Amelioration of premature aging in mtDNA mutator mouse by exercise: the interplay of oxidative stress, PGC-1α, p53, and DNA damage. A Hypothesis.

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Supplement 1. Excess of the "short fragment mutations" in sedentary PolG mice can be explained by non-mutational DNA damage and the presence in the cell of mtDNA subpopulations.

1.1. Excess of the "short fragment mutations" in sedentary PolG mice.

Intriguingly, our own previously published data [1] appear to contradict our current conclusion that exercise does not change mutational load in the muscle of PolG mouse. Previous data showed a decrease of mutational load in the exercised PolG mouse. We believe that the source of the discrepancy is in the methodological approach used. The previous data was generated using the 454 Next Generation Sequencing (NGS), which involves droplet PCR of the ~1kb DNA fragments (short fragment PCR). In contrast, the current study used long-range single molecule PCR of ~16kb DNA fragments. Theoretically, mutational fraction should be the same, whether DNA is amplified using short or a long fragment PCR. This is why we were shocked to discover such differences between our studies. Specifically, there was a clear ~2-fold difference in the mutational load between sedentary and exercised PolG mice in our previous study [1]. In contrast, in our present study, we discovered essentially an equal level of mutations in sedentary and exercised PolG mice (Figure 1A), measured by long-range single molecule PCR of the entire mtDNA. Moreover, in this study, the mutational levels were generally lower than those measured by the 454 NGS. It appeared as if 454 NGS was tapping into an additional pool of mutations in sedentary mice which was not present in the exercised ones and was not assessable by the long single molecule approach.

To confirm that the length of the amplicon indeed was the cause of the discrepancy, we performed the single molecule PCR analysis using a shorter PCR fragment (3 kb long, which is closer to that used in the 454 NGS approach). Interestingly, indeed, merely reducing the amplicon length resulted in a significantly ($p<0.001$) higher apparent mutant fraction being measured in sedentary, but not exercised mice (**Figure S1.B**).

To gain further insight in the source of discrepancy, we also changed the enzyme that was used for PCR, from bacterial Taq polymerase (ExTaq formulation by TaKaRa) to a modified archaeal high-fidelity polymerase Q5 (New England Biolabs). In the long fragment PCR case, this change did not affect the number of mutations scored (**Fig S1.A** represents a mixture of data from the two polymerases; note the narrow error bar). In contrast, when using the short 3kb PCR fragment, the change in polymerase resulted in diminished *apparent* mutant fraction in the sedentary mice (compare red bars in **Fig S1.B and C**). As discussed in section 1.2 below, the change of the enzyme was unlikely to affect the conventional PCR errors. Furthermore, PCR error would equally affect analysis of sedentary and exercised mice, so lower PCR error of the Q5 enzyme cannot explain the decreased *difference* between sedentary and exercised mice (compare the difference between SED and END bars in **Fig S1.B** and **C** vs. the difference between SED and END bars in **Fig S1.B** and **A**). What could have caused the observed changes in mutant mtDNA fraction with the amplicon size and the type of the enzyme?

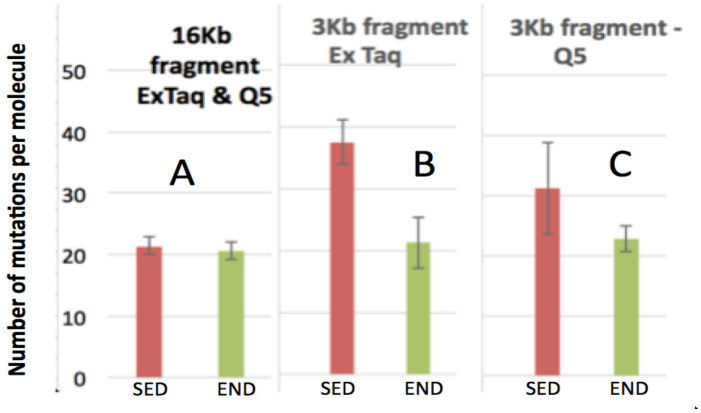

Figure S1. Mutation load in exercised and sedentary PolG mice as measured using long amplicon sequence and different PCR polymerases protocols (details in the text)

1.2. Single molecule mutation analysis is free of spontaneous PCR errors.

Both the 454 NGS approach, and our single molecule PCR approaches (long-range *and* short-range) are highly resistant to *spontaneous* thermostable polymerase error, although both are PCR-based. This is because in all these approaches an individual PCR reaction is performed starting with a single DNA molecule, whether this is emulsion droplet PCR in 454, or single molecule PCR in well of a multi-well PCR plate. As a result, spontaneous PCR errors cannot be present in more than 50% of PCR products [2], because any spontaneous PCR-driven mutation can be created only in no more than one of two DNA strands (the original strand and its PCR copy). Thus mutations created on the first cycle will be 50%; any mutations created on subsequent PCR cycles will have even lower presence, and will be filtered out by data processing. Note that this arrangement assumes that PCR is being initiated from only one single strand, which is correct for the 454 emulsion PCR and mostly correct for the single molecule PCR setting, because only one long strand is amplifiable). If the PCR is started by both strands of a the original double stranded single molecule, the suppression of PCR errors is even more efficient: in this case they represent no more than a quarter of daughter PCR molecules. Of note, the fact that two polymerases with ~50-fold difference in PCR fidelity, ExTaq and Q5, yield essentially identical mutant fractions (**Fig S1**, green bars), is an indication that spontaneous PCR error is not affecting our mutation measurements to a significant

extent. Note that "spontaneous PCR error" means the insertion, during DNA replication, of an incorrect nucleotide across from a *normal, unmodified* template nucleotide. This is different from the DNA *damage-driven* PCR error, which is discussed in the next section 1.3.

1.3. Conversion of non-mutational DNA damage into mutations by PCR is a source of PCR artifacts.

A general rule in the mtDNA mutation analysis field is: if two non-selective approaches yield different mutant fractions, then the approach yielding higher fraction is probably artifact-prone. Both single molecule PCR and the native 454 NGS approach is susceptible to a different type of PCR error, the conversion of non-mutational mtDNA damage (chemically modified nucleotides) into apparent mutations by PCR. We have demonstrated this phenomenon by independently probing the two DNA strands for the presence of mutations decades ago [3]. We discovered that some of the mutations could be detected only in one but not the other DNA strand. Such a pattern could only be a result of conversion into mutations of non-mutational DNA damage, i.e. nucleotide modifications. Unlike true mutations, each nucleotide modification affects only one of the DNA strands. We have proposed that such conversion was a major source of error in mutational analysis of mtDNA [4], and indeed, this has been recently confirmed using the "double stranded" NGS approach [5]. In addition to oxidative nucleotide damage, which we believe drives PCR error in the case of PolG mice, this class of artifacts may result from other types of DNA damage (mots notably deamination of cytosines [6]), as well as unrepaired mismatches, as they will behave similarly to chemically damaged nucleotides in the single strand mutational assay.

1.4. A subpopulation of damaged mtDNA present in the sedentary but not exercised mouse could explain the length dependence of mutation estimates.

How could the conversion of an excess of non-mutational damage in the sedentary mice explain the above observations, i.e. what would be a possible mechanism whereby these "conversion" mutations *only* happen in *shorter* DNA fragments and preferably with Taq but less with Q5 polymerase? We propose that there are subpopulations of mitochondria in the cell, with higher and lower mtDNA damage levels, respectively. Long range, whole genome amplification is highly sensitive to mtDNA damage [7], and will presumably amplify mtDNA molecules only from the less damaged mtDNA subpopulation. This DNA is expected to contain low level of PCR disrupting damage, such as nicks and impassable nucleotide modifications, as well as low level of damage that does not prevent PCR but results in creation of convertant mutations, such as deaminated cytosine, 8-OHdG, etc. The resulting PCR fragments will thus appear "low mutant". In contrast, approaches using shorter fragments (such as short fragment PCR or 454 next generation sequencing) are less sensitive to PCR disrupting damage and thus also tap into the high-damage pool of mtDNA molecules. The short fragments will thus appear to contain higher levels of mutations.

The observed difference by the type of the enzyme is probably related to the differences in the handling of the chemically modified nucleotides by the bacterial and the archaeal DNA polymerases. It is known, for example, that Q5 does not efficiently amplify templates containing uracil (i.e. deaminated cytosine). In fact, the very high fidelity of the archaeal group enzymes in PCR is not compatible with utilization of damaged template nucleotides. DNA sustains significant damage during PCR (e.g. cytosine deamination at high temperature), and if high fidelity polymerases (such as Q5 and pfu) were able to utilize damaged template nucleotides, their extreme fidelity would be compromised [8].

In conclusion, the puzzling observation that mutation load of short mtDNA fragments appears to be higher than that of long ones, specifically in sedentary, but not exercised PolG mouse muscle, is consistent with the hypothesis that a subset of mtDNA molecules in sedentary mouse sustain higher levels of chemically modified nucleotides, which are being converted into mutations during PCR. This is consistent with the idea that mtDNA repair in exercised mouse is assisted by p53, which translocates to mitochondria as a result of exercise (Figure 2).

Supplement 2. Experimental procedures.

2.1. PolG Mutator Mice Breeding, Endurance Exercise Protocol, and Tissue Harvesting.

Heterozygous mice (C57BL/6J, PolgA+/D257A) for the mitochondrial polymerase gamma knock-in mutation were obtained through collaboration with Drs. Gregory C. Kujoth and Tomas A. Prolla, University of Wisconsin Madison and bred for endurance exercise study as previously described [1]. Briefly, at 3 months of age, equal numbers of PolG female and male mice were assigned to the sedentary (PolG-SED) and forced-endurance (PolG-END) exercise groups (n = 10/group; ♀ = ♂). None of the mice had been previously subjected to endurace exercise regimen. The PolG-END mice were subjected to forced treadmill exercise (Eco 3/6 treadmill; Columbus Instruments) three times per wk at 15 m/min for 45 min for a period of 3 months. A 5-min warm-up period and a 5-min cool-down period at 8 m/min were included. The PolG mice were age- and sex- matched with sedentary WT littermate mice (n = 10; ♀ = ♂), which served as controls. At 6 months of age, the mice were shipped to Northeastern Univserity, where they were kept (without exercising) for 3 weeks followed by euthanasia and tissue samples collection for mtDNA mutation analyses. The study was approved by McMaster University's Animal Research and Ethics Board under the global Animal Utilization Protocol 12–03-09, and the experimental protocol strictly followed guidelines published by the Canadian Council of Animal Care.

2.2. Single molecule PCR.

PCR was performed using one of two thermostable polymerase systems. Ex Taq DNA polymerase (TaKaRa) was used according to the manufacturer's recommendations, except the polymerase concentration was halved and LA Taq buffer (TaKaRa) was used instead of the Ex Taq buffer. Q5 Hot Start High-Fidelity DNA polymerase (New England Biolabs) was used according to the manufacturer's recommendations, except the polymerase concentration was halved. PCR was performed in a 15μL volume. For short fragments, DNA was amplified with primers 4008F and 7056R, where the number denotes the 5' nucleotide position and F indicates forward and R reverse. Reactions were run for 50 cycles. For long fragments, the primers were 3092F and 3031R. Ex Taq samples were run for 55 cycles. Q5 samples were run for 50 cycles, then a second stage PCR was carried out wherein 1μL of a 1:10 dilution of Q5 PCR product was added to 14μL of a LA Taq reaction with the primers 3140F and 3003R for an additional 15 cycles. This two-stage amplification was necessary because Q5 alone did not consistently produce enough products for sequencing and the use of a different enzyme on an already-amplified product prevents the introduction of polymerase-based errors.

Single molecule PCR products were sequenced using the Sanger approach at a core facility. The products were tested for being single molecule by the mutational pattern (mixtures of molecules show up as a set of ~50:50 heteroplasmic positions) and mutltiplets were discarded from analysis.

Note that the reason for high mutational count in the "WT" control (blue bar in **Figure 1A**) is that WT mice are actually littermates of the homozygous PolG "cases", and therefore their mothers were PolG heterozygotes, which also suffer increased mutational rates. In this case, the heterozygous mothers belonged to a lineage with three generations of heterozygous PolG, which resulted in a fairly high background of inherited mutational load. All of these mutations are not somatic, they are inherited from previous generation (as evident by their recurrent pattern). Note that similar mutant counts were previously reported for the WT controls by the Prolla laboratory [9].

2.3. mtDNA 8-OHdG immunoblotting.

Total genomic/mitochondrial DNA was isolated from skeletal muscle using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. DNA samples were treated with RNase (Fermentas, Ottawa, ON) to remove RNA contamination. To prevent auto/air oxidation of DNA, ethanethiol (4% v/v, Sigma-Aldrich) was added to RNase-DNase-free water to elute total genomic/mitochondrial DNA from the column. This step is critical in assessing mtDNA oxidation in response to experimental conditions and prevents artifactual oxidation of DNA bases by prooxidant environmental sources that could serve to negate differences between groups . DNA concentration and quality were assessed using Nanodrop 2000 (Thermo Scientific). DNA preparations were digested with *KpnI and DraII* endonucleases (New England Biolabs, Ipswich, MA), followed by treatment with Exonuclease III (New England Biolabs) to degrade any contaminating nuclear DNA. One microgram of resulting mtDNA from each sample was dot-blotted on a nitrocellulose membrane (Amersham, Piscataway, NJ). Immunoblotting was carried out using mouse monoclonal 8-OHdG (N45.1) antibody (Japan Institute for the Control of Aging, Baltimore, MD). Membranes were then incubated with anti-mouse HRP-linked secondary antibody (Bio-Rad Laboratories, Hercules, CA) and visualized by enhanced chemiluminescence (Amersham, Pittsburgh, PA). Relative intensities of the circular dots were digitally quantified by using ImageJ analysis software (version 1.37, Scion Image).

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