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SURVEY AND SUMMARY

Yeast mother cell-specific ageing, genetic (in)stability, and the somatic mutation theory of ageing

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ABSTRACT

Yeast mother cell-specific ageing is characterized by a limited capacity to produce daughter cells. The replicative lifespan is determined by the number of cell cycles a mother cell has undergone, not by calendar time, and in a population of cells its distribution follows the Gompertz law. Daughter cells reset their clock to zero and enjoy the full lifespan characteristic for the strain. This kind of replicative ageing of a cell population based on asymmetric cell divisions is investigated as a model for the ageing of a stem cell population in higher organisms. The simple fact that the daughter cells can reset their clock to zero precludes the accumulation of chromosomal mutations as the cause of ageing, because semiconservative replication would lead to the same mutations in the daughters. However, nature is more complicated than that because, (i) the very last daughters of old mothers do not reset the clock; and (ii) mutations in mitochondrial DNA could play a role in ageing due to the large copy number in the cell and a possible asymmetric distribution of damaged mitochondrial DNA between mother and daughter cell. Investigation of the loss of heterozygosity in diploid cells at the end of their mother cell-specific lifespan has shown that genomic rearrangements do occur in old mother cells. However, it is not clear if this kind of genomic instability is causative for the ageing process. Damaged material other than DNA, for instance misfolded, oxidized or otherwise damaged proteins, seem to play a major role in ageing, depending on the balance between production and removal through various repair processes, for instance several kinds of proteolysis and autophagy. We are reviewing here the evidence for genetic change and its causality in the mother cell-specific ageing process of yeast.

INTRODUCTION

In this article, we will discuss published evidence for different kinds of genetic change which occur in senescent yeast mother cells and compare these facts briefly with the evidence for genetic change in ageing in higher organisms and with the evidence for genetic changes in chronologically ageing (stationary phase) yeast cells. The occurrence of genetic changes in senescent cells does not necessarily mean that these genetic changes are a cause (or the cause) of ageing. Therefore, it is necessary to study the ageing process in cells and organisms after introducing (in a reverse genetics fashion) precisely known mutations which influence the frequency of genetic change and test what influence on lifespan and the ageing process in general they might have. Alternatively, natural variants which display a higher or lower mutation rate can be studied (including interspecies comparisons) or environmental conditions can be used that increase or decrease the mutation rate. Genetic manipulations, which change the intrinsic mutation rate require advanced methods of reverse genetics and have only recently been performed. Experiments to increase mutation rate are documented, however, experiments to the opposite effect (to decrease the very low natural mutation rate) have, to our knowledge, not been performed for yeast mother cell-specific ageing, although those experiments would be the most useful to test the theory. Below, we list several possible kinds of genetic/genomic instability, and of course, all of them must be tested by gene-targeted analysis. Experiments concerning increased mitochondrial DNA mutation rate in the mouse have very recently led to quite unexpected conclusions and have cast doubt on the notion

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that mitochondrial mutations may cause ageing (see also the part on ageing and the mitochondrial genome) (1,2).

The somatic mutation theory of ageing goes back to the 1950s and 1960s (3,4) and was put forward at the same time as the 'free radical theory of ageing' (5). Both theories share the tenet that the main cause of ageing is random damage to cells and organisms and one of these 'random damages' is damage inflicted on the genome. This has been challenged by researchers who are in favour of a 'genetic programme of ageing', which has been selected during organismic evolution over the course of millions of years and has, according to these researchers, a positive selection value, i.e. it would increase the chance of survival of the species. Typical examples of such theories have been put forward, for instance, by Skulachev (6) and de Magalhaes (7). However, following the convincing arguments of Kirkwood and his colleagues (8,9), Vijg and colleagues (10,11), and other groups, we suggest here that the apparent contradiction between 'random wear and tear' and 'genetic programme' can be resolved by considering the role of genetic programmes which do indeed exist and concern the defence against environmental stress that all cells and organisms have developed in order to survive. These are simple Darwinian traits necessary for survival, or in Darwinian terms, for fitness of the individual. We propose, and we and others have actually shown, that cellular and organismic ageing creates intracellular oxidative stress (12) and many other stresses, most notably in the course of the DNA damage response ['replication stress', (13)]. The cell must respond to these stresses, and survival depends on the stress response genes and their alleles, which are present. Certain gene deletions in the stress response pathways can therefore shorten the lifespan, but some others can also increase the lifespan, under laboratory conditions. In this way, genes in the stress response pathways and their interrelated metabolic pathways (for instance basic metabolic pathways for the supply of ATP or NADPH) appear as 'gerontogenes'. It is generally believed that mutations, which increase the lifespan tell us more about the defence pathways relevant for ageing as compared to mutations which shorten the lifespan. In only a few model organisms do the genes in which such ageing mutations have been found, show us a coherent picture of the pathways involved. This is most clear in *Caenorhabditis elegans*, where the pathway including the IGFR (insulin-like growth factor receptor) is very well represented in ageing mutants. It is less clear in yeast mother cell-specific ageing (Figure 1). The IGFR pathway in metazoans is interrelated with stress response and growth control and provides an understandable link to the caloric restriction hypothesis of longevity (14). However, the existence of these gerontogenes does not mean that evolution has positively selected genes that cause ageing or which set up an intrinsic clock that counts away time and tells us when it is time to die.

The somatic mutation theory of ageing was formulated when the effect of mutagenic ionizing radiation on the lifespan of mice was studied (3). The evidence available at that time was hardly convincing for a causal relationship between mutation rate and lifespan shortening in the mice. Moreover, the article did not consider the problem of

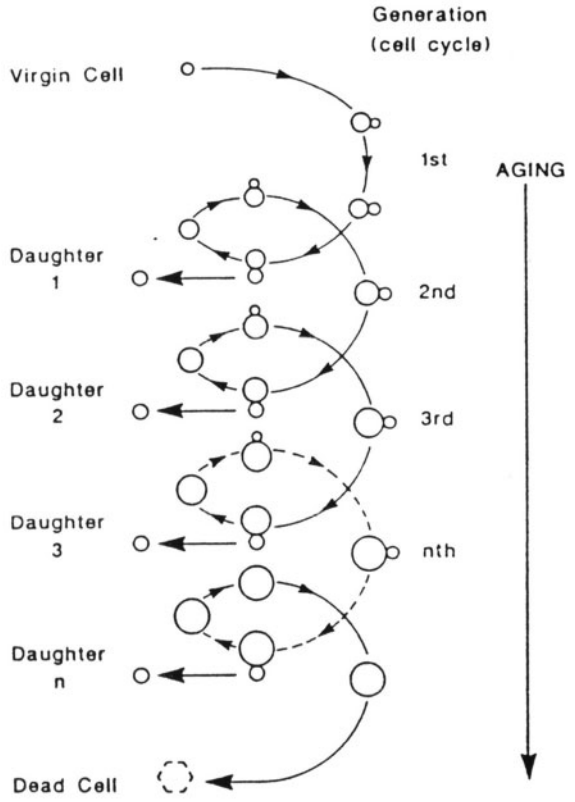

Figure 1. Yeast mother cell-specific ageing is characterized by a limited capacity to produce daughter cells. The lifespan is determined by the number of cell cycles a mother cell has undergone, not by calendar time, and in a population of cells the lifespan distribution follows the Gompertz law. Daughter cells reset their clock to zero and enjoy the full lifespan characteristic for the strain. *Reprinted from Ref. (122), with permission from Elsevier.*

the protection of the germ line from mutagenic damage. If the mutation load indeed accumulates in somatic cells during the lifetime of the individual thereby causing the ageing process, we must assume that germ line cells are specially protected against mutations in chromosomal genes. Otherwise the mutation load would increase in every generation of individuals during the evolutionary history of the species, and the species would die out. The argument obviously depends very much on the germ line hypothesis of Weismann (15,16). Interestingly, to our knowledge, no experimental work exists at present showing that germ line cells are protected from mutagenic insult or display a smaller mutation rate compared to somatic cells. Even if it were true that a high dose of ionizing radiation could cause symptoms that are similar or identical to the natural ageing process (in other words, premature ageing), this would not show that the cause of ageing under natural conditions (with a very low dose of ionizing radiation) is the accumulation of somatic mutations. Nevertheless, the somatic mutation theory of ageing has been investigated and discussed over the years, and we want to critically discuss this hypothesis for the special case of yeast ageing.

Environmental insults (like the mutagenic actions of ionizing radiation) not only attack the genome, they also

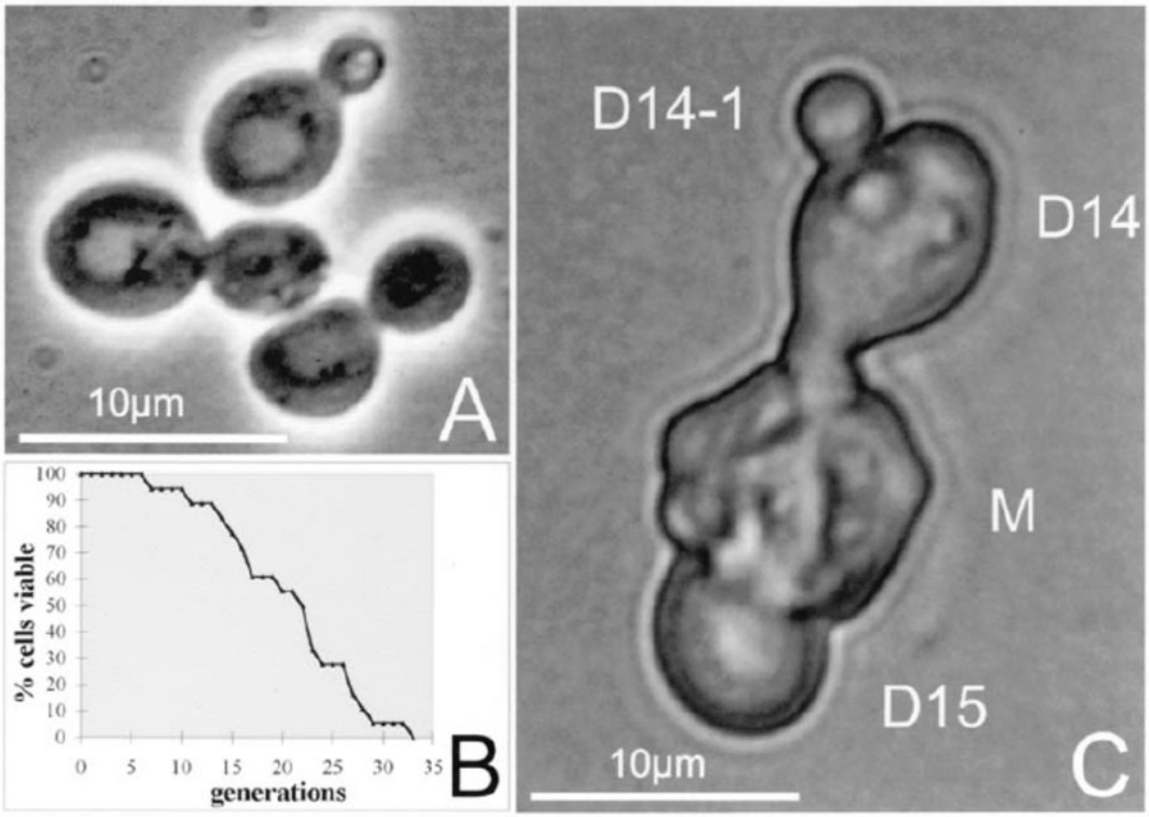

Figure 2. The picture shows in part: (**A**) exponentially growing young yeast cells of strain W303, a commonly used haploid MAT*a* strain, which are included here to show the size and morphology difference to old cells. (**B**) A typical lifespan determination of the same wild-type strain. The number of budding cycles that each of a set of 50 'virgin' cells undergoes before it stops dividing was determined by micromanipulation and counting of budding cycles. (**C**) M is the terminal mother cell after 15 cell cycles. Note the enormous size as compared to young cells and the surface changes. D14 is the second but last daughter that did not completely separate from the mother and did not give rise to new living cells. Also, the surface of D14 is folded or wrinkled. The budding of the 'granddaughter' D14-1 stopped at an early stage of the cell cycle. The budding of last daughter cell, D15, also stopped at an early stage of the cell cycle. *Reprinted from Ref. (20), with permission from Elsevier.*

attack proteins, lipids and other biomolecules. Unlike chromosomal DNA, the other cellular components are not necessarily duplicated by a semiconservative mechanism during the course of a cell cycle. The evidence of oxidative and other damage to these non-DNA components is overwhelming. The problem of 'rejuvenation' (removal of the damaged material) is well recognized by researchers in the field, but very little research has been published up to now dealing with a possible mechanism of this rejuvenation process. In principle, two possibilities exist: asymmetric segregation between mother and daughter cell [this is a major and interesting problem in yeast mother cell-specific ageing; (17)]; and cellular repair processes which remove the damaged material. The repair processes include: protein degradation in the proteosomal pathway, nonsense-mediated mRNA decay, autophagy, mitophagy and others. Finally, in higher organisms, apoptosis is a mechanism for rejuvenation, which removes, unnoticed by the immune system, cells that are damaged beyond a certain threshold and cannot return to a normal cell cycle. As long as the maintenance of cells and tissues works well, damage, in particular DNA damage, triggers a response (the well-researched DNA damage response) which arrests the cell cycle until the damage has been repaired. This is one example of the checkpoint regulation of the cell cycle, which was originally discovered by Hartwell and colleagues (18,19). If there is no repair, or if repair is impossible, the DNA damage response pathway undergoes a molecular switch leading to apoptosis. We have indirect evidence that in very old yeast mother cells this checkpoint mechanism is inactivated, leading to catastrophic cell cycles (20) during which very probably gross chromosomal rearrangements and aneuploidy are produced on a large scale (Figure 2). In our view, the gradual accumulation and partial repair going on in the ageing process, leaves cellular function nearly normal during most of the lifetime of the cell. But the accumulation of unrepaired damage leads to a catastrophic event when the terminal stage is reached, regular cell cycles are no longer possible and the cell dies, typically through apoptosis. We call this final stage 'senescence'. Note that apoptosis serves two different purposes: it is a way of rejuvenation of tissues (mentioned above) but also the way in which old mother cells finally die. This dual function interestingly parallels the activity of a pivotal apoptotic effector, the Endonuclease G, recently studied by the group of Frank Madeo. They have discovered that this endonuclease in apoptosis is transferred from the mitochondria to the nucleus and actually is the enzyme that degrades DNA in chromatin leading

to a 'smear' of nuclear DNA in apoptotic yeast cells and to a positive TUNEL test. However, depending on cellular metabolism (aerobic or anaerobic), the deletion of Endo G can actually promote the death or the life of the cell (21). This points to the second, life-promoting function of Endo G (the 'day job'), which is mitochondrial recombination.

We are now considering the possible genomic changes which are believed to occur in ageing cells and which in part have also been investigated in them. In no model system of ageing have researchers supplied us with a definite proof that these genomic changes do in fact cause the ageing process and we will also consider what would be necessary to show causality in every case.

This is a short list of the kinds of genomic instabilities that have been discussed in the literature and which we will discuss in the rest of this article:

- Extrachromosomal ribosomal circular DNAs (ERCs)
- Loss of heterozygosity, hyperrecombination and aneuploidy
- Telomere length changes
- Mitochondrial DNA changes: (i) point mutations and small deletions; (ii) large deletions

EXTRACHROMOSOMAL RIBOSOMAL DNA CIRCLES (ERCS)

Although mother cell-specific ageing of yeast was discovered by Mortimer (22), and the basic biology of this phenomenon was worked out between 1965 and 1985 by Muller and her colleagues in Germany (23–28), the field became well known in the scientific community only in the 1990s through the work of two American groups, headed by L. Guarente and S.M. Jazwinski. The discovery by the Guarente group of the role of extrachromosomal ribosomal DNA minicircles in yeast mother cell-specific ageing (Figure 3), and, based on this discovery, the elucidation of the role of the histone deacetylase, *SIR2*, in the ageing process, resulted in a renewed interest in yeast ageing research and in the contribution of many more research groups around the world. ERCs in spite of intense searches, have never been found in ageing mammalian cells or in any one of the non-yeast ageing model systems, and must therefore be designated as a 'private' yeast mechanism of ageing (22). The SIR2 (sirtuin) protein family, on the other hand, is highly conserved in eukaryotes (29–32). The link between ERCs, sirtuins and the metabolic regulation of ageing (for instance by so-called caloric restriction) will be explained below. At the same time, Jazwinski and others studied in more detail the role of oxygen toxicity (oxygen radicals), mitochondrial respiration, energy charge and lipid metabolism in yeast ageing (33). Oxygen radicals or rather their follow-up products, have been shown to accumulate in ageing cells in practically all known model systems of ageing, and are therefore a 'public' mechanism of ageing which is very probably also active in human ageing. However, the genetic proof for a causal relationship between oxygen radicals and ageing is not perfect (and will not be discussed in detail here).

The key to understanding this sometimes bewildering multiplicity of proposed 'causes of ageing' is the notion that there is no single most important cause, but depending on environmental conditions, and on the genetic background of the strain or species, different factors can become limiting for lifespan. This is not surprising given the multiplicity of insults to DNA, proteins and lipids with which a cell or organism has to deal during its lifetime and the multiplicity of stress response and signalling reactions of the cells sensing such insults.

ERCs are nucleolar circular DNA species that comprise the 9.1 kb DNA of the nucleolar organizer (coding for the ribosomal RNAs of yeast) or multiples thereof. ERCs have one relatively weak origin of replication per repeat length, but no centromere. Therefore they behave like episomal plasmids, accumulate to a large degree in the nucleoli of mother cells and are very inefficiently transmitted to daughter cells. These are very clear examples of genomic changes associated with mother cell-specific ageing. ERCs can be analysed by Southern blot analysis and they are absent in first generation daughter cells but abundant in old mother cells of several different wild-type laboratory strains that were analysed.

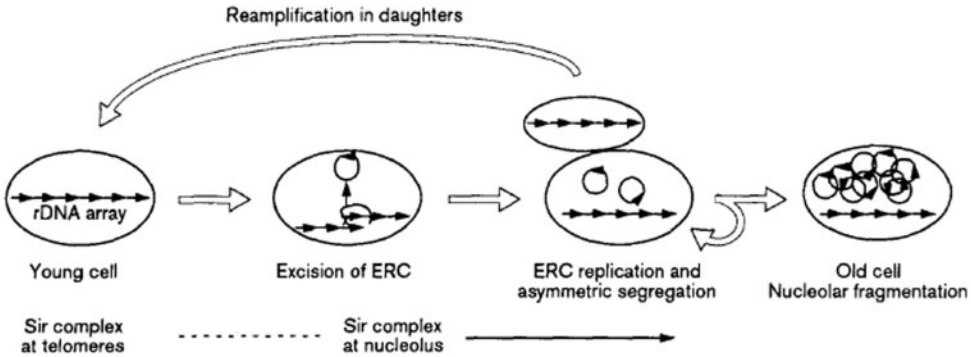

Figure 3. Extrachromosomal ribosomal DNA circle (ERC) model of yeast ageing. Intrachromosomal recombination between repeats within the rDNA array of a mother cell leads to excision of an ERC. The ERC replicates but is not segregated to the daughter cell. The daughter cell presumably restores proper chromosomal rDNA copy number through amplification of the remaining repeats. Continued replication and asymmetric segregation cause ERCs to accumulate exponentially in the ageing mother cell, leading to nucleolar fragmentation and cell senescence. The Sir complex, which silences transcription at the silent mating type loci and at telomeres, translocates to the nucleolus either just before or just after ERCs form, to inhibit their genesis or accumulation. *Reprinted from Ref. (123), with permission from Elsevier.*

Why do these ERCs arise in yeast cells and why do they not arise in higher cells? ERCs in yeast are thought to be a by-product of the special non-allelic recombination mechanism that is needed to maintain sequence identity in about 100–120 copies (in most strains) of the 9.1 kb rDNA repeats on chromosome XII. The genes and gene products involved in this non-allelic recombination process have been well studied (34–40) and mutations are known that prevent the formation of ERCs as well as mutations that promote the production of ERCs. *FOB1* codes for a replication termination protein that is also needed for non-allelic recombination of rDNA. Deletion of *FOB1* is viable and to a large degree prevents ERC formation (41,42) but not homologous recombination elsewhere on the genome [see below; (43)]. It leads to an increase in mother cell-specific lifespan that depends on the strain background. In the original report on ageing in a *fob1*Δ strain (41), a large increase of the median lifespan from 23 to 37 generations was reported, however in the now generally used BY strain background the increase is less dramatic, (44; and our own observations).

Mutations in the *RAD50* series of recombinational repair genes (double-strand break repair genes) completely prevent ERC formation but do not increase (rather they decrease) the mother cell-specific lifespan (45). The tentative explanation is that these mutations (for instance *rad50*, *rad51* and *rad52*) not only influence rDNA-specific non-allelic recombination, which is a very special case of mitotic recombination, but also the more general double-strand break repair, and that this deficiency in double-strand break repair perhaps causes the severe decrease in mother cell-specific lifespan. Interestingly, other DNA repair genes tested (*RAD1*, *RAD7*) had no influence on lifespan.

SGS1p is the homologue of the human WRN protein, mutations of which cause Werner's syndrome, one of the best-studied premature ageing syndromes in humans. A deletion of the yeast *SGS1* gene leads to an increase in ERCs even in young cells and to prematurely ageing yeast (46). This deletion is epistatic to the *fob1* deletion as the double mutant displays the same short lifespan as the *sgs1* mutant (41) in spite of a very low level of ERCs. The same effect is seen in an even more drastic way in the sgs1, rad52 double mutant strain (47). This is a good example for premature ageing, which is not caused by ERCs.

The Sir proteins were originally found to be necessary for transcriptional silencing of the telomeric copies of the mating type information, *HML* and *HMR*, and of the rDNA loci (37,48,49). The Sir2 protein is the only one of the original four Sir (silent information regulator; 48,49) proteins which shows a strong influence on non-allelic recombination of the rDNA repeats (37) and which leads to a strong increase in ERC production when deleted (46,50). That ERCs can cause premature ageing is also shown by the fact that increasing the number of ERCs by genetic constructs that have nothing to do with the natural production of these circular rDNAs also shortens the lifespan considerably. This was achieved by activating the Cre recombinase in a yeast cell that harbours rDNA with appropriate Lox sites to eliminate a centromeric sequence (46). Quite surprisingly, simply introducing an episomal (pRS306) but not a centromeric plasmid (pRS316) that has nothing to do with rDNA also shortened lifespan considerably (46).

On the other hand, the presence of the endogenous 2-micron DNA plasmid, well known for its highly efficient site-specific recombination system that constantly interconverts the circular molecule into its isomer, does not induce any shortening of the host cell lifespan and does not accumulate in old mother cells (51). In fact, due to its self-carried partitioning system encoded by the *STB* locus, the plasmid is very efficiently partitioned at mitosis between mother and daughter cell.

While the ERCs were shown to be nucleolar and the nucleolus of old mother cells is enlarged and morphologically abnormal (46,52), the plasmids mentioned above are nuclear, not nucleolar. The copy number of ERCs and episomal plasmids in old mother cells is estimated to be about 1000. A tentative explanation offered by the authors for the induction of premature senescence by these DNA species is that they titrate away a protein factor essential for life, perhaps a limiting component of the replication machinery (52). In the case of ERCs, there is evidence for this titrating effect: In old mother cells, the Sir protein complex which is needed for silencing of the telomere-proximal copies of mating type information, HML and HMR, is no longer available because it has been titrated away by the ERCs. This leads to expression of the no longer silent mating type copies, and hence, sterility due to a 'pseudo-diploid' state of the old mother cells.

However, the question is much more difficult to answer if in a wild-type strain in complete media, mother cell-specific ageing is indeed caused by the ERCs. Nearly completely prohibiting the formation of ERCs in a *fob1* deletion strain leads to a moderate extension of the lifespan, as mentioned above, which would speak against ERCs as a major cause of ageing in the wild type, on complete media. An experiment which has never been performed, could shed light on this hypothesis: production by genetic engineering of ERCs that lack the binding site for Sir2p or for all of the Sir proteins and testing to see if these ERCs that no longer bind the Sir proteins would still lead to premature ageing.

Why do higher cells in culture or *in vivo* not express ERCs? We do not know, but a tentative explanation could be that the mechanism that ensures sequence conservation of the rDNA repeats is different in yeast and higher cells. One indication that this could be so is that no obvious homologue exists for *FOB1* in higher cells.

The pronounced role of the *sir2* deletion in shortening the lifespan of yeast could also be unrelated to the nucleolus and to the ERCs. The group of Nystrom showed that in the *sir2* deletion strain, the asymmetric distribution of oxidatively damaged proteins between mother and daughter cell is lost and the damaged proteins are found equally in mothers and daughters (17). It is unknown how the loss of Sir2p leads to this loss of asymmetry, but the loss of this histone deacetylase could of course have complex genome-wide consequences. It is also possible that Sir2p has a more direct function in establishing the segregation asymmetry for damaged proteins.

The role of the nucleolus in mother cell-specific ageing, the titrating of the Sir2p protein complex in old mother

cells, and the special role of the Sir2 protein mentioned above, has led the groups of Guarente and of Sinclair to investigate in more detail the role of the NAD^+-dependent histone deacetylase, Sir2p, in ageing. The sirtuins (*SIR2*-like proteins) are highly conserved in sequence and function in eukaryotes and even in archaebacteria. Seven paralogues exist in human cells, one of which (SIRT3) is mitochondrial (32). This family of histone deacetylases is unique because it transfers an acetyl group from defined lysines of histones H3 and H4 to NAD^+, thereby cleaving NAD^+ and creating 2′-O-acetyl-ADP ribose, which is suspected to be a new second messenger.

According to a hypothesis that is attractive but still not proven, the sirtuins are thought to sense the metabolic state of the cell and modulate transcriptional gene expression of the cell according to its metabolic and redox state. Influencing the sirtuins by small molecule pharmaceuticals in order to simulate a metabolic state resembling caloric restriction, and thereby preventing ageing and age-related diseases such as obesity, diabetes and cardiovascular disease, is currently a hotly debated field (53), but outside the scope of this article.

YEAST MOTHER CELL-SPECIFIC AGEING AND TELOMERE-RELATED SENESCENCE

The so-called telomere hypothesis of ageing is based on the observation that somatic cells in culture have a limited potential for cell division (54). This observation was made at about the same time as the formulation of the oxygen or 'radical theory of ageing', which was mentioned in the beginning of this article (5). Hayflick observed that primary human fibroblast cultures could be continuously cultured for about 40–60 generations (cell doublings) but then reached a state called senescence. The senescent cells are very large, morphologically abnormal and still metabolically active (protein synthesis is much slower than in young cells); no apoptosis or necrosis was observed. It may now be added that other cell types, for instance human umbilical vein endothelial cells (HUVEC) show the same phenomenon which is now called the Hayflick limit but do undergo apoptosis when they become senescent (55). During the years following the initial observation, Hayflick and others found that the Hayflick limit (counted in population doublings) continuously decreased when the primary cells were taken from individuals of increasing age. The remaining lifespan of the cultured cells was 'remembered' by the cells even if they were frozen in liquid nitrogen for years, as if an internal clock was set and this setting was relatively stable. This, of course contributed to the thinking of a 'genetic programme of ageing' that was discussed in the beginning of this article. The Hayflick limit of cells from non-human species appeared to be roughly correlated with the total *in vivo* lifespan of the animals. Nuclear transplantation experiments showed that the remaining lifespan of the cybrid was determined by the nucleus of the cells, not by the cytoplasm (56).

The shortening of telomeres of chromosomes in cultured somatic cells was first postulated as a possible cause for the Hayflick limit by Olovnikov (57). This postulate was strengthened when it was shown that telomerase activity was low in cultured cells (also in somatic cells *ex vivo*) and the telomeres of replicatively aged cells were indeed short (58). In the meantime, the gene for telomerase was cloned and the protein was studied in detail (59) and further evidence in favour of the telomere hypothesis was obtained by showing that ectopic expression of telomerase could increase the Hayflick limit of cultured fibroblasts (60). Moreover, immortalization of cultured cells escaping from the Hayflick limit crisis occurs occasionally and is accompanied by an increase in telomerase activity. The immortalized cell clones produce cancers when transplanted in mice. Human cancers sometimes (but not in all cases) show increased telomerase activity. Critically short telomeres that can be achieved by introducing specific mutations in telomerase, activate the DNA damage response (both in yeast and in higher cells) and lead to cell cycle arrest (depending on the checkpoint gene, *RAD9*) and to senescence or apoptosis (in yeast). All this being very convincing, the weak part of the telomere hypothesis is that in biopsies (for instance in muscle biopsies) from healthy centenarians, short telomeres were generally not observed, with the notable exception of T-cells (61). It should be mentioned here that telomerase is not the only means by which telomere maintenance can be achieved: recombinational repair, in particular double-strand break repair can also serve this purpose, both in higher cells and in yeast.

Telomerase elongates telomeres *de novo* by adding the short single-stranded repeats (in yeast: $5'TG_{1-3}\,3'$, in humans: 5′TTAGGG3′) to the 3′ end of one of the two strands; ordinary DNA polymerization is then used to create a complementary strand. The *de novo* added single-strand repeats are synthesized by a reverse transcriptase reaction from the essential RNA component of eukaryotic telomerases. This process is active in the early embryo, but not in cultured fibroblasts. Certainly, telomere maintenance is necessary for the survival of the species over many generations and is part of the above-mentioned rejuvenation process that has to be carried out in the germ line.

Telomere synthesis is not semiconservative, and in principle telomere synthesis could be occurring asymmetrically in the daughter cell of a yeast mother/daughter pair. However, this is not the case and telomeres seem to be maintained in young and old yeast cells. No change in the length of telomeres was found in isolated old yeast mother cells as compared to young cells (62). Therefore, telomere shortening certainly does not apply for yeast mother cell-specific ageing. The phenotype of a deletion of the catalytic protein components of yeast telomerase gene *EST2*, was studied and it was found that a haploid strain devoid of Est2p needs many generations (usually over 100) to reveal the defect of this gene deletion, but then all of the progeny of the original cell carrying the deletion die in a clonal fashion (63). Deletions of other components (encoded by *EST1*, *EST3*, *EST4* and *TLC1*, the RNA component) of the yeast telomerase lead to similar defects (64). Similar phenotypes were also seen after deletion of

KEM1, a G4 DNA-dependent nuclease (65). This has been termed 'senescence' by the authors and is similar to the Hayflick phenomenon in human fibroblasts, but has nothing to do with either mother cell-specific ageing or chronological ageing of yeast. On the lawn of dying cells of the *est2* strain, occasional revertant colonies can be seen that can maintain telomeres by either of two distinct recombination mechanisms (66). The genes needed for this alternative mechanism of telomere maintenance have been analysed by classical genetics and among others, *SGS1* was found, a *recQ* family DNA helicase that was already discussed above. Interestingly, Werner's syndrome patients indeed show a defect in maintaining their telomeres (67). Other genes needed for rescue of telomerase deficient yeast are as expected of the rad50 series of recombinational repair (68 and see below). Still another mechanism of telomere maintenance in yeast was studied by the group of David Lydall and termed the PAL mechanism depending on the exonuclease encoded by *EXO1* (69). Deletion of *EXO1* rescues survival of yeast cells devoid of both telomerase and Rad52p. The chromosomes and their telomeres became highly aberrant in those strains, a phenomenon that is not yet fully studied, but seems highly interesting in the light of karyotype abnormalities after immortalization in cancer cells and the use of yeast as a model for cancer cells. These results are another example for a now intensively discussed topic, the flexibility and greater than expected genomic instability of eukaryotic cells (at least under some conditions). However, this genomic instability is not necessarily connected with ageing. Yeast clonal senescence does not occur in nature, but could be a useful model for clonal ageing of fibroblasts in culture.

RECOMBINATIONAL REPAIR, DOUBLE-STRAND BREAK REPAIR AND INSTABILITY OF THE GENOME DUE TO HYPERRECOMBINATION

In this section, the role of DNA repair by recombination, in the process of cell ageing, will be reviewed and discussed. Since a possible outcome of faulty recombinational repair is the generation of chromosomal aberrations with consequent genomic degradation that can include telomeres, its potential involvement in lifespan shortening will be assessed. In reviewing this field, it becomes noticeable that most of the experimental information derives from studies conducted in mammalian cells, while little work has been done so far with yeast.

Some basic observations must be considered to properly frame the subject of interaction between recombinational DNA repair and cellular ageing. First, it must be considered that genetic alterations increase with the age of organisms, although the basis for this increase is still far from clear. Cellular DNA is continuously being exposed to a variety of environmental and endogenous agents that can cause its damage, throughout the lifespan of a cell. These potentially lethal or mutagenic DNA lesions induce various cellular responses in mitosis, including cell cycle arrest, transcription alteration and processing by different DNA repair mechanisms. The choice of the repair mechanism depends on the structural features of the lesion, and the particular phase of the cell cycle at which it is acting. In yeast, some of these repair mechanisms are based on error-prone DNA recombination, the result of which can be the formation of gross chromosome rearrangements, or GCRs (70).

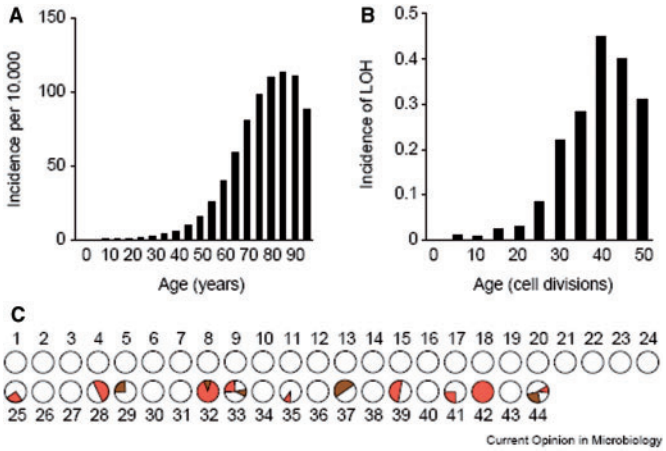

Figure 4. A function of age: human cancer and genetic instability in *S. cerevisiae*. (**A**) The incidence of cancer increases dramatically with age in humans. Shown are probabilities of developing cancer by 5-year interval collected by SEER from 1998 to 2000 for all types of cancer. (**B**) The incidence of LOH in diploid budding yeast cells increases with similar kinetics during replicative ageing. Shown are probabilities of producing a daughter cell that gives rise to a colony containing an LOH event, grouped by 5-division intervals for a cohort of 39 wild-type diploid mother cells (MA McMurray and DE Gottschling, unpublished data). (**C**) Detection of age-induced LOH events by pedigree analysis. Each circle represents the colony produced by successive daughters of a single mother cell; the succession is labelled with numbers. Coloured shapes represent sectors of cells having experienced LOH, where red and brown designate LOH at different loci. *Reprinted from Ref. (124), with permission from Elsevier.*

In a study of *Drosophila* testes, for instance, it was found that the major pathway of DNA repair is altered with age, thus providing a means to dissect the molecular mechanism for age-related genomic changes (71). A most important evidence of a marked increase in genetic defects and genomic instability correlated with the ageing of yeast cells has been given by McMurray and Gottschling (43) using loss of heterozygosity (LOH) as a marker of genetic alteration due primarily to break-induced replication in old cell daughters (Figure 4). Because genomic instability is a major marker of cancer, a valid extrapolation of these findings to a cause–effect relationship between recombinational repair and carcinogenesis in mammalian cells has been suggested. As a matter of fact, the same holds true for another DNA repair pathway active in higher eukaryotes, the process of non-homologous end joining (NHEJ)—exclusively dedicated to the repair of DSBs in which DNA ends are joined with little or no base pairing at the junction—which is prone to make errors, as the end-joining products may be accompanied with insertions or deletions. In one study of the ability of young, presenescent and senescent normal human fibroblasts to repair DNA DSBs, the results indicated that end joining becomes inefficient and more error-prone along with cellular age (72). However, another recent work disagreed

with the generalization of these results by showing that *in vitro* NHEJ activity was significantly lower in adult brain, while young neurons seem to use the system much more frequently (73). Indeed, an age-dependent profile of neuronal utilization of NHEJ indicates that neurons in young brain utilize mostly error-prone NHEJ to repair DNA double-strand breaks accumulated within the genome, and this activity declines gradually with age (73). Therefore, one could conclude that young rat neurons make more genetic errors than old neuron cells, in contrast with the general view that older cells are more error-prone than young ones.

Nucleotide excision repair (NER) is a versatile DNA repair pathway that removes a wide range of DNA lesions, including the main UV-light induced lesions, i.e. cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs), as well as lesions induced by chemicals, like the intra-strand cross-links induced by cisplatin (74). The process of NER is highly conserved in eukaryotes, and in human cells NER reaction requires at least six core protein complexes for damage recognition and dual excision (XPA, XPC-hHR23B, RPA, TFIIH, XPG and XPF-ERCC1) and other factors for DNA repair, synthesis and ligation (PCNA, RFC, DNA polymerase α or δ and DNA ligase) (2,75,76). The nucleotide excision repair pathway seems to play a critical role in the repair and maintenance of telomere integrity and thus in correct cell ageing (77). Moreover, oxidative damage, a major biochemical insult responsible for cell ageing and apoptosis by generalized macromolecular degradation, is mainly processed by the base excision repair (BER), and is also removed in a transcription-coupled NER manner (78,79).

While NER and BER mostly repair lesions that affect only one of the DNA strands, other mechanisms deal with the double-strand breaks (DSBs) which can result as a consequence of ionizing irradiation and exposure to other DNA damaging agents, replication fork collapse, mechanical stress or processing of a single-stranded nicked chromosome. Besides NHEJ, the major mechanism of DNA DSB repair in vegetatively growing yeast cells is homologous recombination (HR), which needs a homologous DNA sequence somewhere else in the cell genome to repair the lesion. HR is a general term that includes multiple mechanisms (80–83) and most of its genes belong to the *RAD52* epistasis group (*RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54, MRE11* and *XRS2*) (80). Molecular and biochemical studies of the Rad52 group proteins have shown that most are required at early steps during recombinational repair (77). Mutations in these genes, among others, affect telomere maintenance leading to their shortening and premature cell ageing. Moreover, the RecQ helicase gene *SGS1* is able to suppress *rad52* deficiency, by promoting telomere recombination and restoration of normal length, with a normalizing effect on the ageing process (68). Branched-DNA molecules have been detected during mitotic S-phase within the tandemly repeated rDNA locus, and formation of these intermediates is dependent on *RAD52*, suggesting that they correspond to recombination intermediates (84). Post-replicative, DNA replication-dependent X-shaped molecules have also been detected between sister chromatids in *Physarum polycephalum* suggesting that resolution of Holliday junctions (HJs) could be essential for chromosome segregation in eukaryotes (85). The presence of these complex DNA molecules could result also from strong oxidation leading to cross-linking, but it is not known if its frequency increases with age. Nevertheless, both *SGS1* and, very recently, *RAD54* seem to be critical for the resolution of these branched DNA molecules (86,87), their impairment indirectly leading to the accumulation of these structures in cells that undergo ageing. This has been demonstrated even more clearly by the recent work of Lee and co-workers (88) in which non-Holliday Junction X-shaped DNA structures have been shown to accumulate at telomeres in a *RAD52* and *RAD53*-dependent fashion in *sgs1*, *tlc1* mutants of ageing *Saccharomyces cerevisiae* cells.

Besides NHEJ, homologous recombination can also result in DNA sequences that differ from the original ones, due to the particular repair pathway that has been activated. Therefore, the accumulation of genetic mutations with age, as a result of faulty recombinational repair, can be due to several combinations of DNA damage and repair interplay: (i) a near-linear increase in genetic errors with time, in the presence of a background level of DSBs and of a steady-state repair mechanism; (ii) an increase in the frequency of DNA DSBs with cell ageing in the presence of a background level of DNA repair; (iii) an increase in the choice of error-prone pathways for DNA repair of steady-level DSBs and, finally, (iv) the interaction of both, an increase in the number of DSBs and in the use and efficiency of error-prone recombinational repair processes during cell ageing. In all cases, it appears evident that the time factor represents the only certain parameter with which the overall likelihood of genetic defect accumulation correlates, rendering nuclear genomes inherently unstable with age (71). Among the most important of these defects, shortening of telomeres is a critical factor responsible for the onset of genome instability (89).

An experimental system using yeast to artificially interfere with genomic homeostasis, and then assess any age-dependent cellular response, has been introduced by the chromosome knockout technology (90). It was demonstrated that inducing a centromere knockout for chromosome V in a diploid strain, leads to chromosome loss followed immediately by its endoreduplication during the successive rounds of cell division, whereas, deletion of chromosome VIII does not show duplication until 21 generations, after which the chromosome is endoreduplicated (91). The influence of these genetic manipulations on the process of ageing have not yet been studied.

Finally, new and exciting developments concerning the role of recombinational repair in cell ageing caused by telomerase deficiency are expected from further studies of secondary DNA structure such as DNA palindromes in yeast models. In fact, these DNA structures, which seem accumulating during postsenescence growth, have proven essential to bypass progressive telomere degradation in recombination-deficient or telomerase knockout yeast

strains, thus allowing their immortalization in the absence of a functional EXO1 gene (69).

AGEING AND THE MITOCHONDRIAL GENOME

The mitochondrial genomes of eukaryotic cells are relatively small circular DNA molecules. In mammals, mitochondrial DNA comprises 16.5 kb and encodes 13 proteins of the complexes I, III and IV of the respiratory chain and complex V (ATP synthetase). In addition, the mitochondrial genome encodes all of the ribosomal RNAs and the necessary 22 tRNAs, considering the hypothesis of 'extended wobble'. The genetic code differs from the universal code in the rule for the start and stop of protein synthesis, and the codons for arginine and tryptophan. The yeast mitochondrial genome is larger in size (85.7 kb), but not in coding capacity, containing introns, some of which are self-splicing, individual promoters for some of the genes, and more than one putative origin of replication. The proteins encoded are only eight in numbers, including subunits I, II and III of complex IV (cytochrome c oxidase), three subunits of the ATPase (encoded by *ATP6*, *ATP8* and *ATP9*), apocytochrome b and the ribosomal protein, Var1p. In addition, several hypothetical proteins are encoded, some of them in introns or as read-through proteins consisting of intron and exon sequences. The two ribosomal RNAs of the mitochondrial ribosome, 24 tRNAs and the 9S RNA which is part of RNAseP, are encoded in the mitochondrial genome (92). The genetic code used is different from the universal code but also slightly different from the code used in mammalian mitochondria. The proteins encoded are not exactly the same as those encoded in mammalian mitochondria, for instance, the subunits of complex I are missing as the *S. cerevisiae* mitochondria do not contain complex I but have instead 'invented' an alternative way, consisting of Ndi1p, of feeding reducing equivalents of NADH into the respiratory chain. No RNAs are imported into mitochondria or exported from mitochondria, but most of the proteins of which mitochondria consist, are encoded in the nucleus, made on cytoplasmic ribosomes and imported into mitochondria postsynthetically. The N-terminal signals in the primary translation products, which lead to import into the three subcompartments of mitochondria, are not uniform and different uptake mechanisms have been found (93).

A special role of mitochondrial physiology in the ageing process of eukaryotic cells is assumed by nearly every researcher in the field. Oxidative damage to lipids, proteins and DNA in ageing cells is a fact that can hardly be overlooked (17,94). The most important source of oxygen radicals, which produce the oxidative damage are believed to be mitochondria. The most important sites of radical production are complex I and complex III. In a side reaction of the respiratory chain, superoxide radical anion is produced as a primary reactive oxygen species (ROS). It is further thought that this process leads to a vicious cycle because the ROS primarily target mitochondrial DNA and produce mutations which further increase radical production, among them point mutations in subunits of complexes I and III (94). However, as has been said repeatedly in this article, such a theory can only be accepted, if experimental results can be presented which are precisely and with no exception showing that the predictions that the theory can make are correct.

Oxidative damage to mitochondrial DNA results in 8-oxo-deoxy guanine (8-oxo-G) and other minor oxidation products. 8-Oxo-G is mutagenic because it changes the base-pairing properties of the G base. It is mostly repaired by the base excision repair (BER) enzyme OGG1 (8-oxo guanine glycosylase). Isoforms of this repair enzyme are produced by alternative splicing leading to the mitochondrial and nuclear isoform, respectively (95,96). Mitochondrial BER is efficient but not perfect leading to mutations, and nucleotide excision repair (NER) is absent in mitochondria (97, and further publications cited therein). The increase of 8-oxo-G in mitochondrial DNA has been shown (97) and the increase in point mutations with age has been shown in several human organs, for instance in brain (98). The question has not been investigated in detail in ageing yeast mother cells. At least some of the mitochondrial point mutations would produce respiratory deficiency.

Mitochondrial genome deletions do accumulate in ageing mammalian cells *in vitro* and *in vivo* and have been studied extensively (99,100), for instance in human heart (101). The deletions, most prominently in the form of the so-called common deletion of 4977 bp, are due to flanking short inverse repeats in the DNA and the presence of an efficient homologous recombination repair system in mitochondria (102). These authors have constructed a reporter system that enables exact measurements of the frequency with which deletions are produced in the mitochondrial genome of yeast based on the loss (through recombination) of $ARG8^m$, a gene for arginine biosynthesis that is engineered to be functionally expressed in mitochondria, with concomitant restoration of *COX2*, making the cell respiratory competent. Measurements show that a high frequency of recombination and loop-out occurs (in the order of $1{:}10^4$), which depends on the length of the inverted repeats. This is not surprising as it has been known since 1949 (103) that yeast has a high (but strain dependent) natural frequency of production of petite mutants which is mostly due to rho^- mutations, which are deletions in the mitochondrial genome (104) followed by formation of tandem repeats from the remaining mitochondrial DNA (105). All rho^- mutations result in respiratory deficiency. The reporter system just mentioned has not yet been applied to ageing yeast mother cells and there is no published investigation of mitochondrial deletions in very old yeast mother cells.

In some strain backgrounds, petite yeast cells show an increase in mother cell-specific lifespan depending on the activity of the retrograde response (106–108). In those strains, non-respiring petite strains are enriched, when very old mother cells are analysed in lifespan determinations (108).

If an increase in mitochondrial point mutations or deletions does indeed occur in ageing yeast mother cells, the mutations are almost certainly heteroplasmic (i.e. a mixture of wild type and mutant mitochondrial genomes

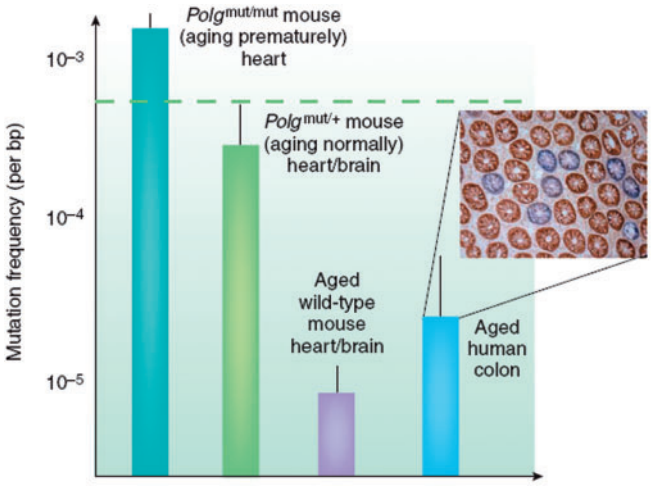

Figure 5. Mitochondrial DNA mutations and ageing. Logic would dictate that mtDNA mutations, when present at levels lower than in phenotypically normal $Polg^{mut/+}$ mice, who heterozygously carry defects in the proofreading exonuclease activity of mitochondrial DNA polymerase (dashed green line), are irrelevant for ageing. However, in aged human colon, the typical histological pattern of mitochondrial defects (blue crypts in the inset) associated with increased mtDNA mutant fractions in individual crypts suggests otherwise. Fractions in colon include clonally expanded mutants only. Error bars represent estimated variation of the data. *Reprinted from Ref. (1), with permission from Elsevier.*

in one cell). The yeast cell contains a few hundred copies of the mitochondrial genome whilst the larger human cells contain a few thousand (109). Cellular respiration is gradually weakened when, through mitotic segregation, the ratio of wild-type to mutant genomes is shifted towards the mutant. Organs such as the heart muscle eventually become a mosaic of respiration-competent and incompetent cells (see also Figure 5). This eventually results in a threshold phenomenon and when cellular respiration is lower than a certain threshold, still unknown signals lead to apoptosis or in the yeast cell, to the petite phenotype. In human somatic cells or in the mouse model system, this mutational process leads to the elimination of damaged cells by apoptosis. It is unknown if, or by which mechanism, the germ line cells are protected from mitochondrial mutagenesis. In yeast cells, the question arises as to how those mitochondrial genomes which are damaged (for instance by deletion) can be discriminated from the wild-type mitochondrial genomes and how they are selectively retained in the mother. It is thought that the daughter cell, which resets the clock to zero, inherits only or mostly wild-type copies of the mitochondrial genome. Again, we have returned to the question of rejuvenation that applies equally to (mitochondrial) DNA and to non-DNA material that is damaged in the old mother cell (17). The segregation problem is even more complicated because in growing cells the mitochondria form a continuous network within the cell that contains a large number of mitochondrial genomes within one organelle. Mitochondrial fission would seem to be necessary for asymmteric segregation to occur. Although mitochondrial fission and fusion has been found to influence the lifespan in fungi (110), the mechanism leading to the observed elongation of lifespan is presently unknown. The ideas described in this paragraph are speculative, because there is no definite proof that mitochondrial mutations cause ageing in yeast.

We will now describe recently published experimental results in the mouse, which cast a heavy doubt on the concept of mitochondrial mutations as a cause of ageing in higher animals (1,2). Trifunovic (111) and Kujoth (112) have presented results that at first sight seemed to confirm a causal role of mitochondrial point mutations in ageing. They introduced a homozygous mutation into the proofreading domain of the only mitochondrial DNA polymerase (DNA polymerase γ), which is encoded in the nucleus, and observed in this 'mitochondrial mutator mouse' both a large increase in mitochondrial mutations (point mutations as well as deletions), reduced lifespan and symptoms of premature ageing including typical age-related pathologies of the heart and nervous system (111). This finding was greeted with much enthusiasm and a second paper (112) confirmed and extended the first one, showing in addition that in the organs inspected (thymus, small intestine, testis) apoptotic markers could be seen much more frequently than in wild type. In the more recent papers mentioned above, a new method for determination of mitochondrial mutations was employed ['random mutation capture'; (113)] which is more accurate because it is not prone to sequence artefacts of the PCR method. It turned out that the spontaneous mutation rate in mitochondria is at least an order of magnitude lower than previously thought. Moreover, the increase of mitochondrial mutation load in cells of the proofreading mutant of DNA polymerase γ is much larger than previously measured (in the order of at least a hundred-fold). The decisive experiment was described by Vermulst (2): the heterozygous mouse showed no pathology or premature ageing, however the mitochondrial mutation burden observed in the heart and brain of the heterozygous animals was very high (Figure 5), at least 20-fold higher than in aged heart of wild-type animals. The mutation burden was also much higher than in aged human colon, where many of the cells are already critically respiration-deficient. As a mutation density, which is much higher than in senescent wild-type animals, does not cause premature ageing in the animals heterozygous for the mitochondrial proofreading defect, the conclusion seems to be obvious that the random mitochondrial mutations that are observed are not a direct cause of ageing. The authors admit that the case is not completely closed for the following reasons: (i) It cannot be excluded that mitochondrial mutations at a specific time in the life history and in specific organs and cells are causing ageing. In the heterozygous mutant, many mutations appear early in life, while in the wild type they increase exponentially during the lifetime and most of these mutants are created only late in life. (ii) It is not clear at the moment if the mouse data can be safely extrapolated to humans (1).

CHRONOLOGICAL AGEING

The chronological lifespan of yeast simply is the lifespan of stationary cells tested either in spent medium or in water by

plating out aliquots of the non-growing cells over the course of days and weeks (114–117). As we have shown by analysing published results of whole genome deletion screening data, there is very little similarity in the two gene sets which on deletion have any influence on chronological versus mother cell-specific ageing (118). Stationary cells in order to survive maintain a minimal metabolic activity (119), they are certainly prone to environmental insult or stress including mutagenic insult and mutations in several DNA repair genes seem to shorten the chronological lifespan of yeast cells (120). This would imply that DNA repair synthesis is probably going on in non-dividing stationary phase wild-type yeast cells. However, this presumed repair synthesis has not been measured and the mutational load of stationary phase yeast cells depending on time in stationary phase has not been studied, or at least it has not been published. Fabrizio *et al.* (121) showed that age-dependent increases in mutations occurred during chronological ageing and are suppressed by deletion of *SCH9*.

CONCLUDING REMARKS

Many reviews on the genetics and physiology of ageing have shown that the process of ageing is far from being understood in molecular detail. Some of the prevailing theories of ageing are not mutually exclusive and actually work together to produce a picture, which is now a little bit more coherent than 10 years ago. For instance, the generally accepted fact of an increasing oxidative stress in aged cells and organisms could easily explain the increase in damaged cellular material and in mutation load (mainly in the mitochondrial genome) with age. It becomes clear more and more that caloric restriction can reduce oxidative stress by an unknown mechanism and can thereby reduce mutation load. However, it is also true that mutations that increase the lifespan in a genetic model system of ageing are not in all cases mimicking caloric restriction and are not in all cases slowing down the metabolism of the cell or the organism. The experimental results discussed here once again show that the causes of ageing are many and one or the other becomes limiting for the lifespan, depending on the genetic background and on environmental conditions. They also do not support the notion of a genetic programme of ageing that was supposedly positively selected during evolution to increase survival of the species. Rather, what appears as a genetic programme of ageing is likely to be a genetic programme or programmes of stress response.

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