besides the well-known mitochondrial source. There is now overwhelming evidence for a

physiological role of ROS (oxygen radicals and the molecules derived from them) in cellular signalling. This adds a number of new aspects to the oxygen TOA. The state of current knowledge is slightly different for the fungal model systems of aging and for the model systems based on mammalian cells and organisms. In mammals, the physiological function of ROS produced by mitochondria and NADPH oxidases (NOXs) has been well researched (Buetler et al. 2004) and these ROS (most probably hydrogen peroxide) control growth and proliferation, and also in some specialized cells, cell differentiation and the production of some specialized molecules, for instance the hormone, thyroxine. In this scenario, overexpression or activation of NOX enzymes can lead to hypermitogenic arrest and to apoptosis, pointing to a direct involvement of ROS generated by NADPH oxidases in the aging process, although many questions remain. In fungi, the signalling function of ROS is less well researched (Takemoto et al. 2007) and in the unicellular hemiascomycetous yeasts (*S. cerevisiae* and *S. pombe*), the current literature view is that an NADPH oxidase does not exist.

Some Experiments Designed to Test the Mitochondrial TOA

Simple theory would predict that supplying the cells with biological antioxidants would increase the lifespan. However, adding biological antioxidants (for instance reduced glutathione) to wild type yeast cells does not increase the replicative lifespan. Addition of the lipophilic antioxidants alpha-tocopherol or ubiquinone reduces the lifespan of *Saccharomyces cerevisiae* (Lam et al. 2010). However, adding glutathione in 55% oxygen does increase the lifespan of a yeast strain to that observed in the wild type in ambient air. One additional step is to use a yeast strain deleted for both catalases, and now the lifespan elongation by glutathione is again observed, even in ambient air (Nestelbacher et al. 2000) (see Fig. 3.1).

The mitochondrial TOA is an offshoot of the oxygen radical TOA. While there is some truth in the oxygen radical or mitochondrial TOA, the above experiments indicate that it is not easy to improve the lifespan of the wild type in ambient air, presumably because the dynamic redox equilibrium of the cell has been set at an optimal value during evolution and deviations that lead to an excess of either oxidants or reductants are detrimental to the cell. The redox equilibrium is determined largely by the most important redox buffer of the cell, which is the GSH/GSSG couple. Other important redox couples of living cells are NADPH/$NADP^+$ and NADH/NAD^+, or the relevant SH groups on redox-active proteins. These are, however, far from equilibrium and do not easily exchange with each other (Schafer and Buettner 2000).

If a number of long-lived mutant strains are tested for resistance to oxidants, most of them are resistant to at least one oxidant, but many exceptions exist, as is obvious from a comparison of whole genome deletion mutant data aimed at oxidative stress resistance (Thorpe et al. 2004), a partial data set for RLS (Kaeberlein et al. 2005; Steinkraus et al. 2008; see also Kaeberlein, Chapter 12, this volume) and a complete data set for CLS (Powers et al. 2006; Fabrizio et al. 2010). However, the correlation

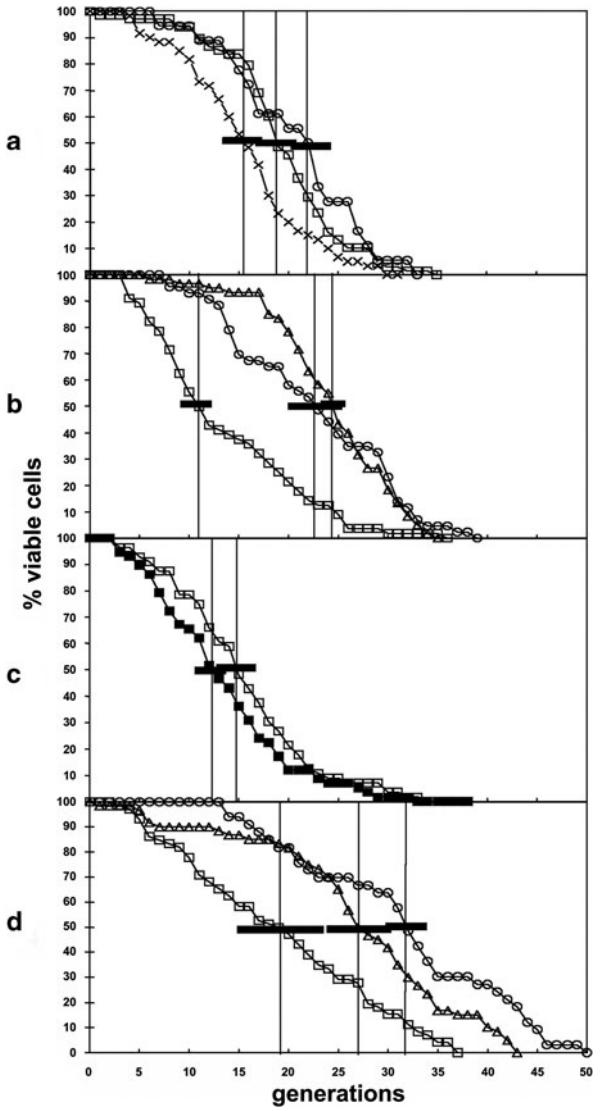

Fig. 3.1 Replicative life-span determination. Percent viable cells are plotted against cell division number (generations). Sample size in all cases was 50–60 virgin cells. *Perpendicular dotted lines* indicate median life spans. SD of the median life spans at a confidence level of 95% which were calculated by applying Kaplan–Meier statistics are indicated by *error bars*. To decide whether two given survival distributions are significantly different at a 95% confidence level, Breslow, Tarone-Ware, and log-rank statistics were used. All experiments were done on SC plates. Additions were as indicated below. **a** Strain W303eA: *circles*, ambient air; *squares*, 55% oxygen; *crosses*, 20 μM of paraquat. **b** Strain W303eA *ctt1:: URA3*, *cta1:: URA3*: *circles*, ambient air; *squares*, 55% oxygen; *triangles*, 55% oxygen, 1 mM GSH. **c** *open squares*, strain W303eA, *ctt1::URA3*; *closed squares*, strain W303eA, *cta1:: URA3*. 55% oxygen. Note that the control experiments for (**c**) are included in (**a**) (isogenic WT, 55% oxygen) and (**b**) (isogenic double mutant, 55% oxygen). **d** Strain JC482: *Circles*, ambient air; *squares*, 55% oxygen; *triangles*, 55% oxygen, 1 mM GSH (from Nestelbacher et al. 2000; with permission from Elsevier)

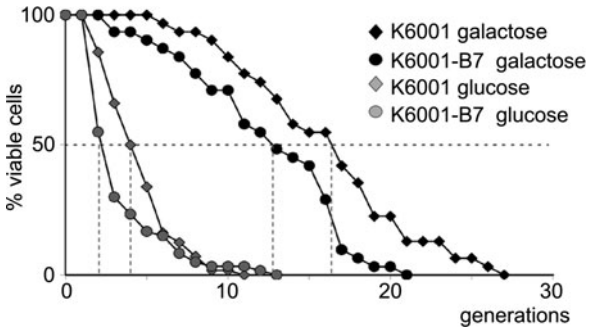

Fig. 3.2 K6001-B7 has a shortened replicative lifespan. Daughter cells from K6001 and K6001-B7 were continuously removed by micromanipulation and counted and analyzed statistically. The shortened lifespan of K6001-B7 in both glucose and galactose media was tested statistically significant using Mantel-Cox, Breslow as well as Tarone/Ware statistics (from Timmermann et al. 2010, with permission from Impact Journals LLC)

between oxidative stress resistance and longevity is better for CLS than for RLS. Comparable correlative evidence exists for long-lived mutants of *C. elegans* (Park et al. 2009).

If, on the other hand, the mutants obtained from an unbiased whole genome screen for resistance to oxidants are tested for their replicative lifespan, many of them are normal or even short-lived. One example analysed in detail was the phenotype of the dominant point mutation B7 in the yeast peroxyredoxin Tsa1, which was found to be short-lived (Timmermann et al. 2010) (see Fig. 3.2). Mutants in triose phosphate isomerase would be another example. They have an increased NADPH/$NADP^+$ ratio, are resistant to oxidants, but are short-lived in both CLS and RLS (Ralser et al. 2006).

Genetics and Biochemistry of Human and Yeast Mitochondria

Mitochondria are the main producers of ATP in most eukaryotic cells, but also house a large number of essential metabolic pathways or, in some cases, parts or steps of pathways. It is unknown why during the evolutionary history of these endosymbiontic organelles, specific parts of metabolic pathways of the endosymbiont had to be retained in these versatile organelles while others were lost to the nucleocytoplasmic system. There is nowadays no serious doubt in the once controversial endosymbiont hypothesis stating that mitochondria evolved by reduction and gene loss from an original hypothetical prokaryotic symbiont in a monophyletic way. Only a small part of the original gene set of the symbiont was retained in the mitochondria while the largest part moved to the nuclear chromosomes. Among the “retained” genes (13 protein coding genes in human cells, 8 in yeast cells)

are genes coding for the largest and most hydrophobic protein components of the respiratory and ATP synthase complexes of the inner mitochondrial membrane. In addition, the ribosomal RNAs and the 22 tRNAs needed for mitochondrial translation had to be retained. The rest of the about 700 (yeast) and about 1500 (mammals) mitochondrial proteins are encoded in the nuclear genome, synthesized on cytoplasmic ribosomes and imported into mitochondria in rather complicated but well-known ways (Schmidt et al. 2010; Baker et al. 2007). Only about 300 of the 4800 viable yeast gene deletions lead to "*petite*" (non-respiring) mutants that are unable to grow on non-fermentable carbon sources and only about half of them are directly involved in respiration (Merz and Westermann 2009). What is puzzling from an evolutionary viewpoint is that some (for instance mammalian) mitochondrial genomes are extremely small and streamlined, while others (yeast, plants) are bigger, very variable in size, and encode slightly different sets of proteins of the respiratory complexes. A microorganism with a much larger number of mitochondrially encoded proteins was discovered (Lang et al. 1997), which is therefore perhaps closer to the original endosymbiont than most modern mitochondria. The genetic code used in mitochondria differs from the universal code, and the mitochondrial code used by humans is not the same as that used by yeast.

Yeast and human cells are inviable without mitochondria, however they are viable without mitochondrial respiration, as exemplified by the so-called rho-zero mutants which by definition are devoid of mitochondrial DNA, but still contain mitochondria. Rho-zero mutant cells of yeast, which can easily be isolated either after spontaneous loss of the mitochondrial genome or after treatment with ethidium bromide, grow slowly on glucose and other fermentative substrates like raffinose, but not at all on "respiratory substrates" like glycerol, ethanol, or lactate. These mutants must import ATP (produced from fermentation) into their mitochondria, which is possible by means of a functional adenine nucleotide transporter. All respiratory deficient mutants of yeast as well as rho-zero cells are chronologically short lived (Fabrizio et al. 2010), but the RLS of these non-respiring cells can be substantially longer than that of the corresponding wild type, depending on the retrograde response in those cells (Kirchman et al. 1999, see the Chapter 4 by Jazwinski, this volume). Certain mutations in mitochondrial ribosomal proteins and in the apparatus regulating mitochondrial translation are also replicatively long-lived (Heeren et al. 2009; see below).

Human rho-zero cells can be isolated by ethidium bromide treatment and can be maintained in cell culture easily, but rho-zero cells or any cells completely devoid of mitochondrial respiration cannot differentiate in the context of a chimeric embryo and cannot form an embryo. We mention these cells because loss or large deletions of the mitochondrial genome as well as mitochondrial point mutations clearly occur during aging in the mouse and in humans. There is no detailed published evidence for the frequency of mitochondrial mutations during replicative or chronological aging of yeast cells (compare, however, Muller 1971), but relatively recently the group of Gottschling showed that the daughters of old mothers indeed tend

to become petite (Veatch et al. 2009). Long-lived respiratory deficient mutations were among the last survivors in replicative aging in certain strains (Kirchman et al. 1999). However, re-examination of this experimental result showed that the respiratory-deficient mutant cells pre-existed in the sample, they were not generated during aging (S.M. Jazwinski, personal communication).

The frequency of chromosomal mutations in replicatively old cells was studied in more detail. The group of Gottschling showed an increase of gene conversions (loss of heterozygosity) at the *MET15* locus in diploid cells during replicative aging (McMurray and Gottschling 2003; Veatch et al. 2009), and the group of Longo showed an increase in canavanine-resistant clones during chronological aging (Longo 1999) and an increase in chromosomal rearrangements caused by mitochondrial oxidative stress leading to DNA lesions and the activation of translesion synthesis repair pathways (Madia et al. 2009; Parrella and Longo 2008) indicating an increase in chromosomal mutations during chronological aging.

Further Evidence for the Role of Mitochondria in the Aging Processes: Mitochondrial Mutations (Point Mutations vs. Large Deletions)

The mitochondrial TOA (see above) was historically conceived because mitochondria were recognized as a source of ROS leading to the idea of a vicious cycle in mitochondrial ROS metabolism. Mitochondrial ROS according to this idea would produce point mutations, which would produce defective respiratory complexes, which would again produce more ROS and so on. This was plausible, although not really proven (Muller et al. 2007). A general decline of mitochondrial function in aging was shown in higher organisms (for instance Boveris and Navarro 2008). In yeast, accumulation of ROS-positive mitochondria (Laun et al. 2001) and a strong decline in aconitase activity in old wild type mother cells (Klinger et al. 2010) was shown in mother cell-specific aging. Similarly, aconitase is deactivated during chronological aging in wild-type and *sod2*-deleted yeast (Fabrizio et al. 2001; Parrella and Longo 2008) and mitochondria showing increased ROS accumulate during chronological aging (Herker et al. 2004; Buttner et al. 2006; Mesquita et al. 2010; Buttner [unpublished data]) (see Fig. 3.3).

It seems clear that a decline of mitochondrial activity takes place during both aging processes observed in yeast, but so far there is only evidence for the accumulation of mitochondrial point mutations or large deletions in replicative aging (Veatch et al. 2009). Mitochondrial mutation would also be worth investigating in chronological aging. There is presently intensive research going on pertaining to the role of mitochondrial mutations in mammalian aging (Khrapko et al. 2006; Trifunovic et al. 2004; Vermulst et al. 2007, 2008; Williams et al. 2010).

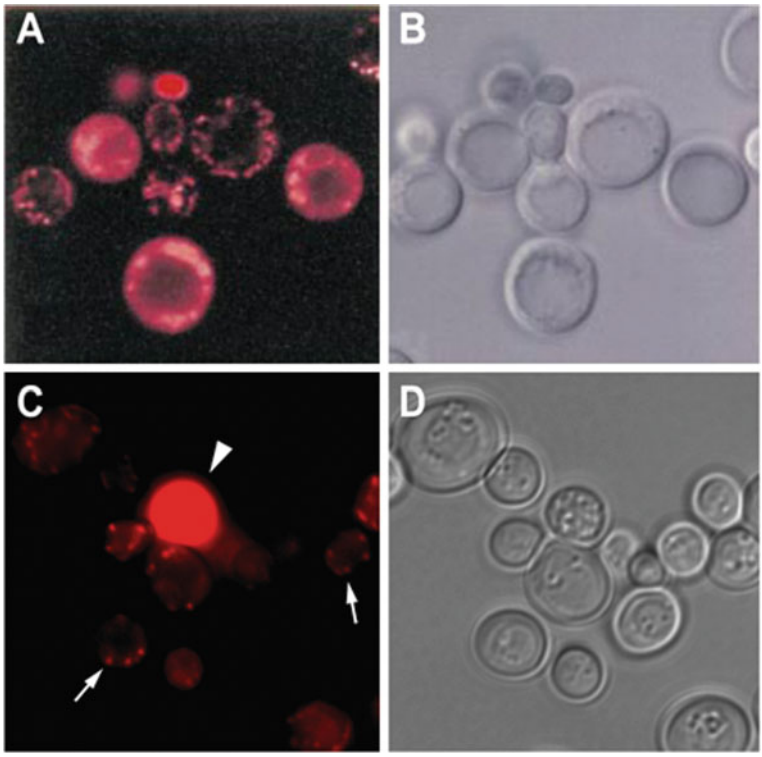

Fig. 3.3 **a** Old mother cells were stained with dihydrorhodamine (DHR, 5 mg ml^{-1}; stock solution 2.5 mg ml^{-1} in ethanol) and viewed and photographed under a confocal laser-scanning fluorescence microscope after 10 min using the rhodamine filter set. The stained cells show typical mitochondrial morphology. **b** The same sample in phase contrast (modified after Laun et al. 2001 with permission from John Wiley and Sons). **c** Chronologically aged yeast cells (day 5) were stained with dihydroethidium (DHE, 2.5 μg/ml in PBS, stock solution 2.5 mg/ml in DMSO) and analyzed using laser scanning confocal fluorescence microcopy. An exposure time of 4000 ms was used to visualize not only lethal doses of ROS (*arrowhead*, necrotic dead cell) but also the generation of sublethal doses of ROS at mitochondria (*arrows*, cells in early stages of apoptosis). **d** The same sample was analyzed using phase contrast

Accumulation of Mutations in the Mitochondrial Genome: Cause or Consequence of Aging?

Although the relevant measurements have not been made in yeast mitochondrial DNA, we should summarize briefly the current state of the situation concerning the role in aging of mitochondrial point mutations and large deletions in the mouse. In two laboratories homozygous mouse knock-in strains were constructed to contain exclusively a mitochondrial DNA polymerase with a defect in the proof-reading

domain (Trifunovic et al. 2004; Kujoth et al. 2005). These mice age prematurely as shown by a shorter lifespan and an increase of practically all diseases of aging of the mouse (for instance, cardiovascular diseases) at an age where the wild type does not show these disease phenotypes, while the heterozygous littermates are normal. However, the heterozygous mice have a normal lifespan and health, although in heart and brain they carry tenfold more mitochondrial point mutations than normally aged very old mice (Khrapko et al. 2006; Vermulst et al. 2007). This would clearly indicate that mitochondrial point mutations cannot be the cause for aging in these mice (see Fig. 3.4).

The matter depends very much on the ability to accurately measure spontaneous mitochondrial point mutations by sequencing. In a later paper (Vermulst et al. 2008) the Loeb group maintains that not point mutations but rather large deletions of mitochondrial DNA may be causative for aging. They argue that the heterozygous mice still have a low level of deletions, not higher than normally aged tissues, and that the formation of deletions (which are also very well researched in age-related pathology in humans) depends on mitochondrial homology-driven repair in the absence of proofreading. Trifunovic et al. contest this notion (Edgar et al. 2009, 2010) and maintain that mitochondrial point mutations are causative for aging. The question is open at present. The issue is complicated because mitochondrial genetic instability, including deletions and point mutations always start as being heteroplasmic, cell

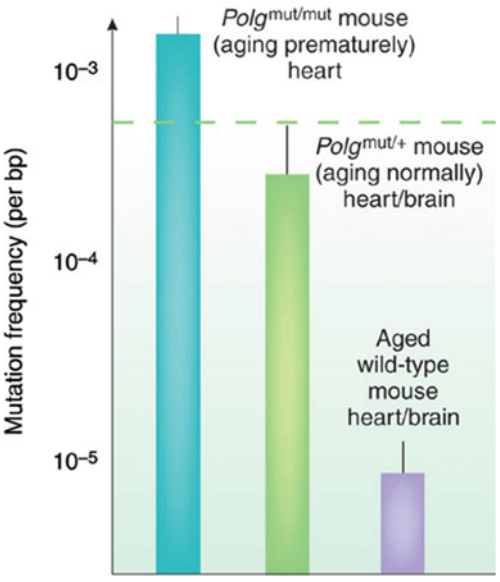

Fig. 3.4 Logic dictates that mtDNA mutations, when present at levels lower than in phenotypically normal *Polg*$^{mut/+}$ mice (*dashed green line*), are irrelevant for aging. Note that very old wild-type mice display a much lower frequency of mitochondrial mutations as compared to the normally aging heterozygotic mice. *Error bars* represent estimated variation of the data (adapted by permission from Macmillan Publishers Ltd: Aging Cell Khrapko and Vijg 2007, copyright 2007)

divisions then lead to segregation of mutant and wild-type mitochondria, and after a number of cell divisions a low threshold value of respiration is reached which causes loss of function of the particular cell concerned. This threshold value may be different in different organs of the mouse and of humans.

Mitochondrial Catalase

Although these very important experiments have been done in mice, and not in yeast, we want to introduce them briefly, because they are feasible and worth doing in yeast as well. In the wild type, mitochondria do not contain catalase. The superoxide produced at a low level in the respiratory chain is converted to hydrogen peroxide by mitochondrial MnSOD and can pass the mitochondrial membranes and can be further converted to ROS. Transgenic mice engineered to express human peroxisomal catalase in their mitochondria showed a significant increase in mean and maximum lifespan and a significant decrease in cardiac and cataract pathology. The authors also showed that a double transgenic mouse expressing human catalase and mouse SOD2 in their mitochondria had an even more enhanced median lifespan. Similar experiments with expression in the nucleus or in the peroxisome of mice did not significantly influence their aging process (Schriner et al. 2005). This constitutes one of the most convincing experiments showing that the combined antioxidative action removing ROS can increase fitness and lifespan and, because of the dependence on subcellular location, the experiment also shows that the mitochondrially generated oxygen radicals (and hydrogen peroxide) can limit the lifespan of the wild type.

A Decline in ATP Production

ATP production during the aging of yeast has never been measured in a systematic way, but the experiment would be worth doing. In higher cells, "AMP-kinase" is a signalling element responsive to the level of AMP/ATP in the cell, which plays an important role in aging. In yeast, the *sch9* deletion mutant is replicatively and chronologically long-lived (Fabrizio et al. 2001). The pathway represented by Sch9 is one of the partially redundant glucose-sensing pathways (together with the RAS/cAMP pathway and the TOR pathway) and it seems to be a general principle that down-regulation of glucose (or nutrient) sensing can improve longevity. This has been stressed in many review articles (for instance Kapahi et al. 2010) and is believed to constitute one of the "public mechanisms" of aging. It is not quite clear what is the gene corresponding to *SCH9* in higher organisms, but it could be S6 kinase, PKB or AMP kinase based on sequence comparisons. PKB (protein kinase B, AKT) of mice, flies and worms has been extensively researched and down-regulation of the IGF (insulin-like growth factor) signalling pathway in which PKB is involved seems to increase lifespan (Longo and Fabrizio, Chapter 5, this volume).

The Increase in Mitochondrial ROS and the Role of Apoptosis in Aging

This increase has been discussed above and has been observed both in RLS and CLS experiments. Problems inherent in the measuring methods are discussed in the chapter on oxidative stress (Aung-Htut et al. Chapter 2, this volume) and a general treatment of the role of apoptosis in aging is given by Laun et al. (Chapter 10, this volume). Increased ROS production is a marker of apoptotic and necrotic yeast cells (Madeo et al. 1997, 1999; Eisenberg et al. 2009). Mitochondria have been identified as a primary source of ROS in programmed cell death (Braun et al. 2006; Ruckenstuhl et al. 2009; Ludovico et al. 2002). Both apoptotic cells and necrotic cells are observed among replicatively as well as chronologically aged yeast cells (Eisenberg et al. 2010; see the Chapter 10 by Laun et al. this volume; Jungwirth et al. 2008; Herker et al. 2004; compare Fig. 3.3).

It is an interesting question whether apoptosis is just a phenomenon finalizing the aging process caused by other means, or if perhaps apoptotic genes and mechanisms are causally involved in the aging process itself. The first of these two possibilities seems to be true for RLS, based on observations with replicatively long-lived mutants. For instance, in the *afo1* mutant (Heeren et al. 2009), mitochondria staining positively for ROS and apoptotic phenotypes do occur in senescent mother cells, but they are seen at a time 50–60% later than in the wild type. This means that the longer RLS is not caused by a defect in the apoptotic process. On the other hand and pointing in the same direction, most of the genes and deletion mutants involved in yeast apoptosis are not replicatively long-lived. However, there may be some causal connection between apoptosis and CLS, because many apoptosis mutants are chronologically long-lived. An example is the yeast caspase deletion mutant, *yca1*Δ, which shows a normal RLS (Laun et al. unpublished data) and a longer than wild type CLS (Laun et al. 2008; Madeo et al. 2002).

Signalling by Mitochondria and Its Role in the Aging Process; Hypoxia and Aging

Mitochondria exchange information or "cross-talk" with the nucleo-cytoplasmic protein synthesis system in order to adapt the cell to a changing environment. A well-known example is the sensing of hypoxia in the environment and responding by degrading mitochondrial components on the one hand and synthesizing isoforms adapted to hypoxic conditions, on the other. This is achieved by the HIF-1 system in higher cells and by a different mechanism involving the *HAP* genes and the *ROX1* gene of yeast. The HIF-1 system is termed "positive regulation", because the transcription factor HIF-1 positively stimulates transcription of hypoxic genes. The HAP system is termed "negative regulation", because the HAP1 and HAP2/3/4/5 transcription factors stimulate mitochondrial biogenesis in high oxygen and repress hypoxic genes. In spite of these differences in transcriptional regulation, adaptation

of respiration to low oxygen works in a very similar way in the two types of cells, by introducing for the standard complex IV subunit cox4-1 (the ortholog is cox5a in yeast) a new isoform subunit cox4-2 (the yeast ortholog is cox5b), which can handle oxidative phosphorylation at low oxygen more efficiently without producing a high level of ROS. The new and exciting aspect is that activating this system (stabilizing HIF-1) under normoxic conditions where it is normally degraded by VHL (the Von Hippel-Lindau tumor suppressor, an E3 ubiquitin ligase), can substantially increase the lifespan of *C. elegans* (Mehta et al. 2009; Kaeberlein and Kapahi 2009; Muller et al. 2009). Similarly, mild inhibition of respiration can also activate HIF-1 and increase the lifespan (Lee et al. 2010) although this treatment actually increases ROS production. Conversely, other authors found that the HIF-1 deletion or RNAi-mediated downregulation of HIF-1 can also increase the worm's lifespan. This effect functionally interacts with CR, since it depends on nutrient availability and is not effective in calorically restricted worms (Chen et al. 2009). Actually lowering oxygen tension has been tested in *Drosophila*. Hypoxia can increase the lifespan of *Drosophila* (Vigne and Frelin 2007). The mechanism by which this works is independent of caloric restriction and IGF-1, but is under control by the TORC1 kinase. Taken together, the role of hypoxic regulation in lifespan determination and its interaction with dietary restriction must await further experimental results to fully explore the regulatory network that is active here.

The hypoxia response is one example of signalling from the nucleus to the mitochondrion leading to a major re-structuring of this organelle. Important aspects of this signalling mechanism remain to be determined, in yeast as well as in higher organisms, for instance the exact nature of the oxygen sensor (Lee et al. 2009). Multiple oxygen sensors seem to exist, one well researched example which, however, occurs only in higher cells, is the proline hydroxylase (Berra et al. 2006; Ratcliffe 2006) which, depending on oxygen tension, is functional in the degradation of HIF-1.

Another example for regulation of mitochondrial biogenesis is the regulation of mitochondrial mass and activity in response to a change in carbon source (Ohlmeier et al. 2004), which is necessary when the yeast cell switches from fermentative (on glucose) to respiratory metabolism on glycerol, ethanol, of lactate as carbon source. A third example is regulation of mitochondrial respiration and ROS production by the RAS/cAMP pathway (Pichova et al. 1997) under the selective influence of the catalytic subunit of protein kinase A, TPK3 (Hlavata et al. 2008; Leadsham et al. 2009; Gourlay and Ayscough 2006), leading to a very short replicative and chronological lifespan of yeast in mutants that over-express TPK3 or express the $RAS2^{ala18,val19}$ allele. The latter effect depends on the yeast-specific extension of the *RAS2* gene (Chen et al. 1990).

However, signalling by mitochondria to control nuclear gene expression, is also possible. The retrograde response is a gene regulation mechanism that controls among many other genes, also those controlling the synthesis of peroxisomal anaplerotic enzymes. These enzymes, for example *CIT2*, are needed for a fully functional amino acid metabolism in non-respiring yeast cells, which do not express

a full mitochondrial tricarboxylic acid cycle (Butow and Avadhani 2004; Liu and Butow 2006). As mentioned before, yeast respiratory-deficient cells (*petite* cells) are chronologically short-lived, but are replicatively long-lived in a nuclear genetic background that enables a strong retrograde response (Jazwinski 2004, 2005a, b, c; Kirchman et al. 1999). The nature of the signal emitted by the non-respiring mitochondria is unknown, but the role of the Rtg1/2 transcription factors downstream of the primary signal is well researched (Jazwinski 2005b; Liao and Butow 1993). The retardation of replicative aging by the retrograde response is independent of the CR effect on the lifespan (Jiang et al. 2000). An important but unanswered question is: does the retrograde response exist in human cells (Jazwinski 2005a), taking into account that during human aging respiratory-deficient cells do occur and may play a role in aging (Khrapko et al. 2006)?

Recent work from our laboratory (Heeren et al. 2009) showed that non-respiring mitochondria command yet another different way of back-signalling to the nucleus,

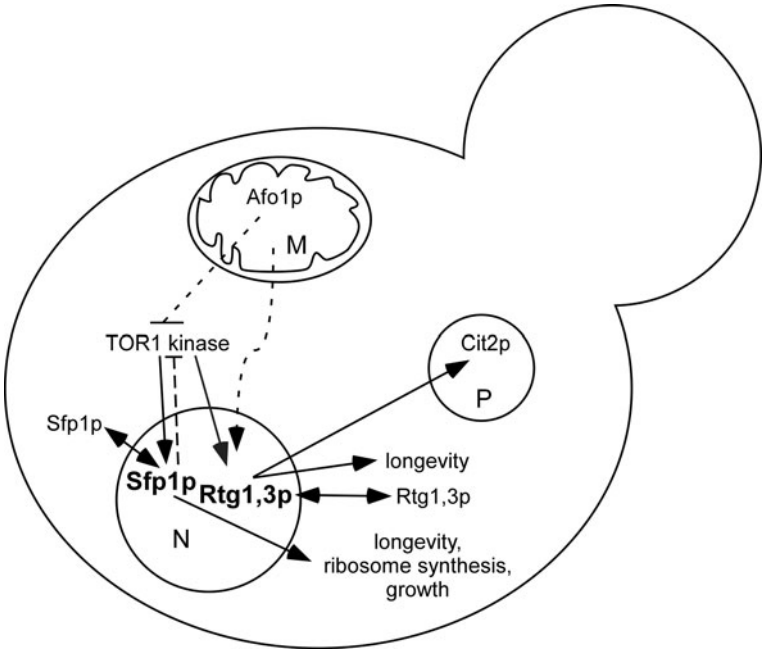

Fig. 3.5 Schematic diagram of back-signalling involving *AFO1* based on the results summarized. *Dashed arrows*: genetic interactions for which a molecular mechanism has not been determined. Both Sfp1p and Rtg1,3p shuttle to the cytoplasm when Tor1p is inhibited by rapamycin. They are indicated in **bold** in the nucleus, where they are active. An activating influence of the *TOR1* kinase complex on the transcription factor *Rtg1/Rtg3* has been postulated by Dann and Thomas (2006). Feedback inhibition of Tor1p by nuclearSfp1p is indicated. The RAS/cAMP and *SCH9* components are omitted for clarity. Their interaction with the TOR pathway is complex. *M*, mitochondrion; *N*, nucleus; *P*, peroxisome (adapted after Heeren et al. 2009, with permission from Impact Journals LLC)

which results in a long replicative lifespan as well as an unusually rapid rate of growth (comparable to the congenic rho-plus wild type) of these non-respiring cells. A deletion of the nuclear-encoded *AFO1* gene encoding a mitochondrial ribosomal protein displays these remarkable features together with resistance to three different oxidants. The *afo1* deletion leads to rapid loss of the mitochondrial genome, but on standard medium (YPD, 2% glucose) where the long lifespan is manifest, the retrograde response is not activated. However, the mutant is resistant to rapamycin and the transcription factor Sfp1 governing cytoplasmic ribosome synthesis remains active and nuclear in 100 nM rapamycin. The long lifespan of the mutant depends on the presence of a functional *TOR1* gene. We conclude that deletion of *AFO1* activates TORC1 in respiratory-deficient cells leading to the observed long replicative lifespan and the rapid growth on glucose. Since Afo1 is mitochondrial under all conditions tested, and since two other deletion mutants in genes coding for mitochondrial ribosomal proteins do not have any effect on lifespan and grow slowly like standard rho-zero yeast cells, we believe that Afo1 is involved in mitochondrial back-signalling, a function important for respiratory deficient yeast, but possibly independent of its primary function, which is mitochondrial translation (see Fig. 3.5).

The Mitochondrial Translation Complex Regulates RLS

Intriguingly, other mutations in components of the mitochondrial translation system can also substantially elongate the replicative lifespan of yeast cells. One example is *sov1Δ*, which has a strikingly similar phenotype to *afo1Δ*. It is respiratory deficient, does not trigger the retrograde response, is resistant to rapamycin (Xie et al. 2005), is resistant to certain kinds of oxidative stress, and replicatively (but not chronologically) long-lived. The mechanism that comes into play here could be similar to the one shown in Fig. 3.5, but a detailed comparison of the two mutants has yet to be done.

The synthesis of intricate mitochondrial structures, such as the respiratory complexes, is a well-coordinated process. In order to carry out the correct assembly of these complexes, proteins encoded by the nuclear genome have to match and be perfectly balanced with the ones that are directly synthesized in the mitochondria. This interplay between the nucleus and mitochondria demands a tight regulation of mitochondrial protein synthesis that, in yeast, requires more than 150 proteins regulating proper translation of mitochondrial genes encoding the respiratory complex subunits (Grivell et al. 1999). In particular, it has been shown that the translation of each of the mitochondrial-encoded genes requires at least one specific translation activator protein that in some cases also helps targeting the newly synthesized protein into the mitochondrial inner membrane (Fox 1996; Naithani et al. 2003; Perez-Martinez et al. 2003). This group of specific translation activators have been clustered and denominated the MTC (mitochondrial translation complex) module (Table 3.1; Perocchi et al. 2006).

Table 3.1 The 14 proteins of the mitochondrial translation complex with the mitochondrial target proteins for which they are specific

MTC subunit	Mitochondrial target proteins and function
Sov1p	Required for translation of *VAR1*
Mss51p Pet54p Pet111p Pet122p Pet309p Pet494p	Required for the translation of the mitochondrial *COX* genes (*COX1, COX2,* and *COX3)*
Cbs1p Cbs2p Cbp6p	Required for translational activation of *COB* mRNA
Aep1p Aep2p	Required for translation of *OLI1*
Suv3p Dss1p (Msu1p)	Form the mitochondrial degradosome, required for turnover of aberrant or unprocessed mtRNA

Interestingly, deletion mutations in at least 9 of the MTC genes results in an increased RLS (Caballero et al. 2011), and this enhanced longevity is totally independent of the lack of respiration, loss of mitochondrial DNA, altered ROS generation or, more interestingly, a global lack of mitochondrial translation, since a deletion in *IMF1,* which encodes a mitochondrial translation initiation factor does not extend life span by itself (Caballero et al. 2011). Furthermore, mutations in MTC module genes that increase RLS do so without activation of the retrograde response. Instead, knock-down mutations in *SOV1* or *CBS1* stimulate *SIR2*-dependent life span extension by elevating nuclear DNA silencing. Although the pathway through which MTC proteins affect nuclear silencing and life span is not fully understood, it is clear that life span extension achieved by deleting *SOV1* requires cAMP/PKA-dependent activation of the stress regulators *MSN2*/4. In addition, the presence of the cAMP/PKA target gene *PNC1* (Caballero et al. 2011), which encodes a scavenger of nicotinamide, a derivative molecule from NAD that inhibits Sir2p activity (Anderson et al. 2003; Gallo et al. 2004) is also required for Sov1p deficiency to extend RLS. Thus, the pathway involved in Sov1p-dependent life span control shares many features and players described previously for life span extension dependent on *TOR1* (Medvedik et al. 2007). At the present, it is not clear whether the MTC proteins have a role apart from translational control in the mitochondria that affects the efficacy of genomic silencing, or if defects in translational control trigger a compensatory response that activates *SIR2*-depedent silencing and life span extension. Nevertheless, it is interesting that a discrete, functionally related, group of mitochondrial proteins regulates yeast life span by affecting the efficacy of genome silencing and that this phenomenon may be independent of previously proposed RLS control circuits exerted by mitochondria in yeast (Ugidos et al. 2010).

Noticeably, some of the MTC proteins are also present in higher eukaryotic organisms; whether these proteins can also control life span is obviously something that remains to be tested.

Autophagy and Mitophagy and the Asymmetric Segregation of Mitochondria

As has been stated elsewhere in this book, during aging the unavoidable attrition and the effects of adverse chemical reactions originating from metabolism pertaining to all cellular components including proteins, lipids and nucleic acids makes degradation or repair of those components necessary for survival, just like the synthesis of those components itself. It appears that the most important of these degradation processes are proteasomal degradation and autophagy, of which several different pathways have been discovered (Deffieu et al. 2009; Bhatia-Kissova and Camougrand 2010). Conventional macroautophagy is believed to be essentially non-selective, meaning that portions of the cytoplasm of a cell are delivered into the lytic compartment (the yeast vacuole) on a random basis (Kissova et al. 2007). However, much more relevant for the present discussion are selective pathways of autophagy (Nair and Klionsky 2005) and in particular, mitophagy (Kim et al. 2007; Kanki and Klionsky 2008; Kanki et al. 2009; Bhatia-Kissova and Camougrand 2010). In the case of mitochondria, selectivity for mitochondria is achieved through the mitochondrial protein Atg32 together with Atg11 (Kanki et al. 2009). It is still not completely clear how non-functional mitochondria are discriminated from functional ones, but the existence of the correct membrane potential across the inner mitochondrial membrane could be essential for protection against mitophagy. This was shown by inducing mitophagy by genetically damaging mitochondria, for instance by depletion of Mdm38, which strongly induced mitophagy (Nowikovsky et al. 2007). However, this artificial situation does not reflect aging mother cells.

Autophagy/mitophagy and the other selective degradation processes do not work with 100% efficiency and they must be complemented by asymmetric segregation of damaged proteins and presumably also damaged mitochondria and other organelles in order to avoid clonal aging and death of the strain, as was first shown by Lai et al. (2002). The process is not specific to yeast, but is a universal one (Nystrom 2007) which, as we hypothesize, is generally necessary for survival of the species preventing clonal senescence. As shown by the Nyström group (Aguilaniu et al. 2003; Erjavec et al. 2008; Erjavec and Nystrom 2007; Liu et al. 2010), oxidized proteins are asymmetrically distributed between mother and daughter cells. The majority of the oxidized proteins were found to be cytoplasmic, but some were mitochondrial. Denatured proteins after heat shock were preferentially transported back to the mother cell in a process depending on the actin cytoskeleton, the polarisome, and on the heat shock protein, hsp104 (Liu et al. 2010).

In a recent paper (Klinger et al. 2010), we have shown that the protein of the mitochondrial matrix, aconitase, which is extremely sensitive to oxidative inactivation due to the 4Fe/4S centre in its active site, is inactivated during replicative aging without applying any external stress. The inactivated protein is preferentially retained in the mother cell, while the still active enzyme is preferentially transported to the daughter cell. This asymmetric segregation works in the direction to the daughter and, like the process just described (Liu et al. 2010), contributes to the rejuvenation process of the daughter cells, which in our view and according to a broad consensus is essential for the survival of the species. As aconitase in yeast remains a mitochondrial protein throughout the whole life cycle of the cells, it is clear from these results, that in the divisions of aging mother cells, whole mitochondria must be discriminated and segregated. The mechanism of this process is unknown at present. The old mother cells, due to internal oxidative stress contain small roundish mitochondria (not the familiar mitochondrial network) and it is quite conceivable that these structures are preferentially transported to the daughter, if they still have a near-normal membrane potential.

Closing Remarks

Overviewing the literature published in recent years, it becomes clear that the role of mitochondria in the aging processes of yeast is diverse and a unifying theme or mechanism has not yet been found. Of the three questions posed at the beginning of this chapter, only one can be (incompletely) answered at present time: Yes, biochemical changes can be found that look similar in aging yeast cells and in higher organisms. They could point to a basic but still unknown unity of the aging process. They may have to do with mitochondrial dysfunction. In the aging processes, any one of the possible metabolic and morphological changes of mitochondria which are listed below play a role for which some experimental evidence exists. These processes are not independent of each other and, probably, multiple simultaneous causes exist:

(i) Decline of ATP production
(ii) Increase in mitochondrial ROS production
(iii) Role of mitochondria in the apoptotic process
(iv) Mitochondrial signalling: the retrograde response; back-signalling
(v) Asymmetric segregation of damaged mitochondria, degradation of mitochondria
(vi) Defects in the fission and fusion processes of mitochondria.

What is rather well established for both replicative and chronological aging is an increase in internally generated cellular oxidative stress with age leading to small roundish mitochondria that stain positively for ROS production, as was described above in the main part of this chapter. Moreover these observations seem to be common also for the aging processes of higher cells. However, it is presently an open question, if mitochondrially generated ROS are a primary cause of aging (inducing,

among other things, the pathways of apoptosis), or, alternatively, the mitochondrial ROS observed in old cells are a by-product of aging.

The retrograde response and the mitochondrial back-signalling process can substantially increase the RLS despite respiratory deficiency, but present knowledge about the genetic mechanism of these effects is fragmentary.

We think that one of the most interesting questions to be further investigated in aging research generally, also relating to RLS only, is the asymmetric segregation of damage between mother and daughter cells in the aging process, and in particular the asymmetric segregation of whole mitochondria according to their functional state. The evidence is strong that processes of this kind are not yeast-specific but very general; however, many open questions remain, including the mechanism of discrimination, the sensing of damage (membrane potential?), the relation between mitophagy and asymmetric segregation, and the mechanism of transport of damaged and of still functional mitochondria. Mitophagy as well as asymmetric segregation are mechanistically largely unknown at present. Both processes could form a link to the aging of stem cell populations of higher organisms. Protein aggregates at the surface of mitochondria ("stress granules") could be involved in these processes (Rinnerthaler et al. 2006; Rinnerthaler et al. unpublished).

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