Decreased Reactive Oxygen Species Production in Cells with Mitochondrial Haplogroups Associated with Longevity

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Abstract
Mitochondrial DNA (mtDNA) is highly polymorphic, and its variations in humans may contribute to individual differences in function. Zhang and colleagues found a strikingly higher frequency of a C150T transition in the D-loop of mtDNA from centenarians and twins of an Italian population, and also demonstrated that this base substitution causes a remodeling of the mtDNA 151 replication origin in human leukocytes and fibroblasts [1]. The C150T transition is a polymorphism associated with several haplogroups. To determine whether haplogroups that carry the C150T transition display any phenotype that may be advantageous for longevity, we analyzed cybrids carrying or not the C150T transition. These cybrids were obtained by fusing cytoplasts derived from human fibroblasts with human mtDNA-less cells (ϕ0 cells). We chose for cybrid construction and analysis haplogroup-matched pairs of fibroblast strains containing or not the C150T transition. In particular, we used, as one pair of mtDNA donors, a fibroblast strain of the U3a haplogroup, carrying the C150T transition and a strain of the U-K2 haplogroup, without the C150T transition, and as another pair, fibroblasts of the J2b haplogroup, carrying the C150T transition and of the J1c haplogroup, without the C150T transition. We have found no association of respiratory capacity, mtDNA level, mitochondrial gene expression level, or growth rate with the presence of the C150T transition. However, we have found that the cybrids with haplogroups that include the C150T transition have in common a lower reactive oxygen species (ROS) production rate than the haplogroup-matched cybrids without that transition. Thus, the lower ROS production rate may be a factor in the increased longevity associated with the U and the J2 haplogroups. Of further interest, we found that cybrids with the U3a haplogroup exhibited a higher respiration rate than the other cybrids examined.

Introduction
A C150T transition in mitochondrial DNA (mtDNA) was found to occur more frequently in centenarians and in twins of an Italian population [1]. The C150T base substitution, which is located in the mtDNA D-loop region and causes a remodeling of the mtDNA 151 replication origin in leukocytes, was found to be homoplasmic in about half of the leukocyte samples in which the base substitution was observed. Because of this homoplasy and because the C150T transition is a commonly occurring polymorphism, it is likely in these cases that the base substitution is an inherited polymorphism rather than a somatically acquired mutation.

In fact, the C150T polymorphism is associated with several haplogroups or subhaplogroups, including J2, D5, M7b, T2, U3, U5, and N9a [2]. Furthermore, observations of associations between haplogroup and longevity have been reported. For instance, DeBenedictis and colleagues found a higher frequency of the J haplogroup in healthy older men from northern Italy [3]. Similarly, Niemi and colleagues found that the haplogroup frequencies in a sampling of very old individuals (vitality 90+) in Finland differed from those in the middle-aged controls, haplogroups U, K (U-K) and J being more prevalent among the old individuals [4]. The subhaplogroups of U were unspecified in that study. More recently, in Finnish and Japanese subjects, 150T and two additional common polymorphisms, 10398G and 489C, of all of three of which occur in the J2, D5, and M7b haplogroups, were associated with longevity [5]. The C150T transition was not associated with longevity in the U5, T2, and N9a haplogroups.

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found to be associated with mtDNA coding region polymorphisms such as 5178A, which defines haplogroup D, occurring in Asian individuals, and 9055A, which defines haplogroup K [6] [7,8]. However, conflicting associations with regard to the 5178A polymorphism have been reported [9]. The aforementioned associations are based on population genetics. The consequences on mitochondrial function of these longevity-associated polymorphisms, if any, are unknown. At present there have been few studies that identify functional effects of DNA polymorphisms [10–15].

In order to understand the effect of the C150T transition or of its associated haplogroup polymorphisms, we have analyzed cybrids obtained by fusing cytoplasts derived from human fibroblasts carrying or not the C150T transition with human mtDNA-less cells (ρ0 cells) derived from an osteosarcoma cell line [14]. In particular, we found, among the fibroblast strains used in the previous study by Zhang and colleagues [1], two pairs that were matched in haplogroup but differed at position 150 (Table 1).

The first pair was of the U haplogroup. One fibroblast strain was of the U3a subhaplogroup and had C at position 150. The other pair of fibroblasts strains was of the J2b (C150T) haplogroup. The members of this pair were of the J1c (150C) subhaplogroups. Hereofore we will refer to the latter as J1c (150C) subhaplogroups. Heretofore we will refer to the two pairs that were matched in haplogroup but differed at position 150 (Table 1).

The first pair was of the U haplogroup. One fibroblast strain was of the U3a subhaplogroup and had C at position 150. The other pair of fibroblasts strains was of the J haplogroup. The members of this pair were of the J2b (C150T) and J1c (150C) subhaplogroups. Hereofore we will refer to subhaplogroups as haplogroups. These fibroblast strains served as mtDNA donors in the construction of cybrids. The use of cybrids made with the ρ0 cell line allows us to observe the effects of mitochondrial polymorphisms without the confounding effects of the varying nuclear backgrounds.

We have searched for phenotype differences between cybrids of different haplogroups. We have analyzed growth rate, respiratory rate, mitochondrial protein synthesis rate, mtDNA level, steady state level of components of the respiratory chain and reactive oxygen species (ROS) production rate. We have found that the one parameter that correlates with the presence of the C150T transition is a lower ROS production rate. ROS have long been thought to play a role in aging [15]. Thus, longevity that is associated with particular haplogroups may be at least partly explained by the relative level of ROS production that is allowed or specified by those haplogroups.

### Materials and Methods

#### Cell line and culture conditions

The following human fibroblast strains, obtained from the American Type Culture Collection and listed in Table 1, were used as donor cells for introducing mitochondria and mtDNA into mtDNA- less (ρ0) cells: AG07309 (150C, LS9-1 in Michikawa et al. [16], AG14421 (150C, LS10-2), AG07309 (150T, LS11-1), AG13152 (150C, LS4-2).

The fibroblasts were grown in high glucose Dulbecco’s modified Eagle’s Medium (DMEM; containing pyruvate, Gibco), supplemented with 10% fetal bovine serum (FBS). The mitDNA-less ρ0 143B.206 cell line [14], derived from the osteosarcoma cell line 143B.TK−, was grown in DMEM supplemented with 10% FBS and 50 μg of uridine per ml. All cybrid cell lines were maintained in the same medium as the fibroblast cell lines.

#### Mitochondria-mediated transformation

Transformation of mtDNA-less ρ0 206 cells was carried out as described by King and Attardi [14], by fusing human fibroblast cells, which had been enucleated by centrifugation in the presence of cytochalasin B, with ρ0 206 cells in the presence of 40% polyethylene glycol 1500 (PEG, BDH). Mitochondrial transformants were isolated in medium lacking pyruvate and uridine, specifically DMEM without pyruvate supplemented with 5% dialyzed FBS. 100 μg/ml BrdU was included in the medium to select against hybrids and unenucleated donor cells and for cybrids carrying the thymidine kinase deficiency marker of the ρ0 nucleus.

#### Growth measurements

All cybrid cell lines were grown in DMEM medium with 10% FBS for seven days before the growth rate experiment was done. Cells were then plated on multiple 10-cm plates at 105 per plate and were counted daily for 7 days. The population doubling time (DT) of the cell lines in DMEM medium, supplemented with 10% dialyzed FBS, was determined from growth curves or by using the formula: DT = (t – 0)log2/logN0 − logN0, where t and 0 are the times at which the cells were counted, and N0 and Nt are the cell numbers at times t and 0, respectively.

#### Analysis of mitochondrial protein synthesis

Pulse-labeling of the cell lines for 45 min with [35S]methionine–[35S]cysteine (ExPress 35S35S, Perkin Elmer) in methionine-free DMEM in the presence of 100 μg/ml emetine and electrophoretic analysis of the translation products were carried out as detailed previously [17]. The dried gel was exposed to a PhosphorImager screen. Quantification of radioactivity in the entire lane of the gel or in individual well-resolved bands was done using ImageQuant software.

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**Table 1. Fibroblast strains and their haplogroups.**

| Fibroblast strain | Name in Zhang et al., 2003 | nucleotide at pos. 150 | Haplogroup | Cybrids |
|-------------------|---------------------------|-----------------------|-------------|--------|
| AG07309           | LS11-1                    | T                     | U3a         | TF3A5  |
|                   |                           |                       |             | TF7G11 |
|                   |                           |                       |             | TF2D9  |
|                   |                           |                       |             | TF4G2  |
|                   |                           |                       |             | TF5A12 |
|                   |                           |                       |             | TF11H2 |
| AG13152           | LS4-2                     | C                     | U-K2        | E8     |
|                   |                           |                       |             | H5     |
|                   |                           |                       |             | G11    |
|                   |                           |                       |             | H9     |
|                   |                           |                       |             | T8     |
|                   |                           |                       |             | E5     |
| AG07135           | LS9-1                     | T                     | J2b         | F8     |
|                   |                           |                       |             | F1     |
|                   |                           |                       |             | P2     |
|                   |                           |                       |             | P1     |
|                   |                           |                       |             | P2P1   |
|                   |                           |                       |             | B3     |
| AG14421           | LS10-2                    | C                     | J1c         | TFA24  |
|                   |                           |                       |             | TFA15  |
|                   |                           |                       |             | TFA7   |
|                   |                           |                       |             | TFA12  |
|                   |                           |                       |             | TFA16  |

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O$_2$ consumption measurements

Rates of O$_2$ consumption in intact cells at 37°C were measured polarographically by using a Clark-type oxygen electrode connected to a computer-operated Oxycalograph control unit (Hansatech Instruments, Norfolk, England). 1–2×10$^5$ cells were suspended in 1 ml of Tris-based, Mg$^{2+}$/Ca$^{2+}$-deficient (TD) buffer (0.137 M NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$, and 25 mM Tris-HCl [pH 7.4] at 25°C) for the analysis, as previously described [14]. Polarographic analysis of digitonin-permeabilized cells, using respiratory substrates and inhibitors to test the activity of the individual respiratory complexes, was carried out as detailed previously [18].

Membrane potential analysis

Cells were stained with 10 nM tetramethylrhodamine ethylester perchlorate (TMRE, Molecular Probes) in DMEM without serum for 45 minutes. Then cells were harvested by trypsinization, resuspended in PBS with BSA (2.5 mg/ml) containing 10 nM TMRE, and analyzed by flow cytometry in a FACSCalibur analyzer (BioRad).

Determination of ROS generation

The intracellular ROS levels of cybrid cells were determined using CM-H$_2$DCFDA (Molecular Probes). For these experiments, cells were collected and incubated with phosphate-buffered saline (PBS) and 1 mM CM-H$_2$DCFDA. After a 30 min incubation period at 37°C, cells were centrifuged and resuspended in PBS. Measurements at 1 min intervals of the levels of fluorescence were begun immediately using Molecular Devices fluorescence plate reader and were continued for 1 h (excitation 485 nm and emission 538 nm). The rates of ROS generation were derived from the data.

Mitochondrial DNA analysis

a. Allele-specific termination of primer extension for identifying cells with the C150T transition has been described previously [1]. Briefly, mtDNA fragment Init-Tra-Rep [16] was amplified from total cellular DNA. To detect the C150T polymorphism, primer extension was carried out with the primer 5′-GCAG-TATCTGTCTTTGATTCCTGC CTC (positions 121–147 in the Cambridge sequence, [19,20]) in the presence of dA, dT, and ddC.

b. Restriction fragment length polymorphism for determining mtDNA haplogroups. The mtDNA sequence was amplified by PCR using the primer pairs and amplification conditions described by Torroni et al. [21]. The PCR segments were digested with specific restriction endonucleases to distinguish haplogroups U, U-K, H, I, and J [21].

c. Total DNA sequencing: Total DNA from cybrid cell lines was extracted by using standard procedures. The complete mtDNA was amplified in 22 overlapping PCR fragments by the use of sets of the light-strand and the heavy strand oligonucleotide primers, listed in Table S1 [22]. Each PCR product was purified and subsequently submitted for sequence analysis. Sequencing was from M13 forward and M13 reverse primers, the sequences of which were incorporated in the PCR primers. The resultant sequence data were compared with the updated consensus Cambridge sequence of mtDNA (GenBank Accession No.: NC_012920) [20]. Identifications of polymorphisms as haplogroup- and subhaplogroup-specific are based on a published comprehensive phylogenetic tree [2]. The single nucleotide polymorphism database of MitoMap (http://www.mitomap.org) [23] and the comprehensive phylogenetic tree (http://www.phylotree.org) [2] were searched to determine whether the non-subhaplogroup polymorphisms that we had found had been reported previously.

Statistical analysis

Except for the protein synthesis data, data are expressed as mean ± standard error of the mean. Statistical significance was determined by unpaired, 2-tailed t-test (http://graphpad.com/quickcalcs/ttest1.cfm).

Results

Generation of cybrid cell lines with different mtDNA haplogroups and growth properties

Fibroblast strains that were used by Zhang et al. [1] were subjected to haplogroup analysis [21]. Strains AG07309 (LS 11) and AG13152 (LS 4) were identified as belonging to Haplogroups U and U-K, respectively, and strains AG07135 (LS9) and AG14421 (LS 10) were identified as belonging to Haplogroup J. Subsequent DNA sequence analysis of the cybrids refined the haplogroup identities to U3a, U-K2, J2b, and J1c, respectively (Table 1). These four fibroblast strains were emulsified and subsequently fused to mtDNA-less human p9 cells (see Materials and methods). The cybrid clones derived from each fibroblast strain are listed in Table 1. We chose three or more transmembranoid cybrid clones from each fusion for analysis. The presence or absence of the C150T transition in each clone was verified by allele-specific primer extension [1]. Later, DNA sequencing of the mtDNA of several of the clones confirmed the base at position 150. Haplo-type-matched C150T transition and “wild type” 150C clones were then used for the biochemical characterization described below.

Figure 1 shows that the cybrid cell lines of the two haplogroups U3a and U-K2 have a nearly identical doubling time, or growth rate. Also, whereas the overall mean doubling time of J1c is greater than that of J2b, the difference is not statistically significant.

Mitochondrial protein synthesis and COXII protein level difference in two haplogroup pairs

Figure 2A shows electrophoretic patterns of the mitochondrial translation products of the cybrids. Patterns of the mtDNA-encoded polypeptides of the 150T-carrying cybrids were qualitatively identical, in terms of electrophoretic mobility of the various polypeptides, to those of the 150C-carrying cybrid cells. Figure 2B shows a quantification of the results of the labeling experiments. The mean rate of labeling of the mitochondrial translation products in U3a (C150T) cybrids was higher than the mean value measured in the U-K2 (150C) cybrid cell lines. In contrast, the labeling rate in J2b (150T) cybrids was lower than that in J1c (150C) cybrid cell lines. However, because of the variability among the cybrid clones, the difference between the U3a and the U-K2 cybrids was not significant; nor was the difference between the J2b and the J1c cybrids. In addition, Western blots revealed that the variations in the steady state levels of complex IV subunit II (COXII) among the individual cybrids reflected, for the most part, corresponding variations in the overall rate of labeling of mitochondrial translation products (data not shown).

Respiration differences between two haplogroup pairs

The respiration rate of intact cells utilizing endogenous substrates was measured by polarography. As shown in Figure 3A, the U3a (C150T) cybrids exhibited higher rates of total O$_2$ consumption, by 85%, relative to the U-K2 (150C)
cybrids. In contrast, the J2b (C150T) cybrids showed a lower level of overall respiratory capacity, by 45%, as compared to the mean value of that of the J1c (150C) cybrids. The effect of the uncoupler dinitrophenol (DNP) on endogenous respiration, as revealed by the ratios of uncoupled to native respiration, was similar in U3a and U-K2 cybrids and similar in J2b and J1c cybrids (Figure 3B). Thus, the differences in endogenous respiration rates are not due to differences in the level of proton leakage of the inner membrane.

In order to investigate differences in individual enzyme complexes of the respiratory chain, O2 consumption measurements were carried out on digitonin-permeabilized cells, using different substrates and inhibitors. As illustrated in Figure 4A, in the U3a (C150T) cybrids, the rate of malate/glutamate-driven respiration, which reflects the activity of complex I, was significantly higher, by 89%, than the average rate in the U-K2 (150C) cybrid cells. Similarly, the rate of succinate/glycerol-3-phosphate (G3P)-driven respiration, which reflects the activity of complex III, was significantly higher in U3a cybrids, by 110%, than the average rate in the U-K2 cybrids (Fig. 4B). Furthermore, the rate of N,N′,N″,N‴-tetramethyl-p-phenylenediamine (TMPD)/ascorbate-driven respiration, which reflects the maximum activity of Complex IV, was higher by 31% in U3a cybrids (Fig. 4C). J haplogroup cybrids showed the opposite trend; i.e., the J2b (C150T) cybrids showed generally lower rates than the J1c (150C) cybrids, i.e lower by ~30% for malate/glutamate-stimulated respiration; by ~30% for succinate/G3P-stimulated respiration; and by ~20% for TMPD/ascorbate-stimulated respiration (Fig. 4). The relative rates of malate/glutamate-driven respiration among the different haplogroups paralleled the rates of respiration observed in intact cells, as expected.

Membrane potential differences in two haplogroup pairs

Maintenance of mitochondrial membrane potential (ΔΨm) is necessary for production of energy (ATP), for protein import, and therefore, for the maintenance of most mitochondrial functions and the preservation of cellular homeostasis. Figure 5 shows the results of flow cytometric analysis in cybrid cells loaded with the ΔΨm indicator tetramethylrhodamine ethylester perchlorate (TMRE). The membrane potential is similar in U3a (C150T) cybrid cell lines and U-K2 (150C) cybrid cell lines. There was variability in the membrane potentials among the individual J2b (C150T) cybrid cell lines and J1c (150C) cybrid cell lines, with F8 and TFA15 cybrid cell lines showing a large deviation from the other cell lines within their respective haplogroups. The reason for this is not clear. The differences in membrane potential between haplogroups are not statistically significant.

Common ROS generation tendency between two haplogroup pairs

The analysis of ROS production showed that the mean ROS production rate of the U3a (C150T) cybrid cell lines was 26% lower than that of the U-K2 (150C) cybrid cell lines (P = 0.0356) (and lower than those of the J2b and J1c cybrid cell lines by 17%, P = 0.0142, and by 36%, P<0.0001, respectively) (Figure 6). Similarly, the mean ROS production rate of the J2b (C150T) cybrid cell lines was 23% lower than that of J1c (150C) cybrid cell lines (P<0.0001). Because ROS production rate differences may arise from differences in ROS generation rate or differences in natural ROS defenses, we investigated whether the expression of ROS defense enzymes also differ. The MnSOD protein level, as revealed by Western blotting, did not differ between the U3a and the U-K2 cybrids, nor did it differ between the J2b and the J1c cybrids (data not shown).

Mitochondrial DNA sequencing

We sequenced the entire mtDNA of TF3A5 (U3a), E8 (U-K2), T8 (U-K2), F8 (J2b) and TFA7 (J1c) cybrid cell lines. Overlapping fragments covering the entire 16.5-kb mtDNA were amplified from genomic DNA. We compared our sequences to the revised version of the Cambridge Reference Sequence. All the variants identified, except those that differ from the H2b- and individual-
specific polymorphisms present in the Cambridge Reference Sequence, are reported in Tables 2, 3, and 4. The mitochondrial genome sequences of the haplogroup cybrid cell lines differed by 33–35 nucleotide substitutions from the revised Cambridge Reference Sequence. Some of the individual-specific polymorphisms are novel (Table 4). Only the TF3A5 cybrid cell line harbors heteroplasmic nucleotide changes, specifically in complex I subunits. The sequence of the U3a haplogroup cybrid revealed that almost all the coding region polymorphisms are U3a haplogroup specific; i.e., there are few individual specific polymorphisms aside from those that specify the U3a haplogroup.

Discussion

Since the report in 2003 [1] identifying the C150T transition as a longevity marker, several hypotheses have been proposed for explaining the advantage of the C150T transition. The C150T transition alters the location of H-strand replication origin. This may affect regulation of replication and in particular, may relax copy number control, allowing more mtDNA to be synthesized [1]. Although we have found no marked difference in the level of mtDNA between several cybrids of the U3a (C150T) haplogroup and cybrids of the J1c (150C) haplogroup (data not shown), there remains the possibility of a small difference in mtDNA level. Another possibility could be that the C150T transition could enhance the immune system in some way, perhaps by slowing the turnover of memory T-cells [24]. It is also possible that it is not the
per se that confers longevity, but rather one or more of the other polymorphisms that occur together with the C150T in a particular haplogroup.

We have carried out a biochemical and molecular analysis of cybrids of four different haplogroups. We measured doubling time, respiration rate, mitochondrial protein synthesis rate, membrane potential, and reactive oxygen species (ROS) production rate. The U3a and the J2b cybrids, which carry the C150T transition, exhibited a statistically significant lower ROS production rate than their haplogroup-matched correlates, which do not carry that transition. Haplogroup U and subhaplogroup J2 have been associated with longevity in population studies [3,4]. No other parameter we measured showed a correlation with the presence of T at position 150 or with the longevity haplogroups. Thus, the lower ROS production rate is due to the T at position 150, and/or to one or more of the polymorphisms defining the haplogroup or specific to the individual.

Cybrids with haplogroup U-K2, which has C at position 150, and which has also been associated with longevity, have a lower ROS production rate, on average, than the cybrids of haplogroup J1c, but the difference is not statistically significant (P = 0.1885). Haplogroup J1 has a negative correlation with longevity [4]. Thus the U-K2-specific mtDNA polymorphisms may confer an advantage for longevity by some means other than by decreasing ROS production.

ROS generation occurs by the leakage of electrons from the respiratory chain directly to O2. Thus, one might expect that a higher respiration rate would be associated with a higher ROS production rate. The respiration rates differed markedly among the cybrid lines, with the U3a cybrids having the highest respiration rates. A high respiration rate associated with U haplogroup had been observed previously [12]. Contrary to expectation, the U3a cybrids had the lowest ROS production rates among the cybrids tested.

Our sequence analysis of cybrid mtDNA confirmed and refined the haplogroup assignment of the cybrid groups (Tables 2, 3). The U cybrid mtDNA sequence revealed that the donor’s subgroup was U3a and that the donor’s mtDNA sequence was nearly

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**Figure 3. Respiration rates.**

A. Average rates of O2 consumption in intact cells, normalized to protein concentration, in individual cybrid clones are shown. Cell lines are grouped by haplogroup. A total of 3–6 determinations were made on each of three or four cybrid clones of each haplogroup. The error bars indicate the standard error of the mean. The horizontal gray lines indicate the mean respiration rate for the haplogroup. The horizontal black bars with asterisk indicate that the difference between the indicated groups is statistically significant; *, P < 0.05; **, P < 0.01.

B. DNP-uncoupled endogenous oxygen consumption rates, normalized to protein concentration, were measured 3–6 times for each cybrid clone. The ratios of uncoupled to endogenous respiration rates are shown.

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The activities of the various components of the respiratory chain were investigated by measuring the respiration rate dependent on (A) malate plus glutamate, on (B) succinate plus G3P (in the presence of rotenone) and on (C) TMPD plus ascorbate (in the presence of antimycin). Cell lines are grouped by haplogroup. A total of 3–4 determinations were made on each of the three or four cybrid clones derived from each fibroblast strain. The mean of those determinations is shown. The error bars indicate the standard error of the mean.

**Figure 4. Analysis of O2 consumption in digitonin-permeabilized cells of the cybrid clones using different substrates and inhibitors.** The activities of the various components of the respiratory chain were investigated by measuring the respiration rate dependent on (A) malate plus glutamate, on (B) succinate plus G3P (in the presence of rotenone) and on (C) TMPD plus ascorbate (in the presence of antimycin). Cell lines are grouped by haplogroup. A total of 3–4 determinations were made on each of the three or four cybrid clones derived from each fibroblast strain. The mean of those determinations is shown. The error bars indicate the standard error of the mean. Horizontal gray lines represent the mean for each haplogroup. Horizontal black bars with asterisks indicate differences between averages are statistically significant; *, P<0.05; **, P<0.01. P = 0.0578 for the difference between the means of respiration rate of the J2b and the J1c cybrids in panel A, i.e. not quite statistically significant. doi:10.1371/journal.pone.0046473.g004

Identical to two sequences recently added to the database [25] and identified as U3a1c [2]. The mtDNA coding region polymorphisms in this cybrid were limited almost exclusively to U- and U3a-specific polymorphisms (Tables 3,4). There were no individual-specific polymorphisms in the protein-coding genes except for three sites of heteroplasmy (Table 4). Thus, if the three heteroplasmys, which occur at about 50%, are neutral, it is possible that the U3a-specific threonine to alanine substitution in ND4L and/or the U3a-specific threonine to methionine substitution in ND5 (Table 3) are important for lowered ROS production and high respiration rates. Alternatively, polymorphisms associated with the U-K2 and J1c haplogroups may be responsible for greater ROS production rates.

A polymorphism that occurs in both 150C-containing haplogroups, which may be responsible for increased ROS production rates, occurs in the cytochrome b gene. The base change at 14798 causes a leucine for phenylalanine substitution and is present in both the U-K and J1c haplogroups. The structure of bovine complex III, including that of cytochrome b has been solved [26] and the amino acids in the following discussion are conserved between cow and man. The 14798 polymorphism changes the 18th amino acid in cytochrome b from phenylalanine to leucine. The phenylalanine occurs at the end of a very short helix near the NH2-terminal end of the protein, at the inner coenzyme Q$_{10}$ (CoQ$_{10}$)-binding site (Q$_i$). It interacts with three of the carbons of ubiquinone [27]. Leucine in the place of phenylalanine would likely interact less well with CoQ and thus might destabilize CoQ binding and thereby inhibit the reduction of CoQ at the Q$_i$ site. The inhibition of the Q cycle would increase the steady state concentration of the radical semiquinone at the outer coenzyme Q$_{10}$ (CoQ$_{10}$)-binding site (Q$_{oi}$), which would lead to ROS generation.

The J2 haplogroup has another cytochrome b non-synonymous cytochrome b mutation, namely that at 15257. This base change substitutes an asparagine for aspartate at the 171st amino acid position. This is near the Rieske iron-sulfur subunit in the complex and may be involved in stabilizing the interaction of cytochrome b with the iron-sulfur protein [28]. An amino acid substitution here could affect the rate of oxidation of CoQ$_{10}$ at Q$_i$ [29]. On the other hand, Fig. 4 shows that complex III-dependent respiration rates are not specifically lower in the J2 cybrids. The J2b-specific polymorphism at 15812 causes a methionine for valine substitution at amino acid 356. The valine is not conserved between phyla and may be involved in stabilizing the interaction of cytochrome b with the iron-sulfur protein [29]. The somewhat bulkier phenylalanine may cause a displacement of one or both helices to accommodate the phenylalanine. However, this amino acid is not directly involved in binding substrate or ligand, thus, the substitution of leucine with phenylalanine may not have any effect on the function of the complex.

Regarding the U-K2 cybrids, there are non-synonymous protein coding polymorphisms besides the one at 14798 that might account for its phenotype differences from the U3a cybrids. These are in the genes for ATP synthase subunit 6 (UK-specific) and ND2 (U-K2a-specific).

Also in the J1c cybrids there are individual-specific non-synonymous changes in the genes for ND2 and cytochrome b. The polymorphism at 15740 causes a phenylalanine for leucine substitution at the 332nd amino acid in cytochrome b. This amino acid occurs in a helix that is in close proximity to another helix. The somewhat bulkier phenylalanine may cause a displacement of one or both helices to accommodate the phenylalanine. However, this amino acid is not involved in binding substrate or ligand, thus, the substitution of leucine with phenylalanine may not have any effect.

There were also Control Region polymorphisms: some were haplotype-specific, and some, individual specific (Table 2). In the U and U-K cybrids, the individual-specific polymorphisms were in either Hypervariable segment 1 (C16301T and T16356G, present in the U3a individual) or in Hypervariable segment 2 (G64T, present in the U-K2 individual). Nucleotides 16301 and 16356 are...
located inside the D-loop region, i.e. the portion of mtDNA that is frequently replicated in an abortive manner to yield 7S DNA \[30,31\]. The C16301T and T16356C polymorphisms have been observed several times, as individual-specific polymorphisms, within several haplogroups, including M, N, and D [2]. The C64T polymorphism is 100 base pairs downstream of the two or three most commonly used origins of H-strand replication [32,33] and 7 base pairs upstream of the origin that is used by cells recovering from mtDNA depletion [31]. The C64T polymorphism has been reported many times [23]. This polymorphism has been observed also in several haplogroup backgrounds, as a branch-specified polymorphism (in haplogroups A2 and R0) or as an individual-specific polymorphism (in haplogroups F1, H1, H57 and K1) [2]. It is conceivable that this polymorphism, C64T, or the other two Control Region polymorphisms, C16301T and T16356C, could affect mtDNA replication and alter the mtDNA copy level. However, a change in mtDNA level would be expected to result in a change in protein synthesis rates, but Figure 2 shows that protein synthesis rates in the U3a and the U-K2 cybrids are not different at a statistically significant level.

The J2b and J1c cybrids also differ in the Control Region at several nucleotide positions. Two of them, C462T (J1-specific) and T482C (J1c1-specific) are not far from the L-strand promoter and thus could conceivably affect the production of transcripts of the L-strand, or, because L-strand transcription plays a role in mtDNA synthesis, DNA replication could be affected. However we could not detect statistically significant differences in protein synthesis rates (Fig. 2), suggesting that transcript levels are not

![Figure 5. Membrane potential.](image1)

Cybrid cell lines were stained with the fluorescent membrane potential indicator dye TMRE. The fluorescence was quantified by flow cytometry. A total of 2–3 determinations were made on each cybrid cell line and the mean values are shown. The error bars indicate the standard error of the mean. Gray bars represent the average for each haplogroup. Cell lines are grouped by haplogroup. No difference between any two groups of cybrids is statistically significant. doi:10.1371/journal.pone.0046473.g005

![Figure 6. ROS generation rate.](image2)

Cells of each cybrid clone were stained with the ROS-activated dye CM-H2DCFDA. Fluorescence data were collected on a Molecular Devices fluorescence plate reader for 1 h. A total of 2–5 rate determinations were made on each cybrid clone. Each bar represents the average fluorescence production rate for the indicated cybrid clone. The error bars indicate the standard error of the mean. The haplogroup is indicated below each group of cybrid clones. Horizontal gray lines represent the average for each haplogroup. Horizontal black bars with asterisks indicate differences between averages are statistically significant, as determined by a two-tailed unpaired t-test; *, P<0.05; **, P<0.01. doi:10.1371/journal.pone.0046473.g006
significantly different between cybrids of the two different haplogroups.

Another Control Region polymorphism occurs in both the J1c and J2b cybrids, namely the C295T transition. This polymorphism occurs in one of the two binding sites for the mitochondrial transcription factor, Tfam, that occur downstream of the L-strand promoter. Suissa and colleagues [13] have demonstrated that a DNA fragment that contains T at that position binds Tfam more tightly and is transcribed at a greater rate in vitro than the control DNA. Furthermore, they found that cybrids carrying the J haplogroup had substantially higher mtDNA levels than (but

### Table 2. Control Region Polymorphisms in Cybrid Cell Lines.

| TF3A5   | E8, T8 | F8      | TFA7   |
|---------|--------|---------|--------|
| C150T   | U3     | T16126C J | T16126C J |
| A16343G | U3     | T16224C J | C295T  J |
| G16390A | U3a    | T16311C J | T489C J |
| T16519C | U3a    | T146C  J2 | C16069T J |
| C16656C | U3a1c  | T152C  J2 | C462T J1 |
| C16301T | C64T   | T152C  J2 | G185A J1c |
|         |        |         | T16193T J2b |
|         |        |         | G228A J1c |
|         |        |         | T482C J1c1 |

The table lists all the Control Region polymorphisms found in the sequences of the mtDNAs of cybrids TF3A5, E8, T8, F8, and TFA7. The haplogroup and subhaplogroup with which the polymorphism is associated is indicated. Most of these Control Region polymorphisms are haplogroup-specific. doi:10.1371/journal.pone.0046473.t002

### Table 3. Coding Region Polymorphisms, Haplogroup-specific.

| TF3A5 | E8, T8 | F8      | TFA7   |
|-------|--------|---------|--------|
| 11467 | syn ND4 | U       | 4216   | Y-H ND1 | R2+JT |
| 12308 | tRNA Leu | U       | 11251  | syn ND4 | JT     |
| 12372 | syn ND5 | U       | 15452  | L-I CYTB | JT    |
| 1811  | A-G 165 | Ubr     | 10398  | T-A ND3 | J       |
| 14139 | syn ND5 | U3      | 12612  | syn ND5 | J       |
| 15454 | syn CYTB | U3   | 13708  | A-T ND5 | J       |
| 4703  | syn ND2 | U3a     | 7476   | tRNA ser | J2   |
| 6518  | syn COI | U3a     | 15257  | D-N CYTB | J2   |
| 9266  | syn COIII | U3a | 5633   | tRNA ala | J2b |
| 10506 | T-A ND4L | U3a    | 15812  | V-M CYTB | J2b |
| 13934 | T-M ND5 | U3a     | 10172  | syn ND3 | J2b1 |
| 2294  | A-G 165 | U3a     | 3010   | G-A 165 | J1     |
| 3010  | G-A 165 | U3a1    | 14798  | F-L CYTB | J1c    |
| 9698  | syn COIII | U8  | 3394   | Y-H ND1 | J1c1  |
| 9055  | A-T ATP6 | UK/U8b | 7184   | syn COI | J1c1b |
| 14167 | syn ND6 | UK/U8b |       |        |       |
| 3480  | syn ND1 | UK/U8b |       |        |       |
| 10550 | syn ND4L | UK   |       |        |       |
| 11299 | syn ND4 | UK     |       |        |       |
| 14798 | F-L CYTB | UK   |       |        |       |
| 9716  | syn COIII | K2  |       |        |       |
| 709   | G-A 125 | K2a    |       |        |       |
| 4561  | V-A ND2 | K2a    |       |        |       |

The table lists all the haplogroup- and subhaplogroup-specifying coding region polymorphisms found in the sequences of the mtDNAs of cybrids TF3A5, E8, T8, F8, and TFA7. The first column in each triplet indicates the nucleotide position of the polymorphism, the second column indicates the gene name and the nucleotide change (in the 16S or 12S rRNA genes) or the amino acid change or syn for synonymous substitution (in the protein coding genes). Polymorphisms that cause an amino acid substitution are in boldface. doi:10.1371/journal.pone.0046473.t003

### Table 4. Individual-specific polymorphisms.

| TF3A5 | E8, T8 | F8      | TFA7   |
|-------|--------|---------|--------|
| 3644  | V-64 ND1 | A-6 G165 | 8911   |
| 4924  | S-sN ND2 |       | 10454  | tRNA arg | 2623   |
| 10704V-VH ND4L | 993 | delA 125 | 3862   |
|        |         |         | 15484  | syn CYTB |       |
|        |         |         | 15740  | F-L CYTB |       |

The table lists all the individual-specific coding region polymorphisms found in the sequences of the mtDNAs of cybrids TF3A5, E8, T8, F8, and TFA7. The first column in each doublet indicates the nucleotide position of the polymorphism, the second column indicates the gene name and the nucleotide change (in the 16S or 12S rRNA genes) or the amino acid change or syn for synonymous substitution (in the protein coding genes). Polymorphisms that cause an amino acid substitution are in boldface. New, previously unreported polymorphisms are indicated in italics. We searched two databases (http://www.phylotree.org [2];http://www.mitomap.org/MITOMAP [23]) and found these polymorphisms absent. doi:10.1371/journal.pone.0046473.t004
similar RNA levels as) cybrids carrying the H haplogroup (which has C at position 295). One possible interpretation of these data is that in the J cybrids, formation of DNA replication primers from nascent L-strand transcripts was enhanced and led to more mtDNA replication. We have compared mtDNA levels between several J3a1a cybrids (295C) and J1c cybrids (295T) and found no significant difference. This discrepancy may be explained by the presence of modifying Control Region polymorphisms occurring in our J or U cybrids or in the authors’ H cybrids.

In conclusion, our experiments identify biochemical phenotypes that may help explain how haplogroup-associated polymorphisms determine longevity. We have discussed several haplogroup-specific polymorphisms and their possible effects on respiration and ROS production. A final determination of the effect of single polymorphisms awaits the development of methods that will allow us to change a single nucleotide in the mtDNA at will.

Supporting Information

Table S1 MtDNA Sequencing Primers. (DOCX)

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Author Contributions

Conceived and designed the experiments: GA A. Chen A. Chomyn. Performed the experiments: A. Chen NR. Analyzed the data: A. Chen A. Chomyn NR. Wrote the paper: GA A. Chen A. Chomyn.

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