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Short Article

# Random Point Mutations with Major Effects on Protein-Coding Genes Are the Driving Force behind Premature Aging in mtDNA Mutator Mice

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## SUMMARY

**The mtDNA mutator mice have high levels of point mutations and linear deletions of mtDNA causing a progressive respiratory chain dysfunction and a premature aging phenotype. We have now performed molecular analyses to determine the mechanism whereby these mtDNA mutations impair respiratory chain function. We report that mitochondrial protein synthesis is unimpaired in mtDNA mutator mice consistent with the observed minor alterations of steady-state levels of mitochondrial transcripts. These findings refute recent claims that circular mtDNA molecules with large deletions are driving the premature aging phenotype. We further show that the stability of several respiratory chain complexes is severely impaired despite normal synthesis of the corresponding mtDNA-encoded subunits. Our findings reveal a mechanism for induction of aging phenotypes by demonstrating a causative role for amino acid substitutions in mtDNA-encoded respiratory chain subunits, which, in turn, leads to decreased stability of the respiratory chain complexes and respiratory chain deficiency.**

## INTRODUCTION

Over the years, substantial evidence has emerged from morphological, bioenergetic, biochemical, and genetic studies showing that mitochondrial dysfunction is heavily implicated in the aging process (Trifunovic and Larsson, 2008). Point mutations and deletions of mtDNA accumulate in a variety of tissues during aging in humans, monkeys, and rodents (Wallace, 2001). These mutations are unevenly distributed and can accumulate clonally in certain cells, causing a mosaic pattern of respiratory chain deficiency in tissues such as heart, skeletal muscle, and brain. In terms of the aging process, their possible causative effects have been intensely debated because of their low abundance and purely correlative connection with aging (Wallace, 2001).

The creation of mtDNA mutator mice have provided the first direct evidence that accelerating the mtDNA mutation rate can result in premature aging, consistent with the view that loss of mitochondrial function is a major causal factor in aging (Trifunovic et al., 2004). The mtDNA-mutator mice were engineered to have a defect in the proofreading function of the catalytic subunit of mitochondrial DNA polymerase (POLGA), leading to the progressive, random accumulation of mtDNA mutations during the course of mitochondrial biogenesis. Studies of mtDNA mutator mice has shown that increased levels of somatic mtDNA mutations can directly cause a variety of aging phenotypes, such as osteoporosis, hair loss, greying of the hair, weight reduction, cardiomyopathy, progressive hearing loss, and decreased fertility (Trifunovic et al., 2004). Surprisingly, increased levels of somatic mtDNA mutations cause the loss of critical, irreplaceable cells through apoptosis and leads to premature aging phenotypes without causing increased ROS production or oxidative stress in mtDNA mutator mice (Niu et al., 2007; Trifunovic et al., 2005).

The mtDNA mutator mice have very high levels of point mutations of mtDNA (9–15/10 kb) and also contain high levels (25% of total mtDNA) of a 12 kb linear deleted mtDNA molecule (Trifunovic et al., 2004). A recent study suggests that an additional mtDNA species, i.e., circular mtDNA molecules with large deletions and not the above-mentioned mutant mtDNA forms, are the driving force behind the premature aging phenotypes in mtDNA mutator mice (Vermulst et al., 2008). Large mtDNA deletions typically span multiple genes and always include one or several tRNA genes (Holt et al., 1988). Studies of human patients with high levels of single large mtDNA deletions show that this type of genetic mutation impairs mitochondrial translation due to the lack of certain tRNAs (Hayashi et al., 1991). Similarly, clonal expansion of deleted mtDNA molecules in individual cells of aging human tissues causes respiratory chain dysfunction most likely due to impaired mitochondrial translation (Fayet et al., 2002). The prediction is therefore that the recently reported circular deleted mtDNA molecules in mtDNA mutator mice (Vermulst et al., 2008) should affect mitochondrial translation if they are at all involved in generating the premature aging phenotypes. Here we demonstrate that mitochondrial translation is unaffected and thus exclude a causative role for mtDNA deletions.

Instead, we present a molecular mechanism for induction of aging phenotypes by demonstrating that point mutations of mtDNA leads to synthesis of respiratory chain subunits with amino acid substitutions, which, in turn, impairs the stability of assembled respiratory chain complexes and thereby causes respiratory chain deficiency.

## RESULTS

### Respiratory Chain Deficiency in mtDNA Mutator Mice

The first premature aging symptoms, e.g., slight kyphosis, alopecia, and impaired weight gain, can be observed in mtDNA mutator mice at ~25 weeks of age (Trifunovic et al., 2004). The onset of symptoms is associated with the occurrence of an increased amount of respiratory chain-deficient cells in both liver and heart of mtDNA mutator mice (Trifunovic et al., 2004). These results are consistent with the suggestion that mtDNA mutation accumulation drives the premature aging phenotypes.

We performed additional experiments to further characterize the nature of the respiratory chain deficiency in mtDNA mutator mice. The oxidation of complex I (NADH: ubiquinone oxidoreductase, EC 1.6.5.3)-linked substrates was monitored in isolated intact mitochondria from liver and heart, and we found a general reduction in all inducible respiratory states, with a slightly lower level of reduction in heart (Figures S1A and S1B) than in liver mitochondria (Figures S2A and S2B). As expected, the basal rate, limited by the mitochondrial membrane proton leak and not by the respiratory chain, was not altered (i.e., the rate after oligomycin addition). To examine the consequence of mtDNA mutations on total electron transport chain (ETC) activity, we induced artificial permeabilization of the mitochondrial membrane with alamethicin, thus eliminating impacts of substrate transport and membrane potential. The maximal ETC activity was significantly reduced, and the observed decrease was slightly lower in heart (Figures S1C and S1D) than in liver (Figures S2C and S2D) mitochondria from mtDNA mutator mice. These results thus expand our previous report of respiratory chain deficiency in hearts of 25-week-old mtDNA mutator mice (Trifunovic et al., 2004) and show that the observed deficiency is a direct consequence of impaired activity of the respiratory chain complexes.

### Combined Complex I, III and IV Deficiency in mtDNA Mutator Mice

We analyzed steady-state levels of single respiratory chain protein subunits in order to obtain further insight into the molecular mechanisms of the observed respiratory dysfunction in the mtDNA mutator mice. Interestingly, only the subunits of complex IV (cytochrome c oxidase [COX], EC 1.9.3.1) were significantly reduced in liver (Figures 1A and 1B) and heart mitochondria (Figure 1C), of mtDNA mutator mice. Levels of COX II, an mtDNA-encoded complex IV subunit, were reduced to 57% whereas levels of COX IV, a nucleus-encoded complex IV subunit, were reduced to 27%–40% in heart and liver, respectively (Figures 1B and 1C). In addition, we detected an almost 2-fold upregulation in the levels of the nucleus-encoded α-subunit of complex V in mtDNA mutator hearts (Figure 2C). Under physiological conditions myocardium meets the bulk of its energy demand by aerobic respiration; therefore, upregulation of the complex V subunits is likely a consequence of the inefficient oxidative phosphorylation and an attempt of the cardiomyocytes to upregulate the ATP production rates.

The observed reduction in steady-state levels of complex IV protein subunits resulted in a corresponding reduction in the levels of fully assembled complex IV in both liver (Figures 2A and 2B) and heart (data not shown) as determined by blue native-polyacrylamide gel electrophoresis (BN-PAGE) analysis of mitochondrial extracts. Additionally, BN-PAGE analysis revealed a strong reduction in steady-state levels of complex I and complex III (Ubiquinol cytochrome *c* reductase, EC 1.10.2.2) in liver (Figure 2B) and heart (not shown) of mtDNA mutator mice, despite the fact that some individual protein subunits of these complexes were present at normal levels. The observed reduction of steady-state levels of complex I corresponded to a reduced complex I in-gel activity in mtDNA mutator mice (Figures 2B and 2C).

The assembly of respiratory complexes in the inner mitochondrial membrane was examined by western blot analysis of different protein subunits on two-dimensional gels (BN-PAGE/SDS-PAGE) of isolated liver mitochondria. Analysis of protein extracts from liver and heart mitochondria demonstrated a general decrease of all early subcomplexes, as well as reduced amounts of the fully assembled complexes I, III, and IV (Figure 2D). These results thus demonstrate a general problem with the assembly of respiratory chain complexes in mtDNA mutator mice, causing a combined deficiency of complexes I, III, and IV both in heart and liver mitochondria.

Animals that are heterozygous for the mtDNA mutator allele (*PolgA*$^{+/mut}$ mice) have been reported to have increased levels of mtDNA mutations (Vermulst et al., 2007). We found normal respiratory chain function and normal steady-state levels of assembled respiratory chain complexes in *PolgA*$^{+/mut}$ mice (Figures S3A and S3B). Our findings thus indicate that proposed elevated levels of mtDNA mutations in *PolgA*$^{+/mut}$ mice are below the threshold to impair respiratory chain function and cause aging phenotypes, consistent with reports of normal life span in these mice (Vermulst et al., 2007).

### Increased Turnover of Mitochondrial Proteins in mtDNA Mutator Mice

The observed decrease in levels of assembled respiratory chain complexes in mtDNA mutator mice (Figure 2B) can be caused by reduced synthesis of core subunits (impaired mitochondrial transcription or translation) or by defective assembly of the complex due to amino acid replacements in normally synthesized core subunits. We therefore investigated steady-state levels of a number of different mitochondrial transcripts in the liver of mtDNA mutator mice (Figures 3A–3C). We could not detect any decrease in levels of mRNAs, but rather observed an increase in levels of several mitochondrial transcripts in mtDNA mutator mice (Figures 3A and 3B). Increased levels of mtDNA transcripts have been reported in liver mitochondria isolated from old rats (Barazzoni et al., 2000). It is well known that respiratory chain deficiency induces mitochondrial biogenesis in mice (Metodiev et al., 2009) and humans (Smeitink et al., 2001), and it is therefore possible that the observed increase in levels of mtDNA transcripts in mtDNA mutator mice is part of a mitochondrial biogenesis response. Consistent with this hypothesis, we found an upregulation of levels of tRNAs transcribed from heavy

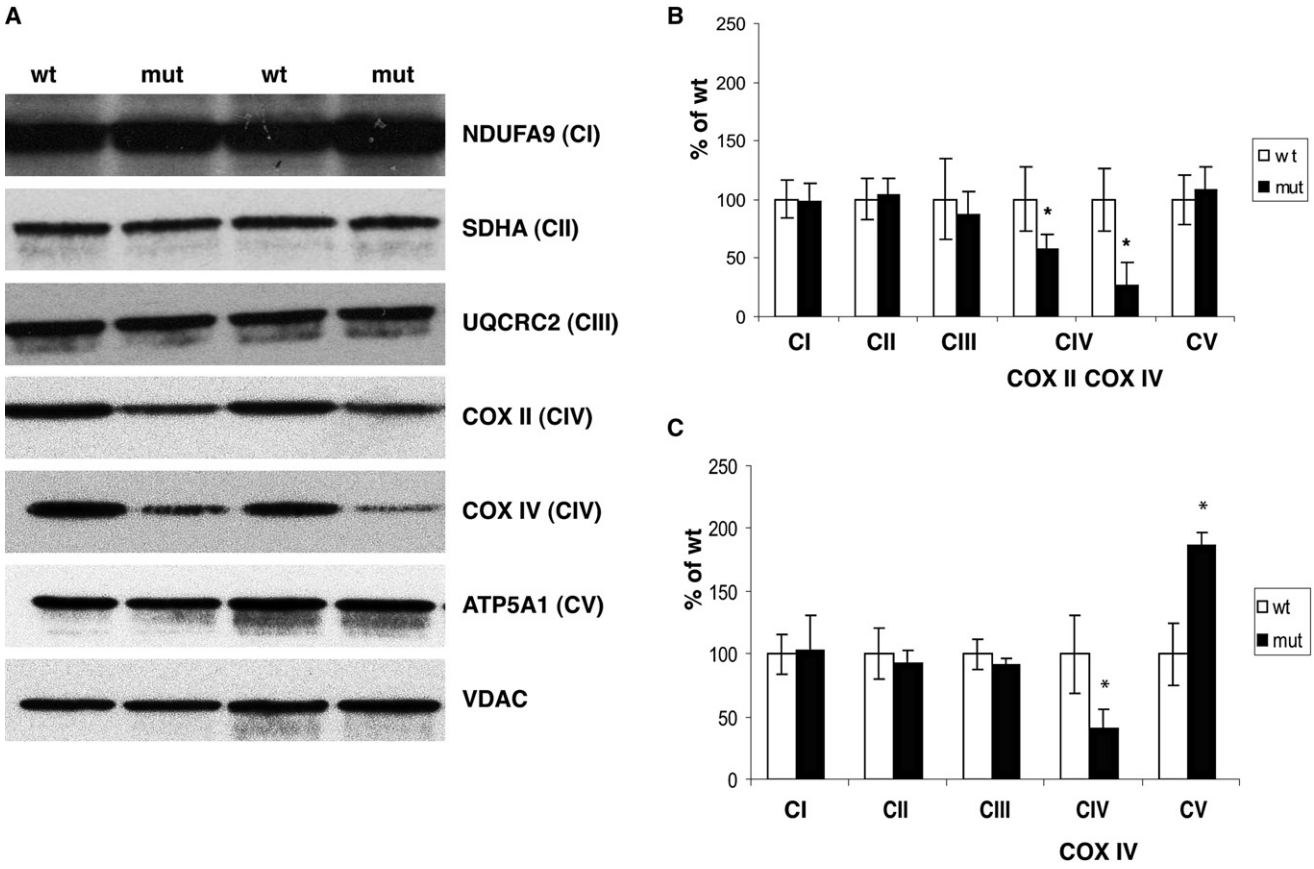


**Figure 1. Levels of Respiratory Chain Subunits in Liver and Heart Mitochondria**

(A) Western blot analyses of subunits of respiratory chain complexes from liver mitochondria of 25-week-old mice. Quantification of the steady-state levels of single protein subunits in liver (B) and heart (C) mitochondria. Bars indicate mean levels ± standard error of the mean (SEM). Asterisks indicate level of statistical significance (*$p < 0.05$, Student's t test) ($n = 6$, liver; $n = 4$, heart).

strand promoter (HSP) (Figures 3A and 3C). The levels of tRNAs transcribed from the other promoter (LSP) were somewhat reduced (Figures 3A and 3C). The overall transcriptional pattern in mtDNA mutator mice is not consistent with an effect caused by large mtDNA deletions because there is no lack of transcripts from a specific longer region of mtDNA. The findings rather suggest a decrease in the activity of LSP or decreased stability of transcripts from this promoter.

Next, we directly monitored mitochondrial protein synthesis by in organello translation studies. We found no difference in the overall mitochondrial protein synthesis rates in liver mitochondria from mtDNA mutator and wild-type mice (Figure 3D). However, we detected increased turnover of mtDNA-encoded respiratory chain subunits after a pulse chase lasting for 3 or 5 hr in mitochondria from mtDNA mutator mice (Figure 3D). Furthermore, we detected a higher turnover of mtDNA-encoded subunits of complex I, III, and IV, whereas subunits of complex V (ATP6 and ATP8) had turnover rate comparable to the one observed in wild-type mice (Figure 3E). This finding is consistent with the proposal that mtDNA mutations lead to the expression of mtDNA-encoded respiratory chain subunits with amino acid substitutions and that these subunits are incorrectly folded and thereby prevent proper assembly of the respiratory chain complexes. Improperly assembled respiratory complexes will be unstable and subjected to an increased mitochondrial protein turnover, as detected in liver mitochondria from mtDNA mutator mice (Figures 3D and 3E). Increased protein turnover despite normal mitochondrial protein synthesis has been described in cells lacking cyt *c* (Vempati et al., 2009) as well as in cells lacking COX 10 causing impaired complex IV assembly (Li et al., 2007). Surprisingly, in both of these cases increased protein turnover was observed also for subunits of other complexes despite the fact that the genetic defect was specific for only one complex, which indicates interdependence of the respiratory complex stability (Li et al., 2007; Vempati et al., 2009).

## Lack of Circular mtDNA Deletions in mtDNA Mutator Mice

We directly investigated the possible presence of circular mtDNA molecules with large deletions in mutator mice by performing long-template PCR analysis with three different primer pairs (Figures 4A–4D). All three primer pairs were designed to amplify mtDNA molecules with deletions in the major arc of mtDNA, between nucleotide 8,800–13,100, of mtDNA, which is the region most commonly affected by deletions. As a positive control, we analyzed mtDNA from transgenic mice engineered

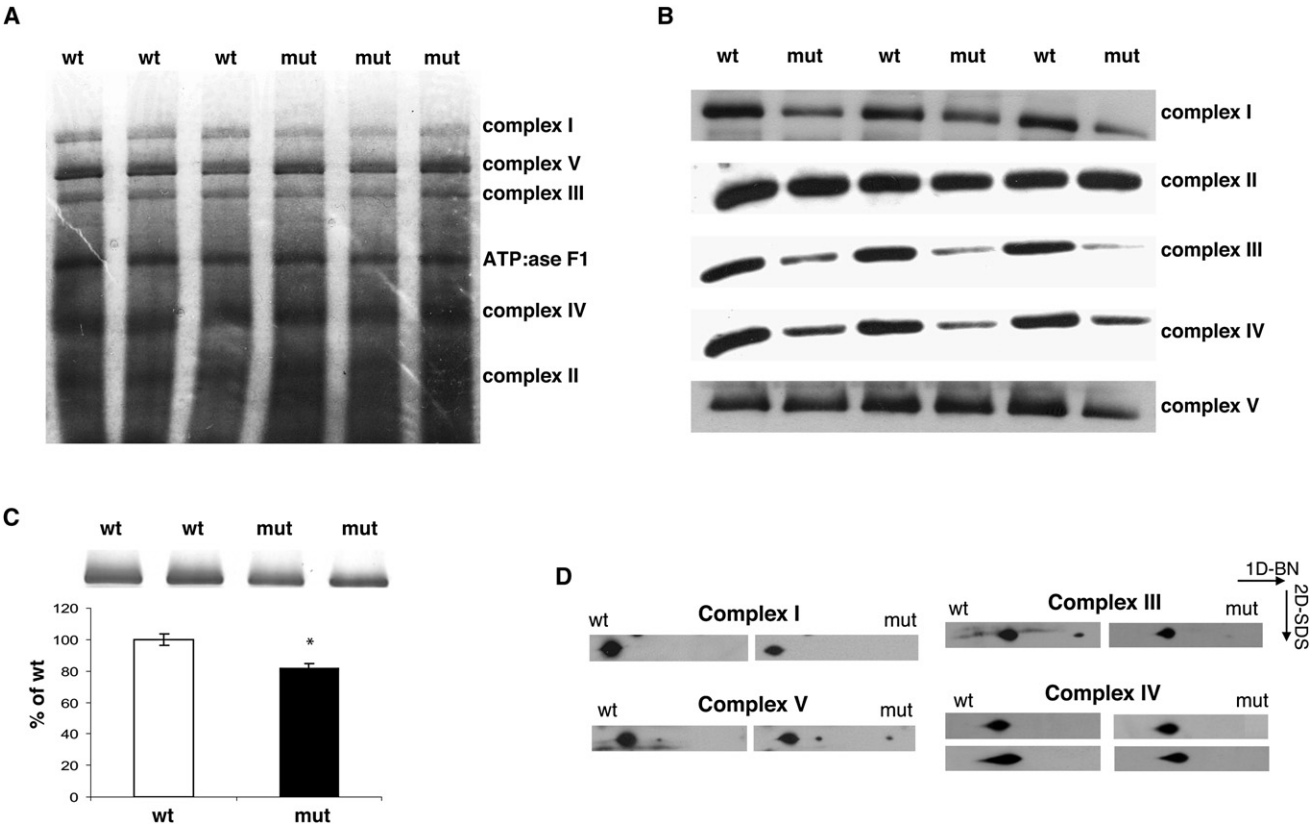


**Figure 2. Steady-State Levels of Respiratory Chain Complexes in Liver Mitochondria of 25-Week-Old WT and mtDNA Mutator Mice (mut)**

(A) Coomassie brilliant blue stained BN-PAGE.

(B) Western blots performed after BN-PAGE.

(C) In-gel activity of complex I. Bars indicate mean levels ± SEM. Asterisks indicate level of statistical significance (*$p < 0.05$, Student's t test) ($n = 2$).

(D) Western blot performed after 2D gel electrophoresis (BN-PAGE/SDS-PAGE).

to harbour mtDNA deletions (Inoue et al., 2000; Tyynismaa et al., 2005). Analyses of mtDNA from control mice showed that both single and multiple mtDNA deletions were robustly detected by our assays (Figures 4B and 4C). Furthermore, by analyzing serial dilution of DNA from these positive control mice, we determined that the assay was very sensitive and detected deleted mtDNA present at a fraction of 0.1% of the total mtDNA (Figures 4B and 4C). Using this sensitive assay, we could detect only full-length mtDNA in both wild-type and mtDNA-mutator mice (Figures 4A–4C), and we therefore conclude that circular mtDNA molecules with large deletions either are absent or present at extremely low levels in mtDNA mutator mice.

## DISCUSSION

In the present study, we have performed molecular analyses to determine the mechanism whereby mtDNA mutations cause respiratory chain deficiency in mtDNA mutator mice. We report that the steady-state levels of mitochondrial transcripts are in general increased and that mitochondrial translation is unimpaired in mtDNA mutator mice. These results strongly argue against any role for large mtDNA deletions in generating the observed respiratory chain deficiency in mtDNA mutator mice. Others have suggested that large circular deletions mapping in the middle region of mtDNA major arc drive premature aging phenotypes in mtDNA mutator mice (Vermulst et al., 2008). Southern blot analysis, a method commonly used to quantify the levels of large mtDNA deletions, with the sensitivity of around 5% of deleted molecules, did not shown the existence of such deletions in 40-week-old mtDNA mutator mice (Trifunovic et al., 2004). Here, we have aimed to detect large mtDNA deletions by using a very sensitive long extension PCR assay. Although this method is not quantitative, we have shown that large single or multiple deletions can be qualitatively detected if present at levels above 0.1% of total mtDNA and are preferentially amplified if present at levels above 5%. However, no circular mtDNA deletions were detected when we analyzed DNA from liver and heart in 40-week-old mtDNA mutator mice. These results thus show that circular mtDNA molecules with large deletions represent only a minor proportion of the total mtDNA in mtDNA mutator mice.

We further show that the assembly and stability of the respiratory chain complexes is severely impaired despite normal mitochondrial protein synthesis. Our results thus show that the observed decrease in steady-state levels of complex I, III, and IV in mtDNA mutator mice are a direct consequence of high levels of mtDNA point mutations that lead to increased turnover of defective protein subunits. It is well known that the assembly

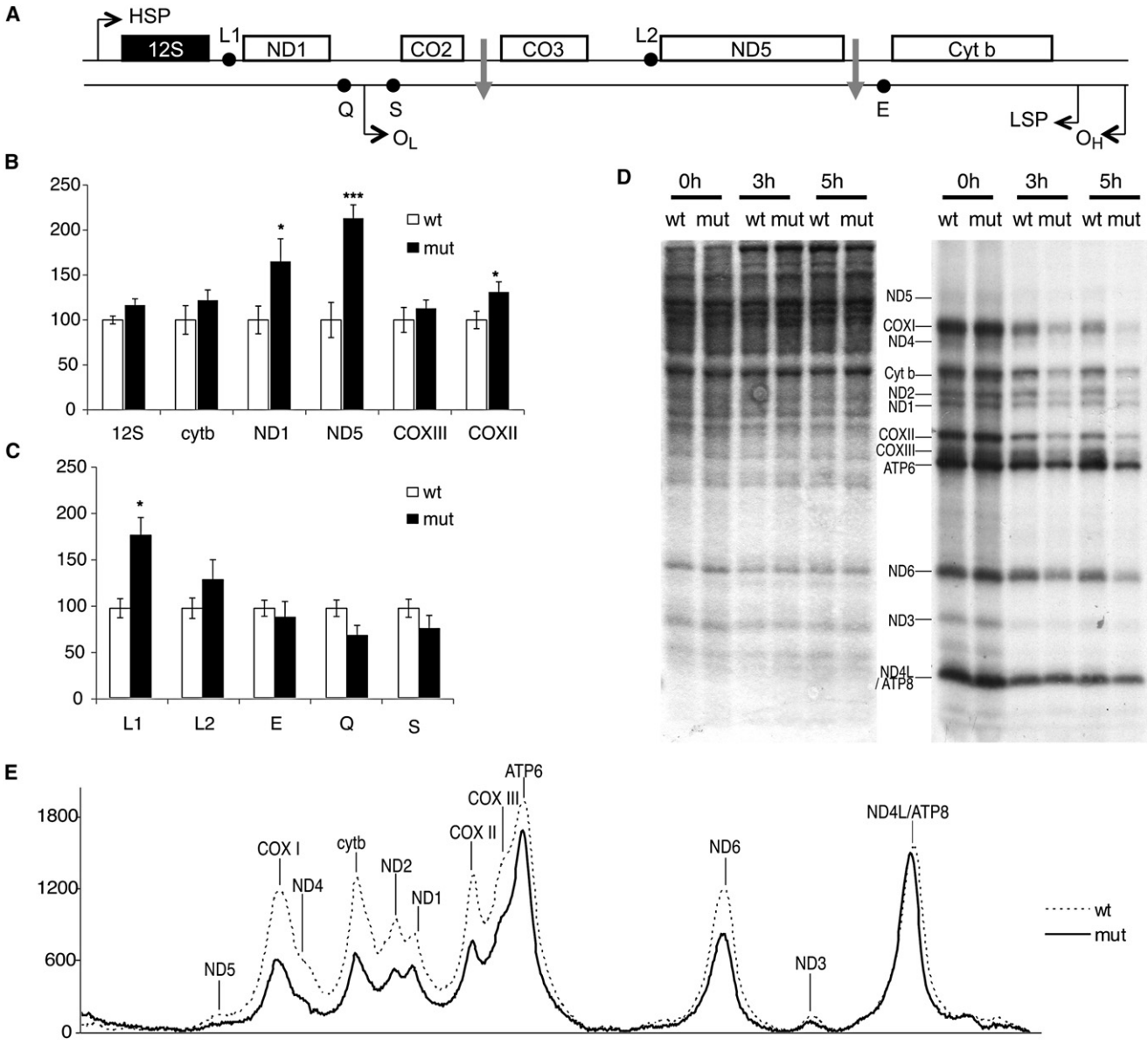


**Figure 3. Transcription and Translation in Liver Mitochondria of 25-Week-Old WT and mtDNA Mutator Mice (mut)**

(A) Map indicating analyzed mitochondrial transcripts and tRNAs: tRNA-leu-(UUR) (L1), tRNA-leu-(CUN) (L2), tRNA-ser (S), tRNA-glu (E) and tRNA-gln (Q). LSP and HSP, and origins of light ($O_L$) and heavy strand ($O_H$) replications are indicated. Gray arrows represent position of the common mtDNA deletions.

(B and C) Northern blot analysis of steady-state levels of (B) mitochondrial transcripts and (C) tRNA levels. Bars indicate mean levels ± SEM. Asterisks indicate level of statistical significance (*$p < 0.05$, ***$p < 0.001$, Student's t test) (n = 5).

(D) Representative gel of in organello translation (n = 4). De novo synthesized proteins are isolated immediately after labeling with $^{35}$S-met (0 hr) or after 3 hr and 5 hr of pulse chase. Coomassie brilliant blue staining of the gel to indicate equal loading of proteins.

(E) Densitometric analysis of de novo synthesized proteins on the representative gel after 3 hr of pulse chase.

of complexes I, III, and IV of the respiratory chain is critically dependent on mtDNA-encoded core subunits (Lamantea et al., 2002; Saraste, 1990; Ugalde et al., 2003). In addition, a decrease in the assembly of complex III or complex IV may directly interfere with the stability of complex I in mtDNA mutator mice by disrupting supercomplex formation. The interdependence of respiratory complexes was first demonstrated when it was shown that assembled complex III is required to stabilize complex I in mammalian cells (Acin-Perez et al., 2004). Furthermore, our results are consistent with our previous observations that some respiratory subunits are more stable (UQCRC2) than others (COX II and COX IV) and sometimes even can accumulate (α-subunit of ATP synthase) despite low levels of repiratory chain complexes (Metodiev et al., 2009; Park et al., 2007). In case of the tissue-specific MTERF3 knockout mice with progressive respiratory chain deficiency in the heart, the levels of COX II

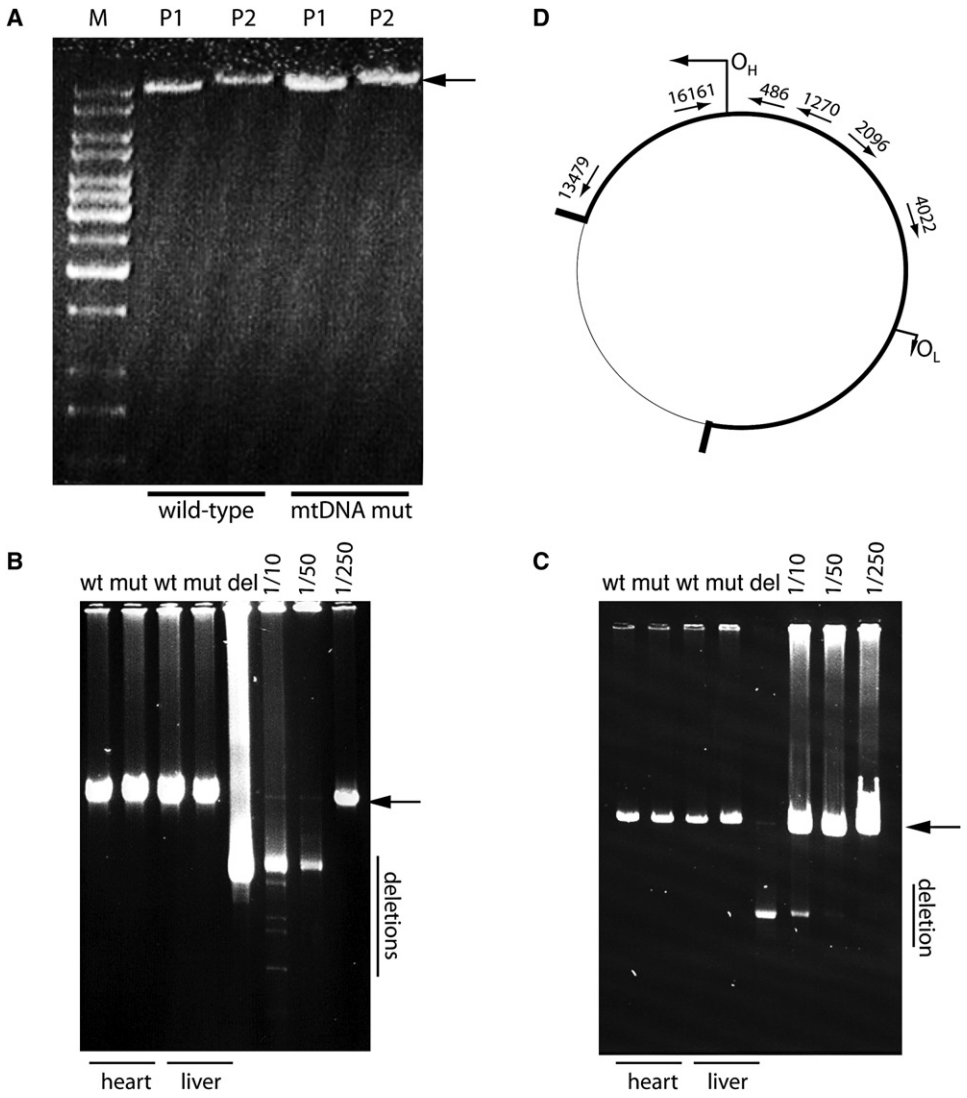


**Figure 4. LX-PCR Analysis of mtDNA from Heart and Liver**

(A) Long-extension PCR (LX-PCR) of heart from 40-week-old mtDNA mutator mice using P1 and P2 primer pairs.

(B) LX-PCR of heart and liver from 40-week-old wild-type (wt) and mtDNA mutator mice (mut) using primers at positions 1270–2069 of mouse mtDNA. Total DNA isolated from skeletal muscle of mice overexpressing mutated form of Twinkle protein (deletor mice) was used as a control (del). Serial dilutions (10×, 50×, and 250×) of deletor mice DNA with DNA isolated from liver of wild-type mice are indicated in the figure. Position of the deleted molecules and full-length mtDNA product (arrow) are indicated on the gel.

(C) The same as in (B), except that total DNA isolated from liver of mice carrying a single large mtDNA deletion (mito-mice) was used as a control (del).

(D) The map of mouse mtDNA with the location of commonly deleted region indicated by the thin line. Arrows indicate the position of the oligonucleotides used.

and COX IV are very low at the final stage, whereas the UQCRC2 levels are basically unchanged despite the fact that assembled complex III cannot be detected (Park et al., 2007).

Our data suggest that the random accumulation of point mutations in mtDNA mutator mice has a deleterious impact predominantly on the protein-coding genes. We have recently followed the maternal transmission of a random set of point mutations generated by mtDNA mutator mice. We found clear evidence for strong purifying selection against nonsynonymous mutations that would change amino acids in respiratory chain subunits, whereas tRNA and rRNA mutations seemed to have less severe functional consequences (Stewart et al., 2008). In patients with mitochondrial diseases, mutations in tRNA genes impairing translation are much more common than point mutations in protein-coding genes of mtDNA, consistent with purifying selection against amino acid substitutions in the mammalian germ line. These data lend additional support to our conclusion that point mutations causing amino acid substitutions are the most deleterious for respiratory chain dysfunction.

In summary, our data demonstrate that accumulation of point mutations of mtDNA causing amino acid substitutions in respiratory chain subunits is the most plausible explanation for the observed respiratory chain deficiency driving the premature aging phenotype in mtDNA mutator mice.

## EXPERIMENTAL PROCEDURES

### Animals

Mice heterozygous for mtDNA mutator allele (+/*PolgA*$^{mut}$) were backcrossed to C57Bl/6 mice for six generations. MtDNA mutator animals were obtained after intercrossing mice heterozygous for *PolgA*$^{mut}$ allele and genotyped as previously described (Trifunovic et al., 2004).

### Isolation of Mitochondria and Oxygen Consumption Measurements

Mitochondria were isolated from 25-week-old mtDNA mutator and wild-type mice and oxygen consumption rates were measured as previously described (Shabalina et al., 2006). Mitochondrial oxidative phosphorylation was measured with 0.5 mg of liver mitochondria or 0.25 mg of heart mitochondria, with addition of 450 μM ADP, 3 μg/ml oligomycin, and 2 μM FCCP. Electron transport activities were measured in 0.3 mg liver mitochondria or 0.1 mg of heart mitochondria, with addition of 10 μg alamethicin, 450 μM NADH and 2 μM cytochrome *c*.

### Western and Northern Blot Analyses

Western and northern blot analysis were performed as described (Park et al., 2007). Primary antibodies used in the following experiments: porin (Calbiochem) and ATP5a1-CV, COX IV-CIV, UQCRC2-CIII, SDHA-CII, NDUFA9-CI (Invitrogen), and polyclonal rabbit antisera raised against COX II-CIV. Antibodies used for 2D gels were raised against NDUFS3-CI, UQCRC2-CIII (Invitrogen), and COXVa-complex IV (MitoScience). All antibodies were used in 1:2000 dilutions, except Anti-SDHA that was used in 1:20,000 dilution.

### Blue Native PAGE and 2D Electrophoresis

Blue Native PAGE (BN-PAGE) and 2D gel electrophoresis were performed as described (Nijtmans et al., 2002).

### In-Gel Complex I Activity

Complex I activity was measured by incubating BN-PAGE gel in NADH 0.1 mg/ml nitrotetrazolium blue (NTB) 2.5 mg/ml in 2 mM Tris (pH 7.4) for 1 hr.

### In Organello Translation

In organello translation was performed as previously described (Cote et al., 1989) with some modifications. Mitochondria were incubated in translation buffer containing 60 mg/ml of each amino acid, minus methionine, with addition of 0.5 mCi/ml of L-[35S]-Methionine (GE Healthcare). Translation was allowed to proceed for 60 min at 37°C. After this period, mitochondrial pellets are either resuspended in SDS-PAGE loading buffer or were washed and incubated 3 or 5 hr in translation buffer containing all amino acids, including methionine.

### Long-Extension PCR

Mouse mtDNA was amplified from 250 ng of total DNA with the primers P1—primers 486–4022, P2—primers 13,479–16,161, and P3—1270–2069 of mouse mtDNA (Expand Long Template PCR System, Roche) by using buffer 2 and PCR conditions 92°C for 10 s, 57°C for 30 s, and 68°C for 10 min, 25 cycles.

## SUPPLEMENTAL DATA

The Supplemental Data include three figures and can be found with this article online at http://www.cell.com/cellmetabolism/supplemental/S1550-4131(09)00191-0.

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