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DNA deletions and clonal mutations drive premature aging in mitochondrial mutator mice

Marc Vermulst[1], Jonathan Wanagat[2], Gregory C Kujoth[3], Jason H Bielas[1], Peter S Rabinovitch[1], Tomas A Prolla[3] & Lawrence A Loeb[1]

Mitochondrial DNA (mtDNA) mutations are thought to have a causal role in many age-related pathologies. Here we identify mtDNA deletions as a driving force behind the premature aging phenotype of mitochondrial mutator mice, and provide evidence for a homology-directed DNA repair mechanism in mitochondria that is directly linked to the formation of mtDNA deletions. In addition, our results demonstrate that the rate at which mtDNA mutations reach phenotypic expression differs markedly among tissues, which may be an important factor in determining the tolerance of a tissue to random mitochondrial mutagenesis.

A longstanding hypothesis postulates that mutations in the mitochondrial genome limit mammalian lifespan[1]. Mitochondrial mutator mice, harboring a proofreading-deficient copy of the catalytic subunit of polymerase gamma (PolgA), provide a unique opportunity to test the validity of this hypothesis and to dissect the role of PolgA in mitochondrial mutagenesis. Homozygous carriers of the proofreading-deficient *Polga* allele (*Polga*$^{mut/mut}$) show several features of accelerated aging, whereas heterozygous mice (*Polga*$^{+/mut}$) do not[2,3]. Using this mouse model, we recently reported that the proofreading domain of PolgA suppresses mtDNA point mutations extensively *in vivo*, and demonstrated that mtDNA point mutations alone do not limit the lifespan of wild-type (WT) mice[4]. Mitochondrial mutator mice also carry an increased amount of mtDNA deletions[3]. Here, we examine the role PolgA plays in deletion mutagenesis, and evaluate to what extent large mtDNA deletions contribute to the premature aging phenotype of mitochondrial mutator mice. To do this, we used a quantitative PCR–based (qPCR) approach to measure mtDNA deletions in the major arc of the mitochondrial genome (**Supplementary Figures 1–3**, **Supplementary Table 1** and **Supplementary Methods** online), and compared the deletion burden of proofreading-deficient PolgA mutator mice to that of WT mice as a function of age. We found that mtDNA deletions accumulated significantly in brain (**Fig. 1a**) and heart tissue (**Supplementary Figure 4** online) of WT[5], *Polga*$^{+/mut}$ and *Polga*$^{mut/mut}$ mice with age. However, they accumulated at an accelerated rate in the tissues of the *Polga*$^{mut/mut}$ mice only (**Fig. 1a**). This resulted in a 7- to 11-fold increase ($P < 0.01$) in mtDNA deletions over those in WT and *Polga*$^{+/mut}$ mice.

Double-strand breaks (DSBs) are an important source of mtDNA deletions. H_2O_2, a powerful mutagen that causes DSBs, induces deletions in normal human dermal fibroblasts (**Supplementary Methods** online), and induction of DSBs by a restriction enzyme in mouse muscle tissue also results in mtDNA deletions[6]. In addition, mCAT mice[7], which harbor a human catalase gene that is targeted to mitochondria in order to increase H_2O_2 scavenging, show a reduced frequency of mtDNA deletions in heart tissue ($P = 0.0487$, Mann-Whitney test; WT $n = 6$, mCAT $n = 3$). Previously, a similar decrease was documented in muscle tissue[7] of mCAT mice. Thus, our results suggest that PolgA has a role in DSB repair. To delineate the role PolgA plays in DSB resolution, we analyzed 223 deleted mtDNA molecules. We found that most mtDNA deletions in WT and heterozygous mice occurred between homologous sequences (**Fig. 1b**), and that most of these occurred between two 15-bp direct repeats that were previously shown to be hotspots for the generation of deletions[5,8]. In contrast, deletions in *Polga*$^{mut/mut}$ mice occurred preferentially between nonhomologous sequences (**Supplementary Table 2** online). Thus, the proofreading domain of PolgA suppresses rearrangements between nonhomologous sequences, implicating the existence of a homology-directed DSB repair mechanism in mammalian mitochondria, similar to recombination[9]. This data provides an explanation for the observation that mtDNA deletions in humans[10] and WT mice[5,8] predominantly occur between direct repeats, as the proofreading domain of PolgA assures that mtDNA rearrangements result from transactions between homologous sequences only.

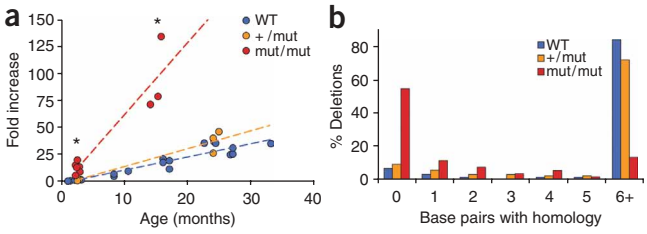

Figure 1 Mitochondrial deletion load and spectrum in WT, *Polga*$^{+/mut}$ and *Polga*$^{mut/mut}$ mice. (**a**) mtDNA deletions in brain tissue as a function of age in WT, *Polga*$^{+/mut}$ and *Polga*$^{mut/mut}$ mice. The average frequency of deletions in young (1–3 months) WT mice is set to 1. Fold increases over that baseline are depicted. An asterisk (*) signifies a statistical difference ($P < 0.01$, two-sided *t*-test) between *Polga*$^{mut/mut}$ and WT animals at 1–3 or 15–17 months of age. (**b**) Number of base pairs with direct homology at deletion breakpoints. Between 51 and 115 deletions were sequenced per genotype.

[1]Department of Pathology, University of Washington, Seattle, 91895 Washington, USA. [2]Department of Gerontology and Geriatric Medicine, University of Washington, Seattle, Washington 91895, USA. [3]Departments of Genetics and Medical Genetics, University of Wisconsin, Madison, Wisconsin 53706, USA. Correspondence should be addressed to L.A.L. (laloeb@u.washington.edu)

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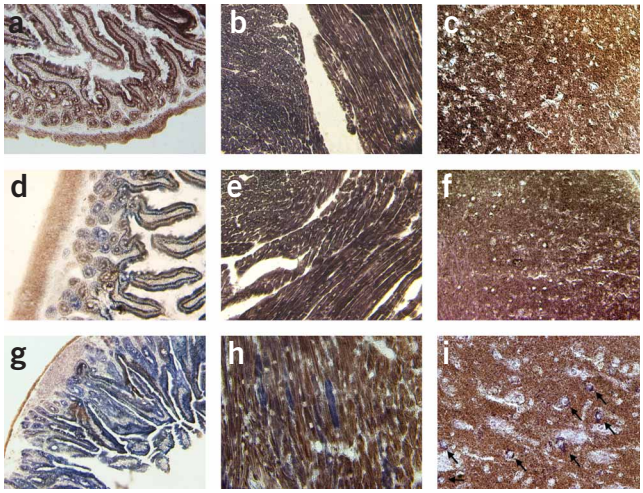

Figure 2 Tissue distribution of COX-negative cells. Tissue sections were sequentially stained for activity of the COX (brown) and succinate dehydrogenase (SDH) complexes (blue) of the electron transport chain. Dark brown staining depicts cells with intact COX and SDH activity; cells in blue are those that have lost activity of the COX-complex but retain SDH-activity, which is encoded by nuclear genes only. (**a**) Duodenum, WT. (**b**) Heart, WT. (**c**) Brain, WT. (**d**) Duodenum, *Polga*$^{+/mut}$. (**e**) Heart, *Polga*$^{+/mut}$. (**f**) Brain, *Polga*$^{+/mut}$. (**g**) Duodenum, *Polga*$^{mut/mut}$. (**h**) Heart, *Polga*$^{mut/mut}$. (**i**) Brain, *Polga*$^{mut/mut}$ (blue cells arrowed). Heart and brain cells of *Polga*$^{mut/mut}$ mice have been magnified ×4 and ×3, respectively, to depict mutant cardiomyocytes and neurons. Depicted heart and brain sections are from the left ventricle and the cortex, respectively.

As direct repeats are hotspots for deletion formation in WT and *Polga*$^{+/mut}$ mice only, we hypothesized that the difference in deletion load between WT and *Polga*$^{mut/mut}$ mice may be larger in regions of the mtDNA that do not contain direct repeats. To test this possibility, we probed two additional sites in the same genetic region using primer combinations that excluded detection of rearrangements between the 15-bp direct repeats. At these loci, we observed a >90-fold increase in mtDNA deletions in *Polga*$^{mut/mut}$ mice compared to WT mice (**Supplementary Figure 5** online). Consistent with our previous results, no difference between WT and *Polga*$^{+/mut}$ mice was observed (data not shown). We conclude that deletions are highly abundant in prematurely aging *Polga*$^{mut/mut}$ mice compared to their *Polga*$^{+/mut}$ counterparts. Because *Polga*$^{+/mut}$ mice age normally, we conclude that this increase in deletions, in addition to an increase in point mutations[4], exceeds the mutation load that is compatible with natural aging. However, as an increase in mtDNA deletions is associated exclusively with prematurely aging mice, and as mtDNA deletions affect thousands of base pairs per event, mtDNA deletions seem to be the most important force behind the shortened lifespan of *Polga*$^{mut/mut}$ mice.

As mammalian cells contain hundreds of copies of mtDNA, clonal expansion of a mtDNA mutation is required to affect cell function. Thus, in order to examine how mtDNA mutations result in pathology, we compared clonal mtDNA mutations in the duodenum, heart and brain of WT and mitochondrial mutator mice. To do this, we used a histochemical stain to detect loss of cytochrome oxidase (COX) activity in tissue sections, which can be used as a screen for clonally expanded mutations that affect mtDNA-encoded COX genes. We did not observe COX-negative cells in the duodenum, heart or brain of 15-month-old WT mice (**Fig. 2a–c**). In contrast, ~20% of the cells in the duodenum of age-matched *Polga*$^{+/mut}$ mice were COX-negative (**Fig. 2d**), consistent with a ~100-fold increase in random point mutations over WT mice (data not shown). In order to confirm that mtDNA mutations cause the COX-negative phenotype of these cells, we microdissected and sequenced seven COX-negative villi from *Polga*$^{+/mut}$ mice. We identified point mutations with high degrees of heteroplasmy in all instances (**Supplementary Figure 6** online). No evidence was found for the presence of mtDNA deletions using a competitive qPCR-based approach (**Supplementary Figure 7** online, data not shown), consistent with the observation that these mice have an elevated frequency of point mutations, but not deletions. Similar clonal expansions of mtDNA mutations, which originate in stem cells, have been observed with age in the digestive tract of humans[11]. Thus, even though these mice do not show a significant change in lifespan, some age-related pathology is present in the duodenum of *Polga*$^{+/mut}$ mice. We previously demonstrated a >100-fold increase in random point mutations in the heart and brain tissue of *Polga*$^{+/mut}$ mice as well. However, we did not detect any COX-negative cells in these tissues (**Fig. 2e**,**f**), demonstrating that a considerable difference exists in the rate at which mtDNA point mutations reach phenotypic expression between cell types. This may be an important factor in determining the tolerance of a tissue to random mitochondrial mutagenesis, and it may contribute to the normal lifespan of *Polga*$^{+/mut}$ mice, as cardiac and neuronal disease correlate better with life expectancy than pathology in the duodenum. Accordingly, we found that, in addition to the duodenum, both heart and brain tissue of prematurely aging *Polga*$^{mut/mut}$ mice contained many COX-negative cells (**Fig. 2g–i**). The accelerated rate at which mtDNA deletions occur in *Polga*$^{mut/mut}$ mice, and the increased amount of cells and tissues affected by clonally expanded mitochondrial mutations provide a rationale for the decreased life expectancy of the *Polga*$^{mut/mut}$ mice. Whether mitochondrial mutations also constrain the lifespan of WT animals is an area of intense debate[12]. However, there is substantial evidence that mtDNA deletions have a causal role in the degeneration of substantia nigra neurons in Parkinson's disease[5,13,14], an important age-related disease. The deletions in these neurons occurred preferentially between direct repeats. Moreover, the number of direct repeats within the mitochondrial genome correlates with maximum lifespan, especially when closely related species are compared[15]. Thus, elucidating the mechanisms that underlie the observations reported here will yield valuable insight into the role of mtDNA mutations in aging and disease.

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

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AUTHOR CONTRIBUTIONS

M.V. carried out the experiments described in **Figures 1a** and **b**, **Supplementary Figures 1–5** and **Supplementary Tables 1** and **2**. M.V. adapted the RMC-assay and wrote the paper. J.W. and M.V. generated **Figure 2** and **Supplementary Figures 6** and **7**. M.V., J.H.B. and L.A.L. conceived the project. J.H.B. performed cell culture and provided technical expertise. G.C.K., T.A.P. and P.S.R. provided animal care, tissues and technical assistance. L.A.L. supervised the experimental work and interpretation of the data. All authors commented on and discussed the paper.

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