

Mitochondrial point mutations do not limit the natural lifespan of mice

Marc Vermulst¹, Jason H Bielas¹, Gregory C Kujoth², Warren C Ladiges³, Peter S Rabinovitch¹, Tomas A Prolla² & Lawrence A Loeb¹

Whether mitochondrial mutations cause mammalian aging, or are merely correlated with it, is an area of intense debate¹. Here, we use a new, highly sensitive assay² to redefine the relationship between mitochondrial mutations and age. We measured the *in vivo* rate of change of the mitochondrial genome at a single-base pair level in mice, and we demonstrate that the mutation frequency in mouse mitochondria is more than ten times lower than previously reported. Although we observed an 11-fold increase in mitochondrial point mutations with age, we report that a mitochondrial mutator mouse³ was able to sustain a 500-fold higher mutation burden than normal mice, without any obvious features of rapidly accelerated aging. Thus, our results strongly indicate that mitochondrial mutations do not limit the lifespan of wild-type mice.

Mitochondria are functionally diverse organelles with a central role in both oxidative phosphorylation⁴ and apoptosis⁵. Accordingly, post-mitotic tissues with high rates of oxygen consumption, such as brain and heart, are exceptionally sensitive to mitochondrial dysfunction⁶. The mitochondrial theory of aging postulates that a lifelong accumulation of mitochondrial DNA (mtDNA) mutations in multiple tissues eventually results in mitochondrial failure, which, in synergy with downstream processes such as apoptosis, results in loss of cellularity and the progressive decline of tissue functioning known as aging^{3,7}. These mutations may compromise the integrity of the electron transport chain and increase the formation of reactive oxygen species (ROS), creating a vicious cycle of mutagenesis that continuously amplifies the production of cytotoxic oxygen radicals^{8,9}.

Although several studies have quantified either tissue-specific mutation frequencies¹⁰ or mutation accumulation as a function of age¹¹, most methods rely heavily on PCR and cloning-based strategies^{12,13}, techniques that are limited in throughput and that may be confounded by polymerase infidelity on damaged templates and by cloning artifacts. Therefore, we explored the relationship between age and mutation accumulation in mtDNA with an adaptation of the random mutation capture (RMC) assay^{2,14}, a quantitative

PCR-based approach that relies on PCR amplification of single molecules for mutation detection (**Supplementary Fig. 1** online) but is not limited by polymerase fidelity^{2,14}. This methodology allows for exact determination of mutation frequencies in high-throughput screens that interrogate millions of base pairs simultaneously^{2,14}. Approximately 150 million bp were screened for mutation detection in this study.

A *TaqI* restriction site (TCGA) located in the gene encoding the 12S rRNA subunit (bp 634–637) was selected for mutation frequency determination. Examination of mtDNA derived from brain tissue of four young mice (1–3 months) yielded an average mutation frequency of $6.0 \times 10^{-7} \pm 0.9 \times 10^{-7}$ per bp (**Fig. 1a**). A gradual increase in mutation burden was observed with advancing age, with mutations accumulating to a mean frequency of $1.1 \times 10^{-5} \pm 0.3 \times 10^{-5}$ per bp in old animals (24–33 months). Mutations started accumulating rapidly after 16 months of age, resulting in a mutation frequency that was ten times higher in old animals (24–33 months) compared with young animals (1–10 months) ($P = 0.0026$, two-tailed *t*-test, **Fig. 1b**). This accumulation was fitted best along an exponential line. We obtained similar results in heart tissue from the same group of animals, with frequencies ranging from 8.5×10^{-7} per bp in a 2.5-month-old animal to 1.3×10^{-5} per bp in a 33-month-old animal (**Fig. 1a**). However, subtle fluctuations from these averages may occur between cell types within tissues. Mutation frequencies determined via the RMC assay in young mice are approximately one to two orders of magnitude lower than previously documented by conventional methods^{7,13}. Although the RMC assay has an increased level of sensitivity over these methods, it registers mutational events at the interrogated restriction site only; thus, it is possible that local sequence context or negative selection at this locus accounts for this discrepancy. However, interrogation of a second *TaqI* restriction site, located at bp 15253–15256 in the mitochondrial cytochrome b gene, yielded similar results: the mutation frequency was 1.2×10^{-6} in 2.5-month-old animals but rose to 7.9×10^{-6} in a 33-month-old animal, suggesting that our results can be extended beyond the local genetic environment of any one site (data not shown). Furthermore, in parallel experiments, we observed mutation frequencies of up to

¹Department of Pathology, University of Washington, Seattle, Washington 98195, USA. ²Departments of Genetics and Medical Genetics, University of Wisconsin, Madison, Wisconsin 53706, USA. ³Department of Comparative Medicine, University of Washington, Seattle, Washington 98195, USA. Correspondence should be addressed to L.A.L. (laloeb@u.washington.edu).

Received 19 October 2006; accepted 25 January 2007; published online 4 March 2007; doi:10.1038/ng1988

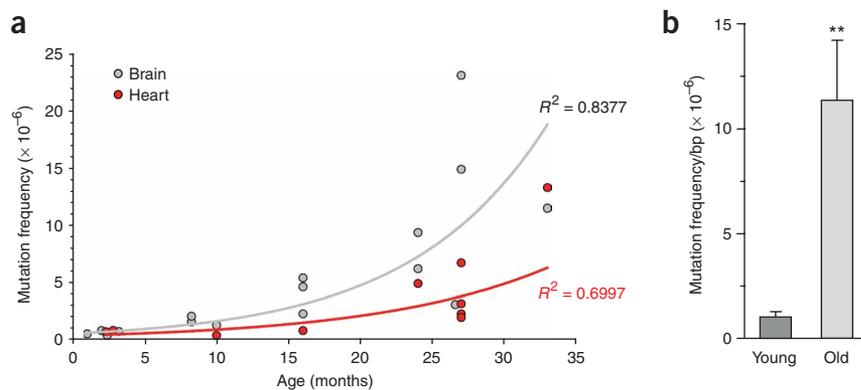


Figure 1 Frequency of mitochondrial mutations as a function of age. **(a)** Mutation frequency was determined at *TaqI* restriction site 634–637 in brain and heart. Each data point represents one animal. On average, 3×10^6 bp were screened per animal. **(b)** Mutation burden (mean \pm s.e.m.) of young animals (1–10 months, $n = 7$) and old animals (24–33 months, $n = 6$). ** $P < 0.01$, two-tailed t -test.

1.5×10^{-3} at both restriction sites in brain tissue and mouse embryonic fibroblasts (MEFs) derived from 2.5-month-old animals completely deficient in the proofreading activity of DNA polymerase γ (*Polg*), the mitochondrial replicative enzyme. Because these animals are healthy at this age and cell lines are viable despite a $\sim 2,500$ -fold increase in mutation burden, these data strongly suggest that negative selection is not an influential factor in determining mutation load at either site. Nevertheless, we cannot exclude the possibility that mutational hotspots that lie outside of our mutational target could raise the overall mutation frequency per bp reported here. However, the contribution of hotspots would be mitigated by their rareness and diluted by the presence of large regions of uniform mtDNA. Thus, we expect their influence to the mutation frequency per bp to be modest, and we conclude that our results indicate that the mutation burden in mitochondria of wild-type mice is more than ten times lower than previously reported. This discrepancy is most likely to be the result of mutations introduced *ex vivo* on (damaged) DNA templates during PCR before cloning steps in conventional assays, as PCR amplification, prior to application of the RMC assay, increased the mutation frequency at least 32-fold (Supplementary Fig. 2 online). In contrast, the RMC assay does not seem to be substantially influenced by DNA damage (Supplementary Fig. 3 online).

mtDNA is located in the vicinity of the electron transport chain, the primary site of ROS production. To investigate whether oxidative damage is responsible for mutation acquisition, we measured the mutation load in MEFs and heart tissue of transgenic animals carrying an extra gene that targets human catalase (mCAT), an ROS scavenger, to the mitochondrion¹⁵. In the hearts of three 26- to 28-month old mice that showed stable expression of the mCAT gene, we measured an average mutation frequency of $1.4 \times 10^{-6} \pm 0.1 \times 10^{-6}$, whereas five age-matched wild-type animals showed an average mutation frequency of $4.0 \times 10^{-6} \pm 1.0 \times 10^{-6}$ ($P = 0.036$, Mann-Whitney test, Supplementary Fig. 4 online). In addition, we measured a mutation frequency of 1.3×10^{-6} in primary MEFs of a wild-type animal but did not find any mutations in a screen of 6.3 million bp from a similar primary cell line derived from an mCAT littermate. We observed a marked difference in the mutation spectrum of wild-type and mCAT-expressing animals (Supplementary Fig. 5 online). Collectively, these findings are in agreement with extensive literature that

proposes a substantial role for oxidative damage in mitochondrial mutagenesis¹⁶.

A compilation of sequencing data collected from wild-type animals identified 81% of all mutation events as GC to AT transitions (Fig. 2), suggesting that a limited number of sources is responsible for mutation acquisition. The detection of five deletions, ranging from 1 to 80 bp, and one insertion (Fig. 2 and Supplementary Fig. 5), attests to the versatility of the RMC assay. GC to AT transitions are the most commonly observed mutations after oxidative stress¹⁷ and are thought to be produced predominantly through cytosine oxidation followed by rapid decomposition of the destabilized base into uracil glycol and 5-hydroxyuracil, both of which mispair with adenine¹⁸. Repair of these lesions creates abasic sites, repair intermediates that are preferentially paired with adenine during Polg-mediated synthesis¹⁹.

However, an altered pattern of mutagenesis in proofreading-deficient Polg mice argues against a substantial role for Polg misinsertions in the absence of DNA damage (Supplementary Fig. 5). The mutation spectrum remained constant between three restriction sites (bp 634–637, 7667–7680 and 15253–15256) and was independent of age or tissue type (brain or heart). However, even though the *TaqI* site is a palindrome, the mutations were not symmetrically divided over all 4 bp (Supplementary Fig. 5); this asymmetry may occur if the mutation rate of the two DNA strands is not equal²⁰. Because DNA oxidation and deamination occur faster on single-stranded DNA than on double-stranded DNA²¹, it is possible that temporary single-strandedness of mtDNA during replication is a driving force in mitochondrial mutagenesis²⁰. Finally, we did not find any evidence for clonal expansions during spectrum analysis, consistent with the post-mitotic nature of the tissues surveyed.

To directly evaluate the hypothesis that the accumulation of mutations in mtDNA is a causal factor in the aging process, we compared the mutation burden of wild-type mice with that of mice that contain

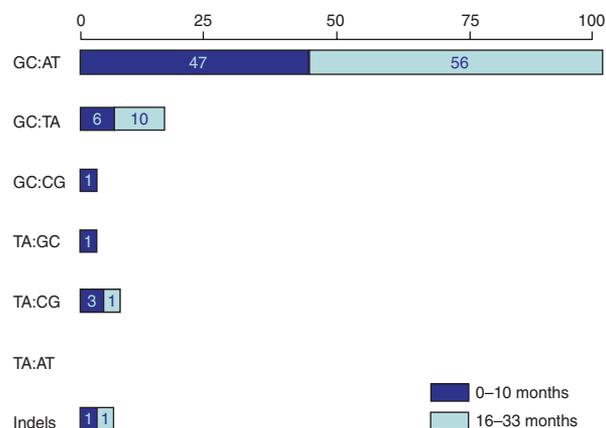


Figure 2 Mitochondrial mutation spectrum in wild-type animals. Mutation spectrum was composed of mutations captured at three restriction sites (634–637, 7667–7670 and 15253–15256) in both brain and heart. Absolute numbers of mutations captured are listed inside the bars. Indels: inversions and deletions.

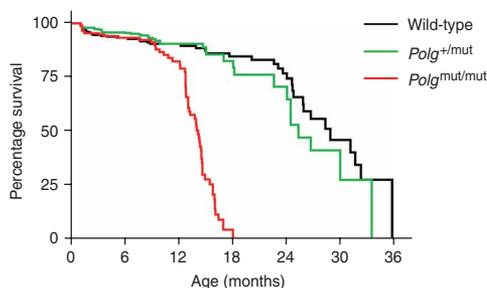


Figure 3 Kaplan-Meier survival curves. Median survival is 423 d for *Polg^{mut/mut}* mice, 758 d for *Polg^{+/mut}* mice and 864 d for wild-type mice. The curves for wild-type and *Polg^{+/mut}* mice do not differ statistically significantly (log rank test, $P = 0.875$).

an allelic substitution in the exonuclease domain of Polg. This substitution abolishes the proofreading activity of Polg, causing it to be error prone. We³ and others²² have recently demonstrated that mice homozygous for this substitution (*Polg^{mut/mut}*) show a marked reduction in lifespan and several features of premature aging. This phenotype could be attributed to an increased rate of mtDNA mutation, which we initially reported to be three- to eightfold higher in homozygous mutant mice compared with wild-type mice, as measured by a standard DNA sequencing approach. Here, we correct this to be $\sim 2,500$ -fold. Because our measurements in young *Polg^{mut/mut}* mice ($1.5 \times 10^{-3} \pm 0.2 \times 10^{-3}$) are equivalent to what was previously reported^{3,22} with conventional assays, this difference can be attributed solely to the increased sensitivity of the RMC assay, which allowed us to correctly determine the very low mutation frequency in wild-type mice. Thus, a key finding of this study is that the exonuclease domain of Polg is a principal caretaker of mtDNA integrity.

Heterozygous animals (*Polg^{+/mut}*) did not show a statistically significant reduction in mean lifespan ($P = 0.875$, Fig. 3), consistent with previous reports^{3,22}. Additionally, no significant increase in age-related pathology has thus far been detected in these animals²² (T.A.P., unpublished data). We re-examined the mutation load of the *Polg^{+/mut}* mice with the RMC assay. Notably, we recorded an average mutation frequency of $3.3 \times 10^{-4} \pm 0.9 \times 10^{-4}$ per bp (Fig. 4a) in brain tissue of young (2- to 3-month-old) *Polg^{+/mut}* animals at the 12S rRNA locus—approximately 500 times higher than in age-matched wild-type animals ($P = 0.008$). Notably, this mutation burden was also 29 times higher than the burden in old wild-type animals (24–33 months, Fig. 4a, $P = 0.001$). The lack of a classic mismatch repair mechanism in mouse mitochondria²³ probably allows for this vast increase in mitochondrial mutagenesis in an exonuclease-deficient background. Because the frequency of intracellular expansions of mtDNA mutations depends directly on the mutation rate²⁴, these data suggest that both heteroplasmic and homoplasmic mutations are markedly higher in *Polg^{+/mut}* cells than in wild-type cells. We confirmed these results in heart tissue of three young *Polg^{+/mut}* mice, which showed an average mutation frequency of $1.6 \times 10^{-4} \pm 0.5 \times 10^{-4}$, approximately 220 times higher than in four young wild-type animals, which carried a mutation burden of $7.1 \times 10^{-7} \pm 0.1 \times 10^{-7}$ ($P = 0.0175$) and 30 times higher than in six old wild-type animals with a frequency of $5.4 \times 10^{-6} \pm 1.7 \times 10^{-6}$ (Fig. 4b, $P = 0.004$). This observation was strengthened by similar results from a probe of three additional *TaqI* restriction sites, randomly dispersed throughout the mitochondrial genome in both brain (Supplementary Fig. 6 online) and heart (data not shown).

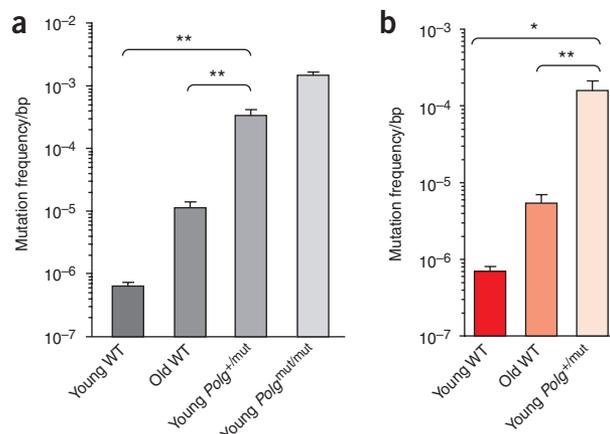


Figure 4 Mutation burden in wild-type and Polg exonuclease-deficient mice. (a,b) Mutation frequency (mean \pm s.e.m.) was determined at *TaqI* restriction site 634–637 in brain (gray) and heart (red) and is plotted on a logarithmic scale. For brain tissue, young wild-type (WT) animals are 1–3 months of age ($n = 4$); old wild-type animals, 24–33 months of age ($n = 6$); young *Polg^{+/mut}* animals, 2.5 months of age ($n = 3$) and young *Polg^{mut/mut}* animals, 2.5 months of age ($n = 2$). * $P < 0.05$. ** $P < 0.01$. For heart tissue, young wild-type animals are 1–16 months of age ($n = 4$), old wild-type animals are 24–33 months of age ($n = 6$) and young *Polg^{+/mut}* animals are 2.5 months of age ($n = 3$).

As the mutation frequency is determined at the same loci in all genotypes, these measurements unambiguously describe the relative differences in mutation rate between them. Because heterozygous mice are born with a 30-fold higher mutation burden than the oldest wild-type animals without suffering a phenotype that resembles premature aging, we conclude that the threshold at which mitochondrial mutations become limiting for lifespan is unlikely to be reached in wild-type mice. Notably, heterozygous carriers of human variants of polymerase γ (POLG) are asymptomatic as well, suggesting that there may be many healthy human carriers of POLG alleles that harbor large numbers of undetected mtDNA mutations. Although our data strongly argue against a causal role for mitochondrial mutations in natural aging, it should be noted that despite our ability to detect small deletions, large mtDNA deletions are not detected by our methods. Large mtDNA deletions have been correlated with the demise of certain specialized tissues such as the substantia nigra^{25,26}. However, *twinkle* transgenic mice, which have an organism-wide increase in the accumulation of large deletions, do not show a decrease in lifespan or a premature aging phenotype²⁷.

The mutation burden rapidly grew after 16 months of age, around the time when reproduction has ceased and the average life expectancy of these animals in the wild has expired. As ROS seem to be the primary source of mutagenesis in mitochondria, this study is consistent with the hypothesis that the production of ROS increases with age²⁸ and that mtDNA is a primary target of ROS, but that the most prevalent result of oxidative lesions, point mutations, are extremely rare and do not determine the rate of aging of wild-type mice.

METHODS

Tissue homogenization and organelle separation. All tissues were harvested within 5 min of death. All animals were cared for according to approved guidelines at the University of Washington. Tissues were sliced in pieces with a scalpel and rinsed in $1 \times$ PBS before homogenization in a Dounce-type glass

homogenizer with 25 firm strokes of a hand-driven glass pestle. Homogenization buffer contained 0.075 M sucrose, 0.225 M sorbitol, 1 mM EGTA, 0.1% fatty acid-free BSA and 10 mM Tris HCl (pH 7.8). Differential centrifugation was performed as described previously¹² to obtain a crudely purified mitochondrial pellet.

DNA extraction and restriction digest. Mitochondrial pellets were digested for 1 h in a buffer containing 0.2 mg/ml proteinase K (Sigma), 0.75% SDS, 0.01 M Tris HCl, 0.15 M NaCl and 0.005 M EDTA at pH 7.8. mtDNA was subsequently isolated using phenol-chloroform extraction (1:1, vol/vol) followed by ethanol precipitation. mtDNA was then diluted and digested in a specialized *TaqI* buffer (New England Biolabs) in the presence of 100 units *TaqI* (New England Biolabs) and 1× BSA (New England Biolabs) for 10 h, with the addition of 100 units of *TaqI* per h.

Mutation detection. PCR was performed using a DNA Engine Opticon Monitor 2, a continuous fluorescence detection system (BioRad), and amplicons were visualized with Stratagene's Brilliant SYBR Green qPCR Master Mix. Primers used for amplification are listed in **Supplementary Table 1** online.

All real-time qPCR reactions were performed in 25- μ l reactions containing 1× Brilliant SYBR Green qPCR Master Mix from Stratagene, 20 pmol forward and reverse primers and 2 units uracil DNA glycosylase (UDG). The samples were amplified as follows: UDG incubation at 37 °C for 10 min and 95 °C for 10 min followed by 45 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1.5 min. Samples were held at 72 °C for 5 min and then immediately stored at -20 °C. All PCR products were either (i) incubated with *TaqI* and then verified by agarose gel electrophoresis to be insensitive to digestion or (ii) isolated with the QiaQuick PCR Purification Kit (Qiagen) and sequenced to identify the mutation at the *TaqI* recognition site.

Cell culture. Mouse embryonic fibroblasts were cultured in DMEM (Gibco-BRL) containing 10% (vol/vol) FBS (HyClone), 1% L-glutamine and 1% penicillin-streptomycin (Gibco). An atmosphere of 5% CO₂ was maintained in a humidified incubator at 37 °C. mCAT and corresponding wild-type cell lines were grown with 2% oxygen.

Note: Supplementary information is available on the Nature Genetics website.

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health grants AG001751 (L.A.L., P.S.R.), CA102029 (L.A.L.), ES11045 (L.A.L., W.C.L.) and AG021905 (T.A.P., G.C.K.). J.H.B. was supported by a research fellowship from the Canadian Institutes of Health. The authors thank G.M. Martin, R.S. Mangalindan, R.N. Venkatesan and C.-Y. Chen for editing this manuscript, technical assistance and discussions.

AUTHOR CONTRIBUTIONS

M.V. carried out all the experiments described and wrote the paper. M.V., J.H.B. and L.A.L. conceived the project. G.C.K. and T.A.P. provided Kaplan-Meier curves and statistical analysis of mouse cohorts. J.H.B., W.C.L., G.C.K., T.A.P. and P.S.R. provided technical assistance, animal care and tissues. L.A.L. supervised the experimental work and interpretation of data. All authors commented on and discussed the paper.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

Published online at <http://www.nature.com/naturegenetics>

Reprints and permissions information is available online at <http://npg.nature.com/reprintsandpermissions>

1. Khrapko, K., Kraysberg, Y., de Grey, A.D., Vijg, J. & Schon, E.A. Does premature aging of the mtDNA mutator mouse prove that mtDNA mutations are involved in natural aging? *Aging Cell* **5**, 279–282 (2006).
2. Bielas, J.H. & Loeb, L.A. Quantification of random genomic mutations. *Nat. Methods* **2**, 285–290 (2005).
3. Kujoth, G.C. *et al.* Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* **309**, 481–484 (2005).
4. Saraste, M. Oxidative phosphorylation at the fin de siecle. *Science* **283**, 1488–1493 (1999).
5. Newmeyer, D.D. & Ferguson-Miller, S. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell* **112**, 481–490 (2003).
6. Wallace, D.C. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* **39**, 359–407 (2005).
7. Trifunovic, A. Mitochondrial DNA and ageing. *Biochim. Biophys. Acta* **1757**, 611–617 (2006).
8. Balaban, R.S., Nemoto, S. & Finkel, T. Mitochondria, oxidants, and aging. *Cell* **120**, 483–495 (2005).
9. Miquel, J., Economos, A.C., Fleming, J. & Johnson, J.E., Jr. Mitochondrial role in cell aging. *Exp. Gerontol.* **15**, 575–591 (1980).
10. Khrapko, K. *et al.* Mitochondrial mutational spectra in human cells and tissues. *Proc. Natl. Acad. Sci. USA* **94**, 13798–13803 (1997).
11. Michikawa, Y., Mazzucchelli, F., Bresolin, N., Scarlato, G. & Attardi, G. Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. *Science* **286**, 774–779 (1999).
12. Copeland, W.C. Mitochondrial DNA: methods and protocols. *Methods Mol. Biol.* **197**, v–vi (2002).
13. Zhang, D. *et al.* Construction of transgenic mice with tissue-specific acceleration of mitochondrial DNA mutagenesis. *Genomics* **69**, 151–161 (2000).
14. Bielas, J.H., Loeb, K.R., Rubin, B.P., True, L.D. & Loeb, L.A. Human cancers express a mutator phenotype. *Proc. Natl. Acad. Sci. USA* **103**, 18238–18242 (2006).
15. Schriener, S.E. *et al.* Extension of murine life span by overexpression of catalase targeted to mitochondria. *Science* **308**, 1909–1911 (2005).
16. Mandavilli, B.S., Santos, J.H. & Van Houten, B. Mitochondrial DNA repair and aging. *Mutat. Res.* **509**, 127–151 (2002).
17. Wang, D., Kreutzer, D.A. & Essigmann, J.M. Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. *Mutat. Res.* **400**, 99–115 (1998).
18. Kreutzer, D.A. & Essigmann, J.M. Oxidized, deaminated cytosines are a source of C → T transitions in vivo. *Proc. Natl. Acad. Sci. USA* **95**, 3578–3582 (1998).
19. Pinz, K.G., Shibutani, S. & Bogenhagen, D.F. Action of mitochondrial DNA polymerase gamma at sites of base loss or oxidative damage. *J. Biol. Chem.* **270**, 9202–9206 (1995).
20. Tanaka, M. & Ozawa, T. Strand asymmetry in human mitochondrial DNA mutations. *Genomics* **22**, 327–335 (1994).
21. Frederico, L.A., Kunkel, T.A. & Shaw, B.R. A sensitive genetic assay for the detection of cytosine deamination: determination of rate constants and the activation energy. *Biochemistry* **29**, 2532–2537 (1990).
22. Trifunovic, A. *et al.* Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429**, 417–423 (2004).
23. Larsen, N.B., Rasmussen, M. & Rasmussen, L.J. Nuclear and mitochondrial DNA repair: similar pathways? *Mitochondrion* **5**, 89–108 (2005).
24. Elson, J.L., Samuels, D.C., Turnbull, D.M. & Chinnery, P.F. Random intracellular drift explains the clonal expansion of mitochondrial DNA mutations with age. *Am. J. Hum. Genet.* **68**, 802–806 (2001).
25. Bender, A. *et al.* High levels of mitochondrial DNA deletions in substantia nigra neurons in aging and Parkinson disease. *Nat. Genet.* **38**, 515–517 (2006).
26. Kraysberg, Y. *et al.* Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. *Nat. Genet.* **38**, 518–520 (2006).
27. Tyynismaa, H. *et al.* Mutant mitochondrial helicase Twinkle causes multiple mtDNA deletions and a late-onset mitochondrial disease in mice. *Proc. Natl. Acad. Sci. USA* **102**, 17687–17692 (2005).
28. Hamilton, M.L. *et al.* Does oxidative damage to DNA increase with age? *Proc. Natl. Acad. Sci. USA* **98**, 10469–10474 (2001).