eLife

Reviewed Preprint
v1 • July 15, 2026
Not revised

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Competing interests: No competing interests declared

Funding: See page 15

Reviewing editor: Julia Promisel Cooper, University of Colorado Anschutz Medical Campus, United States



# Human mitochondrial DNA variants influence telomere length: evidence from a transmitochondrial cybrid model

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## eLife Assessment

This **important** study addresses a timely issue at the intersection of mitochondrial and telomere biology by focusing on the relationship between naturally occurring variants in the mitochondrial genome and telomere length. This work thereby provides a conceptual and experimental framework for investigating communication between mitochondria and telomeres. Using an innovative transmitochondrial cybrid approach, the authors provide evidence that mitochondrial DNA variants influence telomere maintenance through effects on mitochondrial function, reactive oxygen species, and $NAD^+$-dependent repair processes. The evidence supporting the central conclusion that mitochondrial genotype influences telomere-associated phenotypes is **convincing** and is strengthened by the use of complementary functional and rescue experiments. However, some of the mechanistic interpretations and broader conclusions regarding telomere length inheritance in humans would benefit from additional donors and longitudinal analyses following cybrid generation, or more cautious framing.

https://doi.org/10.7554/eLife.111391.1.sa4

## Abstract

Telomere shortening is a hallmark of aging, yet telomere length (TL) varies considerably among individuals and is strongly influenced by inheritance. In mice, efficient mitochondrial function-characterized by low reactive oxygen species (ROS) production-is critical for telomere elongation during early embryogenesis. Since mitochondrial DNA (mtDNA) encodes several subunits of the electron transport chain, it may influence TL at birth by regulating mitochondrial function *in utero*. To explore the relationship between mtDNA and TL, we used a transmitochondrial cybrid approach, introducing mitochondria from donor platelets with varying telomere lengths into mtDNA-depleted cells. This revealed an inverse correlation between donor blood TL and mitochondrial ROS levels measured in the resulting cybrids. Under the specific conditions of

cybrid formation, which involve a metabolic shift from glycolysis to oxidative phosphorylation, mtDNA variants associated with reduced complex I (CI) activity induced rapid telomere shortening, further implicating mitochondrial metabolism in TL regulation. Notably, this effect was prevented by antioxidant treatment and supplementation with $NAD^+$ precursors, supporting a critical role for CI in sustaining PARP1 activity through maintenance of the $NAD^+$/NADH balance to preserve telomere integrity under acute oxidative stress. Collectively, these findings suggest that specific mtDNA variants may contribute to the maintenance of long telomeres in humans by enhancing mitochondrial fitness, thereby highlighting potential therapeutic opportunities for mitochondrial replacement strategies.

## Significance Statement

Telomere length at birth influences aging trajectories and disease risk later in life, yet the mechanisms governing this trait remain incompletely understood. Using a transmitochondrial cybrid approach, we show that single-nucleotide variants in the mitochondrial genome of healthy donors directly affect mitochondrial metabolism and reactive oxygen species production. In addition, mitochondrial ROS levels measured in cybrids inversely correlate with blood cell telomere length in donors. Mitochondrial DNA variants associated with reduced complex I activity promote telomere shortening during an i*n vitro* metabolic shift toward oxidative phosphorylation, an effect that is reversed by antioxidant treatment or $NAD^+$ precursor supplementation. Together, these findings establish a direct link between mitochondrial genetics, redox homeostasis, and telomere maintenance in human cells.

## Introduction

Telomeres form protective caps at chromosome ends thanks to the binding of a six-member protein complex termed shelterin (1). With cell divisions, telomeres of normal somatic cells progressively shorten, a process that likely contributes to cellular aging and the development of various age-related pathologies (2,3). Accordingly, cohort studies established that shorter leucocyte telomere length (TL) was associated with a higher risk of diseases affecting specific organs, such as the lung or the liver, and an increased incidence of esophageal cancer, as well as lymphoid and myeloid leukemia (4).

While aging is a key determinant of TL, genetic variation also plays a significant role in the variability of leukocyte TL among healthy individuals, as evidenced by the identification of nuclear genomic SNPs associated with TL (5). Heritability estimates for human TL reach 82% (6). Consistently, TL in the zygote appears to play a key role in determining TL later in life (5), indicating that it is largely established during early embryonic development. Telomere length resetting during embryogenesis is a telomerase-dependent process that occurs at the morula-to-blastocyst transition (7). Recent findings in mice show that experimentally disrupting mitochondrial function in zygotes significantly impairs telomere elongation between the 8-cell and blastocyst stages, ultimately leading to shorter telomeres in the offspring (8). Similar outcomes have been observed in offspring from reproductively aged or obese female mice -two conditions known to compromise oocyte mitochondrial integrity (8). Collectively, these findings underscore the critical role of proper mitochondrial function in telomere regulation during embryogenesis. Further evidence comes from individuals with mitochondrial disorders such as MELAS syndrome and Leber's hereditary optic neuropathy (LHON). These individuals exhibit significantly shorter leukocyte telomeres at birth, likely reflecting impaired TL establishment during early development (9).

While damaged mitochondria are associated with shorter TL in mouse embryos and may impair TL regulation in patients with mitochondrial diseases (9), the impact of naturally occurring mitochondrial genome SNPs on TL remains largely unexplored. With a total of 29 haplogroups and a myriad of subhaplogroups characterized by distinct variants compiled in the Mitomap database (10), human mtDNA genomes display large heterogeneity. Some variants within the coding regions of mtDNA, such as the NADH dehydrogenase subunits (ND1, 2, 3, 4, 4L and 5) of complex I (CI),

Cytochrome b, the COXI, II and III subunits of CIV or the ATP6 and 8 subunits of the ATP synthase (CV) lead to distinct ETC properties that impact mitochondrial metabolism and ROS production. In line with the hypothesis that the mitochondrial genome may contribute to the observed stronger maternal influence on TL at birth (6,11) -given that mtDNA is maternally inherited-, we hypothesized that non-pathogenic variation in the mtDNA sequence could affect TL.

Building on reference curves for TL in the Belgian population, obtained through fluorescence *in situ* hybridization (FISH) combined with flow cytometry (Flow-FISH), we identified mtDNA variants associated with either very long or short leukocyte TL. We leveraged a transmitochondrial cybrid approach to directly test the effects of these mtDNA variants on mitochondrial function in mtDNA-depleted recipient cells and uncovered an inverse relationship between leukocyte TL in the donors and mitochondrial ROS levels measured in the cybrids. Our results further highlight the critical role of mtDNA-encoded CI activity in sustaining $NAD^+$ metabolism and telomere integrity, particularly under oxidative stress conditions arising during the metabolic transition from glycolysis to oxidative phosphorylation in early cybrid development. Collectively, these findings reinforce the link between mtDNA-encoded mitochondrial function and telomere regulation in human cells.

# Results

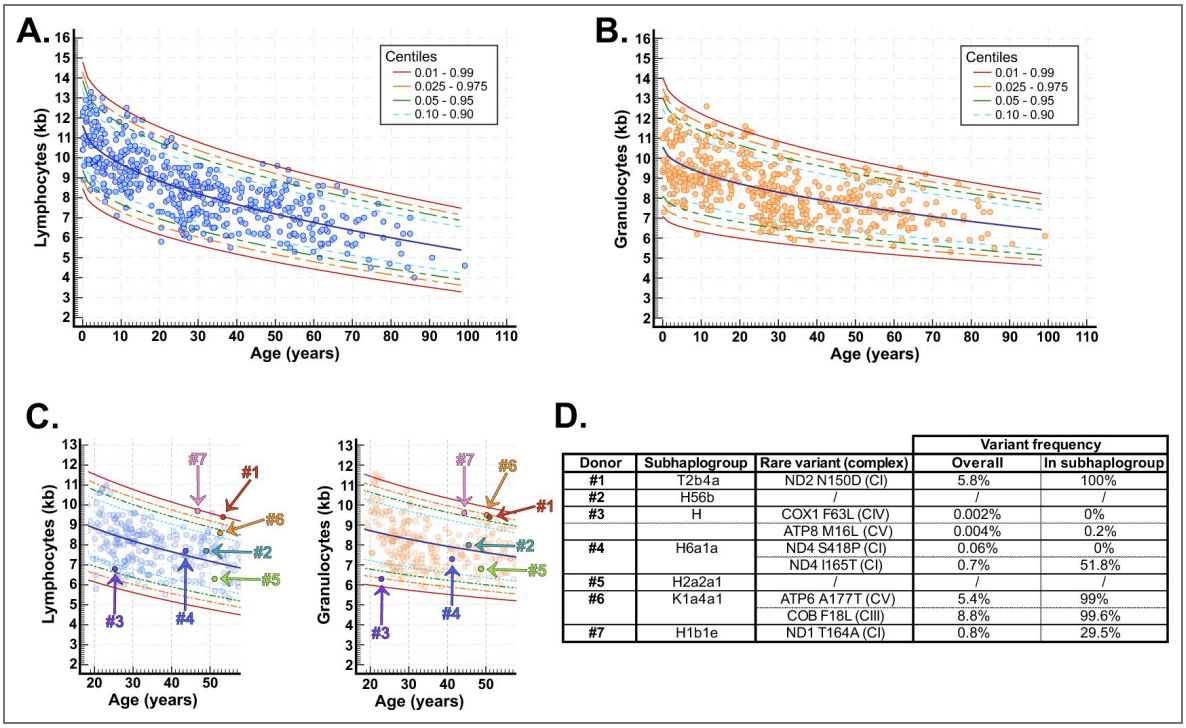
## Establishment of reference curves for TL using Flow-FISH

To establish reference curves for TL in Belgium, we applied the gold standard Flow-FISH approach (12) on leukocytes isolated from a cohort of 491 healthy donors aged 7 days to 99 years old. Percentile curves for TL in lymphocytes (Fig 1A) and granulocytes (Fig 1B) were comparable to those obtained previously (13,14) (Fig S1A) and showed large heterogeneity among individuals of similar age. Although still controversial, obesity may negatively impact TL (5,15). In our cohort, average BMI was not statistically different between donors with telomeres below the $5^{th}$ or above the $90^{th}$ percentile (Fig S1B). Given previous evidence supporting a stronger maternal inheritance of TL in humans (6,11), with similar trend observed in birds (16), we sought to investigate the potential contribution of the human mitochondrial genome to TL heterogeneity. To this end, we selected individuals with long, average or short telomeres from our Flow-FISH curves (Fig 1C). All were of European origin, thus excluding a possible bias linked to African ethnic groups (5), and the seven donors belonged to distinct mitochondrial subhaplogroups (Fig 1D). Strikingly, donor #6, with lymphocyte telomeres at the $95^{th}$ percentile, turned out to belong to the K1a subhaplogroup that encompasses the centenarian-enriched A177T variant (m.G9055>A) in the ATP6 subunit of ATP synthase (CV) (17,18) with an allele frequency of 5.4% in Mitomap database (Fig 1D).

## Distinct mitochondrial genomes differently modulate TL in transmitochondrial cybrids

To evaluate the impact of distinct mitochondrial genomes on TL in human cells, we used a transmitochondrial cybrid strategy in which mtDNA-depleted $Rho^0$ ($\rho^0$) cells are fused with donor platelets, thereby restoring functional mitochondria carrying donor-specific mtDNA variants (19). The presence of variants in cybrids was checked by sequencing and mtDNA content was evaluated by qPCR (Fig S2). Southern blot analysis revealed that telomeres in 143B $\rho^0$ cells were shorter than in parental 143B cells (Fig 2A). To rule out clonal artifacts, mtDNA depletion was independently repeated, and multiple $\rho^0$ clones were isolated, all of which exhibited a consistent reduction in TL (Fig S3A). This telomere shortening was not associated with decreased expression of the telomerase subunits h*TR* or h*TERT* (Fig S3B), but correlated with a reduction in telomerase activity measured *in vitro* using cell extracts (Fig S3C).

In line with a role for mitochondrial function in supporting telomerase activity, fusion of 143B $\rho^0$ cells with platelets from donors #6 and #7 (Cyb6 and 7) restored telomerase activity and rescued TL in $\rho^0$ cells to levels comparable to those of parental 143B cells (Fig 2A-B and Fig S3D). Telomere elongation in these cybrids was accompanied by a reduced frequency of short telomeres,




| Donor | Subhaplogroup | Rare variant (complex) | Variant frequency | |
|---|---|---|---|---|
| | | | Overall | In subhaplogroup |
| #1 | T2b4a | ND2 N150D (CI) | 5.8% | 100% |
| #2 | H56b | / | / | / |
| #3 | H | COX1 F63L (CIV) | 0.002% | 0% |
| | | ATP8 M16L (CV) | 0.004% | 0.2% |
| #4 | H6a1a | ND4 S418P (CI) | 0.06% | 0% |
| | | ND4 I165T (CI) | 0.7% | 51.8% |
| #5 | H2a2a1 | / | / | / |
| #6 | K1a4a1 | ATP6 A177T (CV) | 5.4% | 99% |
| | | COB F18L (CIII) | 8.8% | 99.6% |
| #7 | H1b1e | ND1 T164A (CI) | 0.8% | 29.5% |

**Figure 1. Establishment of Flow-FISH reference curves for telomere length**

**A.** The age-related reference interval and centiles were calculated after telomere length measurement by Flow-FISH in lymphocytes obtained from 491 healthy donors. The P50 curve is shown in dark blue. Other percentile curves are shown as indicated in the legend. **B.** Same as A in granulocytes. **C.** TL in lymphocytes (left) or granulocytes (right) of eight selected donors. **D.** Mitochondrial subhaplogroups of the eight selected donors with an overview of the variants showing both a change in the amino acid sequence and an overall frequency of less than 10% in Mitomap database in the mtDNA coding regions of the donors. The variant frequency in the subhaplogroup of each donor is indicated in the last column.

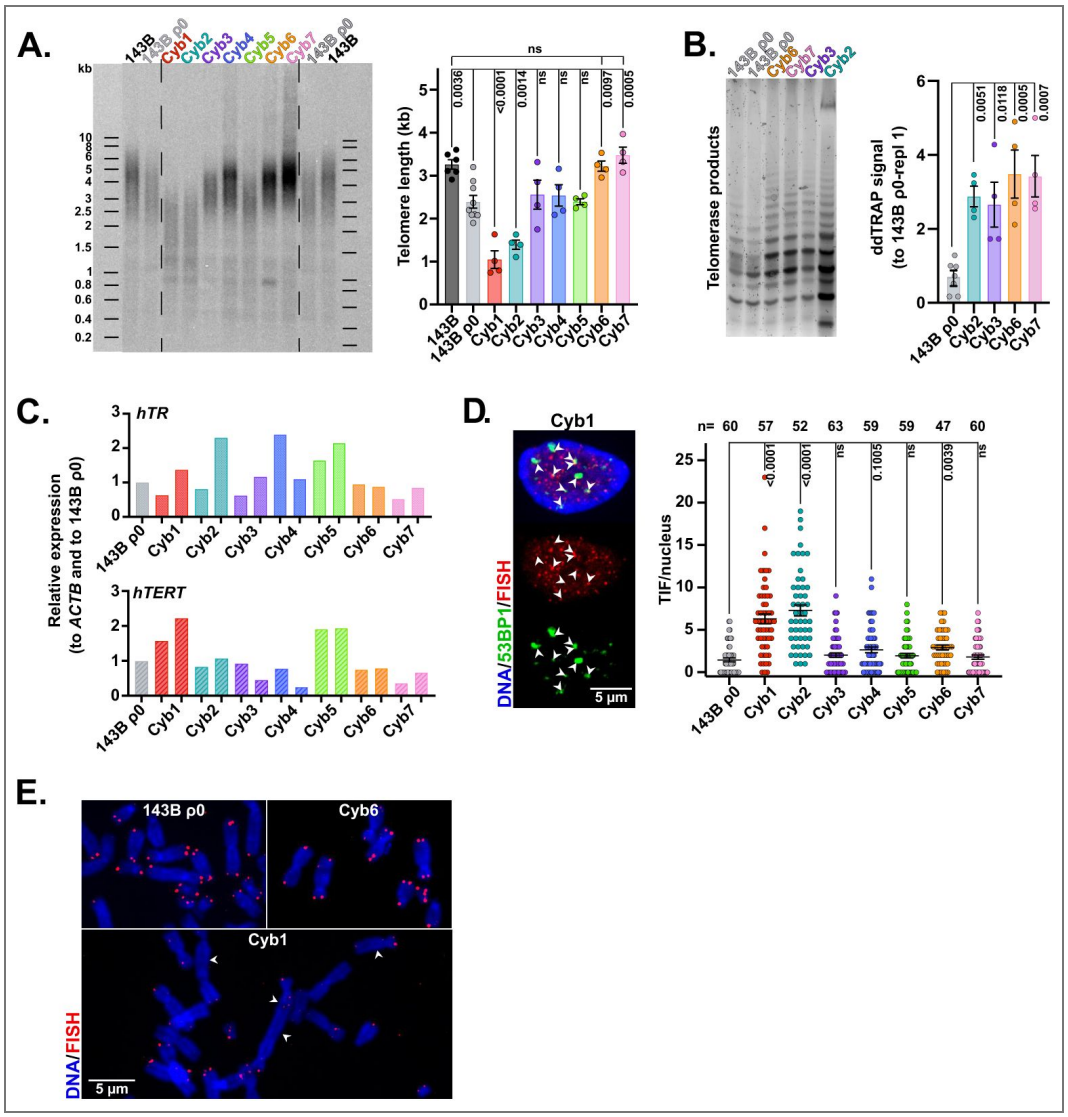



**Figure 2. Mitochondrial genomes modulate telomere length and integrity of ρ$^0$ recipient cells.**

**A.** Left: Representative Southern blot analysis (TRF) of telomeres in 143B, 143B ρ$^0$ recipient cells and in one cybrid clone from each donor at the first PDs post-selection. Right: Quantification of TRF on four independent cybrid clones for each. Mean ± SEM. One-way ANOVA. ns: p>0.05. **B.** Left: TRAP assay products for the indicated cell lines. Right: ddTRAP quantification of telomerase activity in the indicated cell lines. Copy number concentrations were normalized to the first replicate of 143B ρ$^0$ cells (repl 1). Mean ± SEM. One-way ANOVA. **C.** qRT-PCR analysis of h*TR* and h*TERT* transcripts. Values were normalized first to *ACTB* mRNA levels and then to 143B ρ$^0$ ratio. **D.** Left: Representative pictures of telomere dysfunction-induced foci (TIF) detection by co-localization of telomeres (red FISH probe) with 53BP1 DNA damage marker (green). White arrowheads indicate TIF. Blue: DAPI. Scale bar: 5 µm. Right: TIF quantification in clone #1 of all cybrids (first PDs post-selection) and in 143B ρ$^0$ cells. n=47-63 nuclei. Mean ± SEM. Kruskal-Wallis test. ns: p>0.05. **E.** Visualisation of telomere fusions (white arrowheads) in Cyb1 cells at PD7. 143B ρ$^0$ and Cyb6 cells are shown as controls. Telomeres are detected by FISH (red) and DAPI stains DNA (blue). Scale bar: 5 µm.

as assessed by TeSLA (Fig S3E). Unexpectedly, TL was not restored in Cyb1-5 cybrids. Instead, fusion with platelets from donors #1 and #2 induced a marked and reproducible telomere shortening in $\rho^0$ recipient cells (Fig 2A and Fig S3D). This effect was not associated with reduced telomerase activity or decreased h*TR* or h*TERT* transcript levels, but rather with pronounced telomeric damage and telomere fusions following fusion (Fig 2B-E).

Together, these findings demonstrate that mitochondrial DNA sequence variation exerts a strong influence on telomere length in 143B $\rho^0$ recipient cells, ranging from telomere elongation to severe shortening.

## Low complex I activity shortens the telomeres of $\rho^0$ recipient cells

We next investigated why different mtDNA sequences exerted such divergent effects on TL in 143B $\rho^0$ recipient cells. When mitochondria are replenished in $\rho^0$ cells during the formation of transmitochondrial cybrids, a shift from glycolysis to OXPHOS occurs (Fig S4A-B) and cells need to adapt to this metabolic change. Complex I (CI) plays a crucial role in regulating the $NAD^+$/NADH balance and this is crucial for the activity of $NAD^+$-dependent poly (ADP-ribose) polymerase 1) (PARP1) repair enzyme, an enzyme required for the repair of telomeres undergoing oxidative stress in cultured cells (20–22). We thus hypothesized that optimal levels of CI activity may be required for telomere protection in cells experiencing acute oxidative stress during the *in vitro* metabolic shift before cellular adaptation. Consistent with this, we found significantly lower CI activity in Cyb1 and Cyb2 cybrids (Fig 3A) and a strong positive correlation between TL measured in the cybrids at the first passages and CI activity (Fig 3B). Supporting a lower CI activity in in Cyb1 and Cyb2 cybrids, we noticed a significant up-regulation of the $NAD^+$ salvage pathway genes *NAMPT* and *NAPRT1*, likely reflecting a compensatory mechanism (Fig 3C).

Building on the idea that, in the context of lower CI activity, telomeres may not be able to resist oxidative stress-induced shortening, we repeated the fusion of donor #2's platelets to 143B $\rho^0$ cells in the presence of N-acetyl-L-cysteine (NAC) antioxidant and/or nicotinamide riboside (NR), a precursor of $NAD^+$. Strikingly, while telomeres were shortened in all clones (5/5) selected from cybrids obtained in the presence of N-acetyl-L-alanine (NAA) used as control, only one out of the eight selected cybrids obtained in the presence of both NAC and NR showed telomere shortening (Fig 3D-E). Treatment with either NAC or NR alone showed intermediate phenotype (Fig 3D-E). The detection of telomeric parylation signals further supported a role for $NAD^+$-dependent PARP1 activity in repairing oxidative damage at shortened telomeres during the process of cybrid formation (Fig 3F).

Altogether, we propose that the *in vitro* process of cybrid formation exacerbates the impact of low CI activity on telomeres during the acute oxidative stress linked to a metabolic shift from glycolysis to OXPHOS.

## Lymphocyte TL inversely correlates with ROS levels in transmitochondrial cybrids

Having identified CI activity as a strong regulator of TL during *in vitro* cybridisation, we asked whether mtDNA-encoded CI activity could also account for the differences in TL observed in donors' blood cells. However, analysis of age-adjusted lymphocyte TL *versus* CI activity revealed no association between these parameters (Fig 4A).

In mouse oocytes, impaired mitochondrial function, associated with elevated mitochondrial ROS production, adversely affects telomere elongation (8). In human cells, experimentally induced mitochondrial dysfunction was also reported to lead to telomere damage through the production of ROS (23). We therefore tested whether human mitochondrial genomes, through their impact on ROS levels, may modulate TL in blood cells. As transmitochondrial cybrids receive the donors' mitochondria but no other genetic information, this cellular model allows to specifically assess the impact of the mitochondrial genome on ROS production. Mitochondrial superoxide production was measured by electron paramagnetic resonance (24) in clones #1 of all cybrids (Fig 4B). Differences in ROS levels across the cybrids were not linked to distinct changes in oxidative *vs*

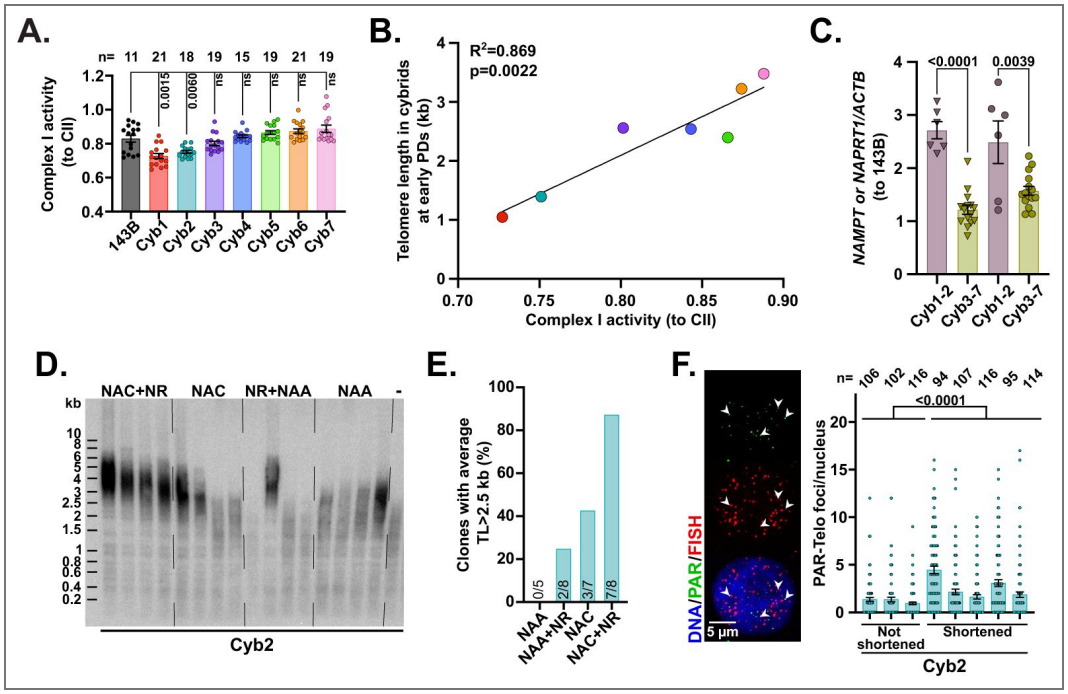



**Figure 3. Telomere shortening in cybrids from donor with low complex I activity is counteracted by antioxidant and NAD$^+$ precursor.**

**A.** Complex I (CI) activity in 143B and in the cybrids. Values were normalized to the activity of complex II (CII) which is entirely nuclear-encoded. n measurements from 2 biological replicates on clone #1 from all: at PD 4-7 or PD 16-24 post-selection. Mean ± SEM. Kruskal-Wallis test. ns: p>0.05. **B.** Correlation between TL in the cybrids at early PDs and average ratios of CI/CII activity. Color code as in A. **C.** Expression of *NAMPT* and *NAPRT1* genes from the NAD$^+$ salvage pathway in cybrids (PD≥5 post-selection) from donors with either low (Cyb1 to 2) or normal (Cyb3 to 7) CI activity. Values were normalized to *ACTB* and to the ratios measured in 143B cells. Color code as in A. n=3 biological replicates. Mean ± SEM. Two-tailed Student's *t* tests. **D.** Representative TRF analysis of TL in cybrids obtained by fusion between donor #2's platelets and 143B $\rho^0$ recipient cells in the presence of the indicated treatments or in the absence of added drug (–). 5 mM N-acetyl-L-cysteine (NAC), 5 mM N-acetyl-L-alanine (NAA, control) and/or 3 mM nicotinamide riboside (NR) were added at the time of the fusion and kept until clone isolation. **E.** TL was measured in the following numbers of independent clones: 5 (NAA), 7 (NAC), 8 (NAC + NR or NAA + NR) and the percentage of clones with average TL>2.5 kb was calculated. **F.** Representative images and quantification of telomeric parylation in Cyb2 cells comparing cells that did or did not exhibit telomere shortening during the initial PDs following cybridisation (from panel D-E). Parylation was detected by IF (green) in the presence or PARG inhibitor; telomeres were detected by FISH (red) and DNA was stained with DAPI (blue). Scale bar: 5 µm. n=94-116 nuclei were counted for each clone. Mean ± SEM. Two-tailed Mann-Whitney test.

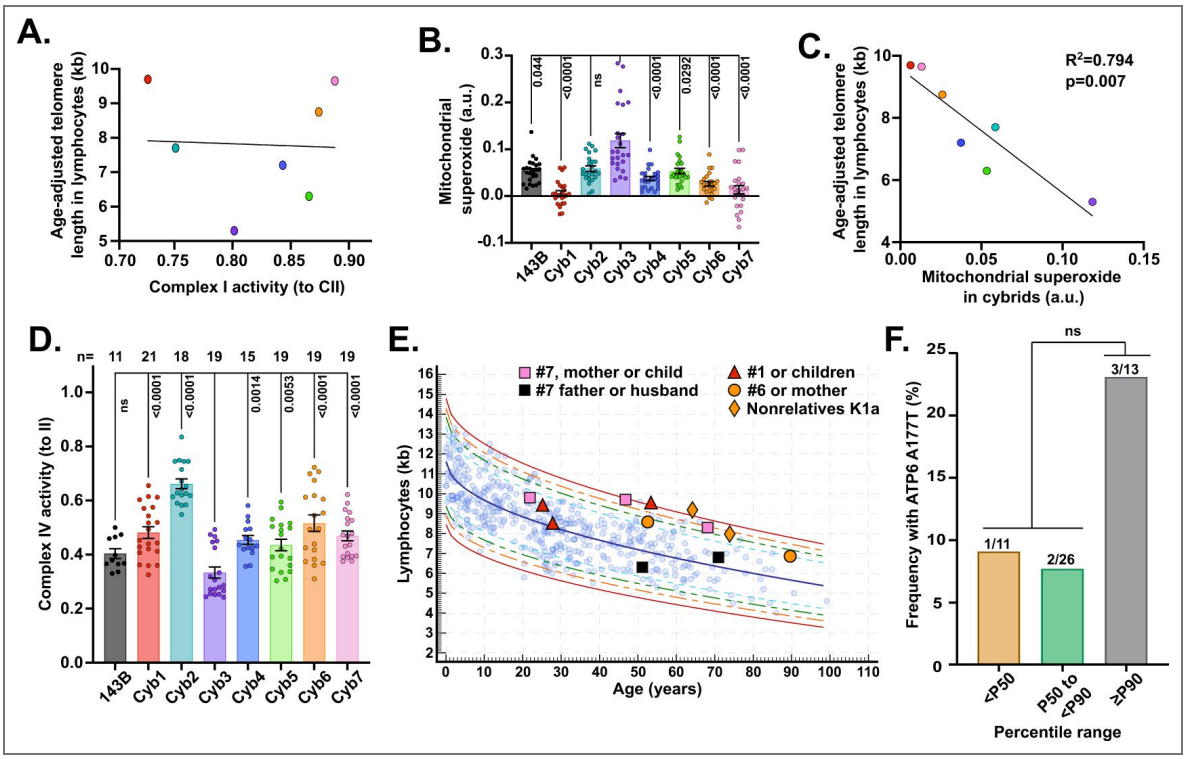



**Figure 4. Mitochondrial superoxide production in cybrids inversely correlates with TL in lymphocytes.**

**A.** Lack of correlation between lymphocyte TL (in kb, adjusted to 50 yo) and average CI activity measured in cybrids from the corresponding donors and normalized to CII activity (CI/CII). **B.** Mitochondrial superoxide production measured by electron paramagnetic resonance and corrected for background using PEG-SOD2 in clone #1 from all cybrids. n measurements from 2 biological replicates with 3 technical replicates. Mean ± SEM. Kruskal-Wallis test. ns: p>0.05. **C.** Correlation between lymphocyte TL (in kb, adjusted to 50 yo) and average mitochondrial superoxide production measured in cybrids from the corresponding donors. **D.** Complex IV activity in 143B cells and in the cybrids. Values were normalized to CII activity. n measurements per cell line from 2 biological replicates. One-way ANOVA. ns: p>0.05. **E.** TL of donor #7 (H1b1e), donor #1 (T2b4a), donor #6 (K1a4a1) and their relatives is shown as indicated in the legend. Orange triangles show TL in unrelated K1a11b and K1a4a1a2b donors. **F.** Frequency of donors with the ATP6 A177T variant among n selected donors with lymphocyte TL in the indicated ranges (<P50, between P50 and <P90 and ≥P90). Chi-square test.

glycolytic metabolism in the cybrids that were characterized by similar lactate/glucose and ATP/ADP ratios (Fig S5A-B). Strikingly, ROS levels in the cybrids inversely correlated with the age-adjusted TL measured by Flow-FISH in the corresponding donors' lymphocytes (Fig 4C). Elevated levels of ROS were notably detected in Cyb3 cybrid. The mtDNA from donor #3 harbors the extremely rare F63L variant (m.T6090>C; Mitomap frequency: 0.002%) in the COX1 subunit, located within a transmembrane helix in close proximity to the heme A group and associated with reduced CIV efficiency (Fig 4D and Fig S6). Together, these findings support a role for mitochondrial ROS in human TL regulation *in vivo*.

## Rare mtDNA variants may account for the maternal inheritance of long telomeres

To further assess the relevance of our findings to a potential role of mitochondria in TL inheritance in humans, we searched for evidence of maternal inheritance of TL within families. Such analyses are most informative in families with extremely long (>P90) or short (<P10) telomeres. We therefore focused on three individuals with lymphocyte TL above the 90th percentile: donors #1, #6, and #7 (Fig 1C).

Despite donor #1 exhibiting lymphocyte TL at the 99$^{th}$ percentile, TL in lymphocytes from both of this donor's children was close to the 50$^{th}$ percentile or only slightly above, arguing against a dominant maternal inheritance of the exceptionally long telomeres observed in donor #1 (Fig 4E). Donor #6 belongs to the K1a subhaplogroup Identified by the MitoAging project as one of the four mtDNA variants significantly associated with longevity, with the A177T variant also showing protective effect against Parkinson's disease (25). Although we could not assess TL in donor #6's progeny, as she does not have children, the fact that her mother showed telomeres at the 90$^{th}$ percentile and that two European male donors, identified in independent cohorts (26) as having telomeres at, respectively, the 97.5$^{th}$ and above the 99$^{th}$ percentile (Fig 4E), were found to display K1a mtDNA, support the notion that this subhaplogroup is associated with long telomere inheritance. This led us to screen additional unrelated donors with varying TL for the presence of the m.G9055>A variant. The variant was identified in 1 of 11 donors with lymphocyte TL below the 50$^{th}$ percentile, 2 of 26 donors with TL between the 50$^{th}$ and 90$^{th}$ percentiles, and 3 of 13 donors with TL at or above the 90$^{th}$ percentile (Fig 4F and Fig S7), suggesting a trend toward higher frequency of the m.G9055>A (ATP6 A177T) variant among individuals with lymphocyte TL at or above the 90$^{th}$ percentile ($p$=0.153).

Donor #7 does not belong to the K1a haplogroup; nevertheless, both her mother and her daughter exhibited telomere lengths at the 95$^{th}$ and near the 90$^{th}$ percentile, respectively (Fig 4E). In contrast, the telomere lengths of her husband and her father were slightly above the 10$^{th}$ and around the 50$^{th}$ percentile, respectively (Fig 4E). These findings pointed to a maternal inheritance pattern associated with the H1b1e mtDNA subhaplogroup, identified through blood sequencing in these women but not previously linked to longevity. While the causal link between the rare m.A3796>G variant in donor #7 (ND1 T164A, CI) and the long telomere phenotype remains to be determined, this variant was absent in all 49 unrelated donors with varying telomere lengths that we screened.

Collectively, these findings support the idea that multiple mitochondrial genome SNPs may contribute to the inheritance of telomere length.

# Discussion

While long telomeres are often viewed as markers of good cellular health, shorter leukocyte TL is linked to a higher risk of diseases affecting organs like the lungs and liver, as well as various cancers (4). Although aging is a major factor influencing TL, genetic variation also plays a key role and helps explain why TL established in the zygote strongly influences TL throughout life (5).

Unraveling the mechanisms that determine TL in the zygote is therefore highly relevant, particularly for optimizing *in vitro* fertilization strategies. A recent study in mice suggests that mitochondrial dysfunction impairs telomere elongation during embryonic development-a defect

that was largely reversed by antioxidant treatment, highlighting the harmful effect of ROS on TL regulation in the embryo (8). In humans, prenatal exposure to psychological stress has also been linked to shorter leukocyte TL in offspring (27). Given the established connection between psychological and oxidative stress (28,29), these findings point to a similar influence of mitochondrial function on TL regulation during human embryogenesis. Building on these findings -and previous evidence suggesting stronger maternal inheritance of TL in humans (6,7)-we hypothesized that the mitochondrial genome may influence human TL. To investigate this, we employed a transmitochondrial cybrid model using platelets from seven donors with known leukocyte TL and distinct mitochondrial subhaplogroups. Notably, we observed an inverse correlation between donor leukocyte TL and mitochondrial ROS levels in the cybrids, indicating that mitochondrial subhaplogroup affects mitochondrial function and suggesting a potential role for mtDNA variation in shaping leukocyte TL *in vivo*. Our findings further implicate CI activity in this process, as, while donors #6 and #7, with signs of maternal inheritance of their long telomeres, both display mitochondria with high CI activity, donor #1, with long telomeres but low mtDNA-encoded CI activity, did not transmit her long telomeres to her progeny. In summary, our data indicate that low levels of mitochondrial ROS, combined with potent CI activity, may be key for a mtDNA-driven maternal inheritance of long telomeres. The Japanese m.C5178A longevity-associated variant in the *ND2* gene (30) further supports an important role for CI in promoting mitochondrial fitness, and possibly TL regulation.

Whether and how the centenarian-linked K1a mtDNA contributes to reduced ROS levels awaits further investigation. As a defective aerobic production of ATP, linked to the ATP6 A177T variant, was proposed to account for the lack of mtDNA K haplogroup in Finnish elite endurance athletes (31), we hypothesize that, by reducing ATP aerobic production, ROS production decreases, thus providing a possible explanation to this longevity-associated mtDNA (32). Consistent with this, Cyb6 cybrids showed a distinct response to FCCP uncoupling agent as, unexpectedly, respiration could not be restored in cells with oligomycin-inhibited ATP synthase, suggesting a defective ability in increasing oxygen consumption rate to face an energy demand increase (Fig S4A-B). This could be related to the location of the A177T variant in a crucial region of ATP6, along the proton pore (33). Strikingly, 32.7% of the genetically homogeneous population of Ashkenazi Jews express the ATP6 A177T variant (10). As telomeres from Ashkenazy centenarians blood cells are longer than in control groups (34), it will be interesting to test the link to the m.G9055>A variant encoding ATP6 A177T.

Experimentally induced mitochondrial dysfunction leads to telomere damage (23). Mechanistically, the *TTAGGG* repeats of telomeres are exquisitely sensitive to 8-oxo-guanine formation, a lesion recognized by the base excision repair pathway that, after removal of the oxidized base, generates single-strand breaks eventually recruiting NAD+-dependent PARP1 repair enzyme (21,35). If left unrepaired in replicating cancer cells, these breaks can convert into double-strand breaks, resulting in distal telomere loss and chromosomal fusions (22). In normal fibroblasts and epithelial cells, telomeric 8-oxoG lesions induced by a chemoptogenetic tool were sufficient to activate p53-dependent senescence (36), highlighting the harmful effects of oxidative stress at telomeres on cellular function. During the generation of transmitochondrial cybrids, cells undergo a metabolic shift from glycolysis to OXPHOS, accompanied by increased ROS production. Before cells adapt to this shift, oxidative stress can be particularly damaging. Supporting the essential role of CI in maintaining PARP1 activity *via* regulation of the $NAD^+$/NADH balance, mitochondrial platelets with low CI activity triggered significant telomere shortening in recipient cells during early cybrid formation -a loss that was prevented by co-treatment with an antioxidant and an $NAD^+$ precursor. Although the observed telomere loss may be exacerbated by the *in vitro* oxidative stress conditions -since donors with low CI activity did not show short leukocyte telomeres-our findings are consistent with reports that $NAD^+$ supplementation can alleviate telomere dysfunction in cells from dyskeratosis congenita patients (37) and improve hematopoietic function and telomere integrity in $Tert^{-/-}$ mice (38). Data are also consistent with the observation that mice lacking PARP display telomere shortening and chromosomal instability

(39). Together, these data bring support to the hypothesis that, when extremely pathogenic and associated with high oxidative stress levels, such as in MELAS or LHON patients (40), mutations in CI may impair telomere length resetting *in utero*.

Our study also unexpectedly revealed a reduction in telomere length in 143B cells following mitochondrial depletion. This telomere shortening was associated with decreased telomerase activity, as measured by ddTRAP, but not with reduced expression of the telomerase subunits h*TERT* or h*TR*. 143B osteosarcoma cells are deficient in thymidine kinase 1 and therefore lack an effective pyrimidine salvage pathway. In the absence of oxidative phosphorylation, the *de novo* thymidine synthesis pathway is also compromised, rendering 143B $\rho^0$ cells dependent on uridine supplementation for survival. Although this hypothesis requires further investigation, we propose that the shorter telomeres observed in 143B $\rho^0$ cells may be linked to the emerging role of thymidine nucleotide metabolism in the regulation of human telomere length, potentially through an allosteric effect of dTTP on telomerase activity (41–43).

Altogether, our findings that certain mitochondrial genomes may support telomere elongation are relevant to three-parent *in vitro* fertilization, a technique that uses donor eggs to prevent the transmission of mitochondrial diseases from mother to child (44). Future work will be necessary to investigate how mitochondrial activity may be involved and whether the nucleotide metabolism that recently emerged as an important regulator of telomerase activity may play additional role.

# Materials and Methods

## Human study participants

Blood samples were obtained from a total of 491 healthy donors. Cohort characteristics are as follows: 261 females and 230 males, median age of 26.3 y (7 days-99.2 y). Protocols were approved by the CHEF institutional Ethics committee and written informed consents were obtained from all 18 year-old or over volunteers. For children below 18, left-over blood samples were obtained through the Cliniques universitaires Saint-Luc after screening for inclusion.

## Leukocyte purification

Blood samples were collected in EDTA tubes and kept at room temperature for a maximum of 48 h before processing. Leukocytes were purified after erythrocyte lysis with ice-cold 0.155 M $NH_4Cl$ (Sigma-Aldrich), 0.01 M $KHCO_3$ (Sigma-Aldrich) and 0.1 mM EDTA (Sigma-Aldrich) solution as described (12). Leukocytes were then resuspended in hybridization buffer (5 % dextrose/10 mM HEPES/0.1 % BSA), and frozen at −80 °C in 80 % FBS (Cytiva)/20 % DMSO (Sigma-Aldrich).

## Flow-FISH

The average telomere length (TL) was measured in peripheral blood leucocytes by flow cytometry combined with FISH (Flow-FISH), using bovine thymocytes as reference as previously described (12). TL of bovine thymocytes was measured by telomere restriction fragment (TRF) analysis (see below). TL was evaluated in lymphocyte and granulocyte populations distinguished by LDS751 staining of DNA as described (12). The telomeric PNA probe was as follows: FITC-OO-CCCTAACCCTA ACCCTAA (Panagene). Values obtained for TL in lymphocytes and granulocytes are provided in Table S1.

## Genomic DNA extraction

Genomic DNA was extracted by overnight digestion at 45°C with 100 µg/mL proteinase K (Sigma-Aldrich) in lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 1 % SDS; pH 8.0) followed by DNA purification with 25:24:1 phenol–chloroform–isoamyl alcohol (Sigma-Aldrich) and precipitation in isopropanol (Thermo Fisher Scientific) and 0.3 M sodium acetate; pH 5.5 (Sigma-Aldrich). gDNA was subsequently treated with 0.2 mg/mL RNAse A (Thermo Fisher Scientific) for 1 h at 37 °C, purified, and precipitated again as described above. DNA concentration, purity and integrity were evaluated with Nanodrop (Thermo Fisher Scientific).

## MtDNA subhaplogroup identification

PCR reactions were performed on 20-50 ng total DNA using GoTaq® G2 DNA Polymerase (Promega) and the primers provided in Table S2 so as to cover the entire mtDNA sequence (45). PCR program was as follows: 3 min at 95 °C followed by 40 cycles of 95 °C for 45 s, 47/50/55 °C for 45 s, and 72 °C for 1 min, with a final step at 72 °C for 5 min. PCR products were loaded on 1 % agarose gel (Eurogentec), stained with 0.5 µg/mL ethidium bromide (Sigma-Aldrich) and extracted from the gel using SmartPure Gel Kit (Eurogentec), according to the manufacturer's instructions, followed by Sanger sequencing (Azenta/Genewiz). MtDNA subhaplogroups were identified using MITOMASTER tool on *mitomap.org* website.

## MtDNA content quantification

MtDNA content quantification was performed by qPCR on 60 ng of total DNA, using KAPA SYBR FAST (Sigma-Aldrich) and primer sequences (45) provided in Table S2.

## Cell culture and treatments

143B cells and cybrids were grown in DMEM high glucose (4.5 g/L) supplemented with 10 % FBS (Cytiva) and 1 % Penicillin/Streptomycin (Capricorn Scientific). 143B $\rho^0$ cells were grown in DMEM high glucose supplemented with 0.11 g/L pyruvate (Thermo Fisher Scientific), 10 % FBS (Cytiva), 50 ρg/mL uridine (Sigma-Aldrich) and 1 % Penicillin/Streptomycin (Capricorn Scientific). For the subcloning of 143B $\rho^0$ population, cells were treated exactly as if they would undergo fusion with platelets and kept throughout the cloning process in $\rho^0$culture medium. All cell lines were regularly checked for mycoplasma contamination. For this, 100 µL of culture medium were heated at 95°C for 5 min before spinning down. qPCR reactions were performed on 2 µL of the supernatant as described previously (46) using primers listed in Table S2.

## Isolation of human platelets

Blood was collected in 8.5 mL-CPDA tubes (Sarstedt) and centrifuged at 330 x *g* for 20 min at 22 °C. The upper platelet-rich plasma fraction was treated with 4 µg/mL eptifibatide (GlaxoSmithKline) and 1 U/mL apyrase (Sigma-Aldrich) on ice before centrifugation at 800 x *g* for 10 min at room temperature. The pellet was resuspended into 5 mL physiological saline solution containing 4 µg/mL eptifibatide and 1 U/mL apyrase. Platelets were counted and diluted with the saline solution to obtain $10^5$ platelets/µL and stored for 30-45 min at 37 °C. $40 \times 10^6$ platelets were then transferred into a 2 mL Eppendorf tube, centrifuged at 800 x *g* for 10 min at room temperature and directly processed for fusion with 143B $\rho^0$ cells.

## Cybrid formation

Cybrids were obtained as previously described (19). A detailed protocol is provided as Supplementary information.

## Generation of new 143B $\rho^0$ clones

New 143B $\rho^0$ clones were generated as previously described (47). Briefly, $1 \times 10^5$ cells were seeded in 10-cm dishes and allowed to adhere overnight at 37 °C. Cells were then cultured in DMEM, High Glucose, Pyruvate (Gibco, 41966029) supplemented with 10% FBS, 1% penicillin–streptomycin, 50 µg/ml freshly added uridine (Sigma, U3003), and 50 ng/ml ethidium bromide (Invitrogen, 15585011). The treatment was maintained for 4 weeks prior to clonal isolation. MtDNA depletion in the resulting clones was checked by qPCR as described above.

## Mitochondrial superoxide assessment by EPR spectroscopy

The electron paramagnetic resonance (EPR) assay is based on the oxidation of Mito-TEMPO-H, a diamagnetic cyclic hydroxylamine that accumulates inside the mitochondria and is transformed into a paramagnetic nitroxide. To isolate the contribution of superoxide to the oxidation of the

probe, PEG-SOD2 was used as a control as described previously (24,48–50). A detailed protocol is provided as Supplementary information.

## Seahorse analyses

Oxygen consumption rates (OCR) were measured using a Seahorse XFe96 plate reader (Agilent). All assays were carried out using a seeding density of 10,000 cells/well in non-buffered DMEM, adjusted at pH 7.4. Mitochondrial function parameters (*i.e.* basal and maximal respirations and ATP production-linked OCR) were evaluated, with the XF Cell MitoStress Test (Agilent), after sequential treatment with 1 µM oligomycin, 1 µM FCCP and 0.5 µM rotenone/antimycin A, as previously described (51). ETC complex activity was assessed as previously reported (52), with slight modifications. Briefly, $10^4$ cells per well were first permeabilized with 1 nM Seahorse XF Plasma Membrane Permeabilizer (Agilent) and respiration was stimulated by adding 10 mM pyruvate, 5 mM malate, and 2 mM ADP. After the baseline scan, 100 nM rotenone was then added to inhibit complex I and 10 mM succinate was injected to establish complex II respiration. Last, 4 µM antimycin A was added to inhibit complex III and 100 µM N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride (TMPD) + 10 mM ascorbate was injected as a complex IV substrate. OCR data were then normalized according to protein content in each experimental well.

## Telomere restriction fragment (TRF) length analysis

TRF analyses were performed as described previously (53) on 2-5 µg of RNAse-treated genomic DNA digested with 20 U *Hinf*I and 20 U *Rsa*I and using [$\gamma$-$^{32}$P] ATP-labelled $(TAACCC)_4$ probe. Smart Ladder (Eurogentec) was run together with the samples, stained with ethidium bromide and imaged with a ruler. TRF were quantified using the online available WALTER (Web-based Analyser of the Length of Telomeres) software (*https://www.ceitec.eu/chromatin-molecular-complexes/rg51/tab?tabId=125#WALTER*).

## TeSLA analysis

The procedure described previously (54) was followed for TeSLA analysis. Primer sequences are provided in Table S2.

## Immunofluorescence and fluorescence *in situ* hybridization

Immunofluorescence and FISH experiments were performed as described previously (53) using the following antibodies and telomeric probe: rabbit anti-53BP1 (Novus Biologicals), Alexa 488 goat anti-rabbit (Invitrogen), mouse anti-Poly(ADP-ribose) (Millipore), Alexa 488 Goat anti-mouse (Abcam), 5'(TYE 563)GGGTtAGGGttAGgGTTAGGGttAGGGttAGGGtTA(TYE 563) (small letters indicate LNA$^{TM}$ modified bases) (Qiagen). For the detection of parylated proteins, cells were grown in the presence of 1 µM PDD00017273 PARG inhibitor (Sigma-Aldrich). For telomeric FISH on metaphase spreads, cells were treated as described previously (55) and telomeres were probed with 5'(TYE 563)GGGTtAGGGttAGgGTTAGGGttAGGGttAGGGtTA(TYE 563). Images were acquired with the Cell Observer Spinning Disk confocal microscope (Zeiss) with 100× objective and analyzed using ImageJ software (National Institute of Health), while maintaining the same threshold for samples from the same experiment.

## Liquid chromatography-mass spectrometry analysis of metabolites

6-well plates with cultured cells were rapidly washed twice with ice cold water and submerged in liquid nitrogen. 300 µL of a solution of 90 % methanol and 10 % chloroform were added, lysates were scraped and transferred to microcentrifuge tubes for centrifugation for 15 min at 4 °C and 22, 000 x *g*. The supernatants were dried in a Speed-Vac vacuum concentrating system and resuspended into 50 µL of 50:50 water:methanol solution. Analyses by LC-MS were performed as previously described (56) based on a method by Coulier *et al.* (57). A detailed protocol is provided as Supplementary information.

### Dosage of extracellular glucose and lactate

Glucose and lactate concentrations were measured in extracellular media by using specific enzymatic assays and an ISCUSflex microdialysis analyzer (M Dialysis), as previously described (51).

### RNA isolation and reverse transcription quantitative real-time PCR

RNA was extracted using TriPure Isolation Reagent (Sigma-Aldrich) according to the manufacturer's instructions. For cDNA synthesis, 1 µg of RNA was retro-transcribed with 200 U MMLV-RT (Thermo Fisher Scientific), 0.2 µg random hexamers (Thermo Fisher Scientific), 20 U RiboLock RNase inhibitor (Thermo Fisher Scientific) and 2 mM dNTP mix. qPCR reactions were performed using KAPA SYBR FAST (Sigma-Aldrich) and the following program: 95°C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 30 sec. Primer sequences are provided in Table S2.

### Telomerase repeat amplification protocol (TRAP) and droplet digital TRAP (ddTRAP)

For telomerase activity assessment *in vitro* using the TRAP assay, 500,000 cells were lysed in 100 µL CHAPS lysis buffer (TRAPeze S7700, Millipore) on ice for 30 min and proteins were quantified by Bradford assay (Bio-Rad). 500 ng of the lysis mixture were subjected to the TRAP assay following the Millipore kit instructions and TRAP products were separated on TBE/acrylamide:bisacrylamide (19:1) gel and visualized by staining with SYBR Gold Nucleic Acid GelStain (Invitrogen) with a PhosphoImager (Fujitsu). For ddTRAP (58), 500 ng of the lysis mixture were added to 50 µL of 1× TRAP buffer (200 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 2.5 mM each dNTPs, 200 mM TS primer (Table S2), and 0.4 mg/ml BSA), and extension reaction was performed by incubating the mix at 25°C for 40 min, followed by deactivation at 95°C for 5 min. For the ddPCR reaction, 3.75 µL of lysis-extension mix were added to 1x EvaGreen QX200 Super-mix (Bio-Rad), 50 nM TS primer, 50 nM ACX primer (Table S2) and $H_20$ up to 25 µL. PCR reaction was performed as follows: initial denaturation for 5 min at 95°C followed by 40 cycles of 95°C/30 s, 54°C/30 s, and 72°C/30 s. Data were analyzed on Quantasoft software.

### Statistics

Statistical analyses were performed using PRISM 8 software. Normality of the data was always checked before statistical tests. Details are provided in the Figure legends. Nomograms for Flow-FISH-based measurements of TL in the healthy population were drawn using the Age-related reference interval function of MedCalc Software (Belgium) based on methods described previously (59–61).

## Data availability

Table S1 contains the numerical data used to generate the Flow-FISH curves of Figure 1A and Figure 1B.

## Acknowledgements

We are grateful to all volunteers for providing blood samples. We thank Sandrine Horman, Marie Octave and Valentine Robaux for help with platelet purification and Géraldine Aubert and Mary Armanios for guidance in Flow-FISH. We are grateful to Victoria Van Regemorter and Caroline Huart for sharing unpublished data on TL in their cohort. We thank Chloé Despontin and Antoine Fattaccioli for technical support, Céline Coquette for help with blood collection and Mélina Vaurs, and Nausica Arnoult for critical reading of the manuscript. This work was supported by the Fonds National pour la Recherche Scientifique (FNRS) (Belgium), The Belgian Foundation against Cancer

and WELBIO (Walloon Excellence in Life Sciences and Biotechnology) (grant X250624 to CC). Patrick Revy's team is Labellisé Ligue (France). We are grateful to the de Duve Institute for constant support.

# Additional information

## Author contributions

M.M., J.-P.D. and A.D. generated the Flow-FISH curves. G.L., I.S., B.B. and A.D. recruited volunteers and helped collecting blood. M.M., B.M. and B.G. contributed to ROS measurement. M.M., E.R. and C.C. contributed to OCR and ETC complex activity measurement and to glycolytic flux evaluation. I.H. and G.B. contributed to metabolite analyses. E.F. and P.R. contributed to ddTRAP analyses. M.M. and T.A. contributed to transmitochondrial cybrid formation. G.L.B. contributed to PAR-FISH analysis. M.M. performed TRF, TeSLA, RT-qPCR, IF/FISH and qPCR analyses. A.D. and C.C. designed and supervised the study. A.D. wrote the paper.

## Funding


| Funder | Grant reference number | Author |
|---|---|---|
| Fonds National de la Recherche Scientifique, FNRS | | Anabelle Decottignies |
| Fonds National de la Recherche Scientifique, FNRS, Belgium | PDR-WEAVE: T.W017.23 | Guido Bommer |
| Fonds National de la Recherche Scientifique, FNRS, Belgium | PDR: T.0034.24 | Guido Bommer |
| Fonds National de la Recherche Scientifique, FNRS, Belgium | CDR J.0084.22 | Bernard Gallez |
| Fonds National de la Recherche Scientifique, FNRS, Belgium | CDR J.0135.18 | Bernard Gallez |
| Belgian Foundation against Cancer | | Cyril Corbet |
| WELBIO | X250624 | Cyril Corbet |
| Labellisé Ligue | | Patrick Revy |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# Additional files

***Supporting text, Figures S1 to S7, SI Reference, Tables S1 to S2***

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# Peer reviews

## Reviewer #1 (Public review):

Summary:

This is an interesting study that addresses whether mitochondrial DNA (mitoDNA) variants impact telomere length (TL), which may be relevant to potential maternal inheritance of TL in offspring. The study addresses this question using a cybrid model approach in which mitochondria from donor platelets from 7 individuals that vary in TL and differ in mitoDNA variants are introduced into 143B cells that lack mitochondria. MitoDNA variants that exhibited reduced complex I activity showed telomere shortening in cybrids and increased telomere dysfunction. Interestingly, these phenotypes could be reduced with NAC antioxidant and NAD+ supplementation, suggesting that ROS and oxidative DNA damage at telomeres contributed to the telomere shortening. They further showed that cybrids with lower levels of ROS correlated with longer TL in the lymphocytes of the mitochondrial donors.

Strengths:

This study provides compelling evidence that mtDNA variants influence TL through a mechanism involving mitochondrial-derived ROS, potentially causing telomeric oxidative damage. The data are robust, and the manuscript is well written. However, the study could be strengthened by addressing the following questions and minor weaknesses below.

Weaknesses:

(1) Introduction. Line 81, the relationship between TL and the risk of lymphoid and myeloid leukemia is not straightforward. POT1 variants associated with long TL increase the risk for lymphoid and myeloproliferative neoplasms (see PMID: 41564438 for example).

(2) Figure 1. Since sex also influences TL, it would be good to know the sex of the selected individuals or explain why this is not necessary.

(3) Please include a description of the 143B cells that were used for cybrid formation in the Results section when introducing the cybrids.

(4) Lines 155-156. The authors note that cybrids from donors 1 and 2 show "pronounced" telomere damage. This result indicates an increase in 53BP1-positive telomeres, which could be indicative of telomere dysfunction or damage. Quantification of the increased chromosome end fusions for cybrids 1 and 2 would strengthen the result. Do the increased fusions correlate with an increase in telomere signal-free ends? These should be apparent in the telomere FISH images of metaphase chromosomes.

(5) Lines 168-169. What is the evidence that the "in vitro metabolic shift" causes acute oxidative stress?

(6) Why did the elevated ROS in cybrid #3 (Figure 4C) not translate to shorter telomeres in the cybrid (Figure 2A)? Perhaps there is a difference between factors that determine TL in the cybrid vs the donor's lymphocytes? In Figure 4B, it appears that the statistical comparisons for mitochondrial superoxide are all relative to Cyb3. If so, why are the comparisons not with the parental 143B rho0 cell line? Please clarify.

(7) Given the heterogeneity in TL and mtDNA variants in the human population, the conclusions could be further strengthened by increasing the number of donors and cybrids analyzed. However, there are admittedly practical factors. Overall, these findings are compelling and provide a solid foundation for expanding this analysis in the future. This is more of a comment than a weakness.

https://doi.org/10.7554/eLife.111391.1.sa3

## Reviewer #2 (Public review):

Summary:

The authors aim to determine whether mitochondrial genotype influences telomere length. By generating cybrids harboring different mitochondrial backgrounds, the authors seek to establish a mechanistic link between mitochondrial status and telomere biology.

Strengths:

A major strength of the study is the use of cybrid technology, which provides a great approach to investigate the role of mitochondrial DNA independently of the nuclear genome. The authors also employ multiple complementary assays to assess telomere-related phenotypes associated with mitochondrial dysfunction. Together, these experiments generate an interesting dataset that will be of value to researchers interested in the intersection between mitochondrial biology, genome stability, aging, and development. These results also build on previous work supporting roles for ROS/mitochondria in driving telomere shortening.

Weaknesses:

The data support the conclusion that mitochondrial background is associated with differences in telomere length and telomere-related phenotypes. However, some of the mechanistic interpretations would benefit from additional evidence. In particular, the manuscript discusses mitochondrial influences on telomere shortening, yet telomere length in some experiments is assessed at a single time point. Consequently, the current data do not directly address the rate of telomere attrition. Differences observed between cybrid lines could potentially arise from events occurring during cybrid formation, clonal selection, or subsequent cell expansion. Longitudinal analyses across multiple passages, ideally beginning immediately after cybrid generation and controlling for population doublings, would help establish whether mitochondrial function directly affects telomere shortening dynamics. Some experimental results would also benefit from additional quantification, clarification, and some biological replicates are missing.

Overall, this study provides interesting evidence linking mitochondrial background to telomere biology. The cybrid models represent a useful resource for the field, and the work raises important questions regarding mitochondria-telomere communication.

https://doi.org/10.7554/eLife.111391.1.sa2

## Reviewer #3 (Public review):

Strengths:

Mahieu and colleagues address an interesting and underexplored question: whether non-pathogenic variation in the mitochondrial genome contributes to the inter-individual variability of human telomere length (TL). Using a Belgian Flow-FISH reference cohort (n=491) to identify donors at TL extremes, they generate transmitochondrial cybrids from platelets of seven donors of distinct mtDNA subhaplogroups and characterize the resulting cells with a broad and well-executed toolkit (TRF, TeSLA, ddTRAP, EPR-based mitoROS, Seahorse with permeabilized-cell ETC dissection, LC-MS metabolomics, telomeric PAR-FISH). The most compelling finding is that cybrids derived from donors with low complex I (CI) activity undergo rapid telomere shortening during the glycolysis-to-OXPHOS transition of cybrid formation, and that this is largely prevented by co-treatment with NAC and the $NAD^+$

precursor nicotinamide riboside, supporting a model in which CI sustains the $NAD^+$ pool required for PARP1-mediated repair of oxidative damage at telomeres. The authors further report an inverse correlation between donor lymphocyte TL and mitoROS in the corresponding cybrids, and provide preliminary evidence that the K1a-defining ATP6 A177T variant (m.G9055>A) may be enriched in long-telomere individuals.

Weaknesses:

(1) Statistical support and donor sampling for the central in vivo correlation (Figure 4C).

The inverse correlation between donor lymphocyte TL and cybrid mitoROS ($R^2$=0.794, p=0.007) is the principal in vivo claim of the paper, but it is built on seven donors deliberately selected from the extremes of the Flow-FISH distribution. Sampling at the tails of the outcome variable can substantially inflate apparent correlation strength and significance. I would encourage the authors to (i) explicitly state this sampling structure where the correlation is introduced, (ii) report a leave-one-out sensitivity analysis to confirm the relationship is not driven by one or two donors (Cyb3 and Cyb6 appear to anchor the line), and (iii) where feasible, extend the analysis to additional donors with intermediate TL to test whether the relationship holds across the full distribution. Even a modest expansion (e.g., 4 to 5 additional donors at P25 to P75) would substantially strengthen this central claim.

(2) Reconciling the cybrid CI / TL relationship (Fig 3B) with the absence of a CI / TL relationship in donor lymphocytes (Figure 4A).

Figure 3B shows a strong correlation between CI activity and TL in cybrids ($R^2$=0.87), while Figure 4A shows no correlation between donor CI activity (measured in the same cybrids) and donor lymphocyte TL. The authors acknowledge this, but the manuscript subsequently builds toward a CI-centric model of in vivo TL regulation, which seems to outrun the data. The most internally consistent interpretation is that the cybrid CI phenotype reports a sensitized in vitro response to the acute oxidative stress of the metabolic shift, rather than a steady-state determinant of leukocyte TL. I would suggest reframing the abstract, significance statement, and Discussion to make this distinction clearer. The in vitro CI / $NAD^+$ / PARP1 axis is a strong finding on its own, while the in vivo role of CI activity (as opposed to ROS more broadly) is not yet established here. Donor #1's profile (very long lymphocyte TL, low CI activity, severe shortening in cybrids, no telomere inheritance in offspring) is informative in this regard and could be discussed more directly as a case that helps delineate where the cybrid model does and does not recapitulate in vivo biology.

(3) The K1a / ATP6 A177T inheritance claim.

The proposal that K1a (and specifically ATP6 A177T) contributes to maternal inheritance of long telomeres is intriguing but currently rests on three pedigrees (one of which, donor #1, does not support the hypothesis) and a chi-square test that does not reach significance (p=0.153, Figure 4F). The supporting evidence is also limited by the fact that platelet-mediated mitochondrial transfer delivers donor mitochondrial proteins, lipids, and residual mtRNA in addition to mtDNA, making it difficult to attribute the cybrid phenotype of donor #6 specifically to the ATP6 A177T variant. I would recommend either: (a) extending the genotyping screen to additional unrelated donors and, if feasible, confirming the effect of ATP6 A177T through an isogenic approach (e.g., mtDNA base editing in a clean background), or (b) softening the relevant statements to "suggestive trend warranting larger studies," and presenting the K1a observation as hypothesis-generating rather than supportive. The Ashkenazi-centenarian connection raised in the Discussion is an excellent direction for follow-up and could be framed accordingly.

https://doi.org/10.7554/eLife.111391.1.sa1

## Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *This is an interesting study that addresses whether mitochondrial DNA (mitoDNA) variants impact telomere length (TL), which may be relevant to potential maternal inheritance of TL in offspring. The study addresses this question using a cybrid model approach in which mitochondria from donor platelets from 7 individuals that vary in TL and differ in mitoDNA variants are introduced into 143B cells that lack mitochondria. MitoDNA variants that exhibited reduced complex I activity showed telomere shortening in cybrids and increased telomere dysfunction. Interestingly, these phenotypes could be reduced with NAC antioxidant and NAD+ supplementation, suggesting that ROS and oxidative DNA damage at telomeres contributed to the telomere shortening. They further showed that cybrids with lower levels of ROS correlated with longer TL in the lymphocytes of the mitochondrial donors.*
>
> *Strengths:*
>
> *This study provides compelling evidence that mtDNA variants influence TL through a mechanism involving mitochondrial-derived ROS, potentially causing telomeric oxidative damage. The data are robust, and the manuscript is well written. However, the study could be strengthened by addressing the following questions and minor weaknesses below.*

We thank the reviewer for this very positive evaluation of our work.

> *Weaknesses:*
>
> *(1) Introduction. Line 81, the relationship between TL and the risk of lymphoid and myeloid leukemia is not straightforward. POT1 variants associated with long TL increase the risk for lymphoid and myeloproliferative neoplasms (see PMID: 41564438 for example).*

We appreciate the reviewer raising the important link between pathogenic *POT1* variants and lymphoid malignancies driven by elongated telomeres. We would like to clarify, however, that our introduction focused not on the pathological telomere attrition characteristic of telomere biology disorders, but rather on the natural, non-pathological variations observed in individuals with baseline telomere lengths on the shorter end of the spectrum.

Nevertheless, we will include this observation regarding patients with long telomeres in the introduction to underscore the complex relationship between telomere length and tumorigenesis.

The two following publications by the Armanios lab will be added:

DeBoy EA et al. Familial clonal hematopoiesis in a long telomere syndrome. 2023. N Engl J Med 388, 2422-2433.

Davidson-Swinton HR et al. Lymphoid malignancy and clonality in the POT1-mediated long telomere syndrome. 2026. Blood 147, 2226-2237.

> *(2) Figure 1. Since sex also influences TL, it would be good to know the sex of the selected individuals or explain why this is not necessary.*

Because this study investigated the potential maternal inheritance of TL via the mitochondrial genome, our cohort consisted predominantly of female donors (6/7). Donor #5, the husband of Donor #7, was the only male included. We will update Figure 1D to include the sex of each donor and clarify this rationale in the text.

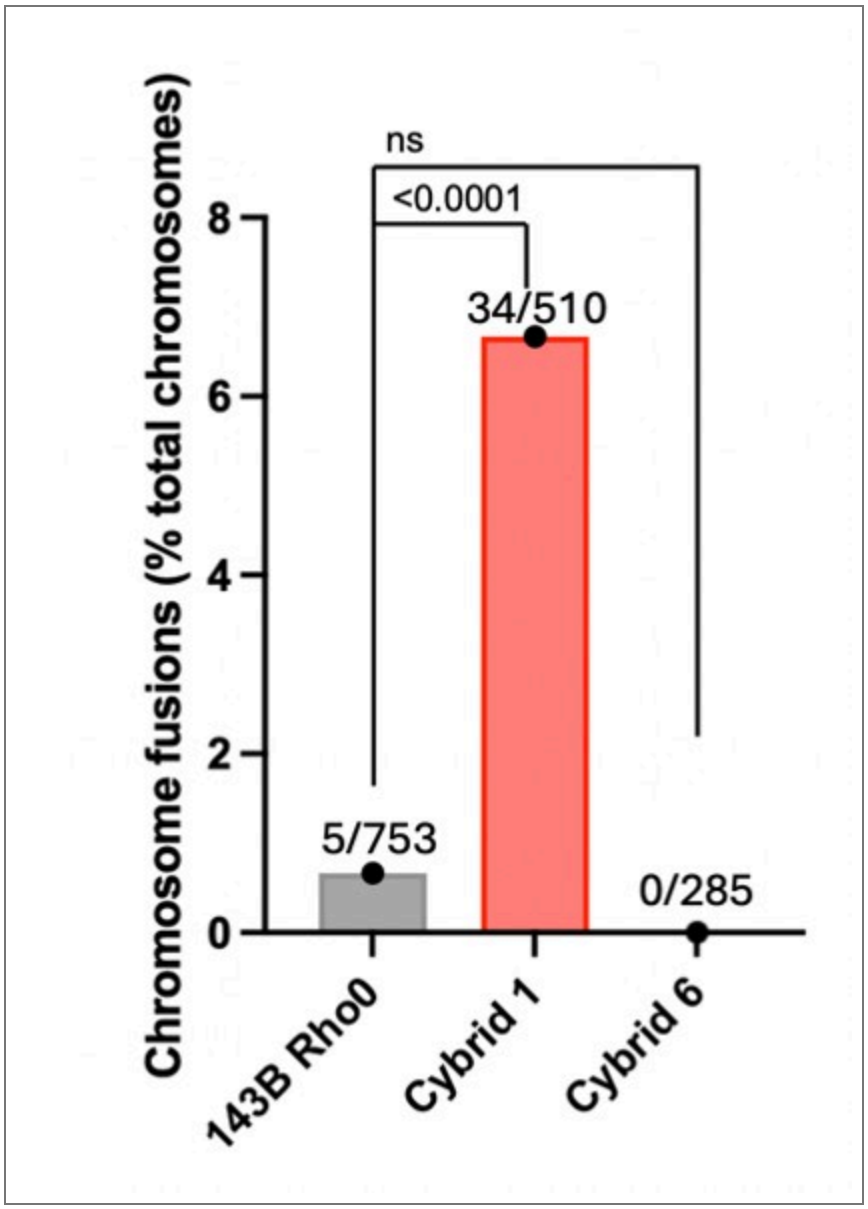
Notably, our analysis revealed minimal influence of sex on TL, which cannot account for the observed differences between the extreme groups.

> *(3) Please include a description of the 143B cells that were used for cybrid formation in the Results section when introducing the cybrids.*

We will do so.

> *(4) Lines 155-156. The authors note that cybrids from donors 1 and 2 show "pronounced" telomere damage. This result indicates an increase in 53BP1-positive telomeres, which could be indicative of telomere dysfunction or damage. Quantification of the increased chromosome end fusions for cybrids 1 and 2 would strengthen the result.*

We thank the reviewer for this suggestion. Following their advice, we used our metaphase spread FISH analyses to quantify chromosome end fusions in the parental 143B Rho0, Cybrid 1 and Cybrid 6 cells. However, because Cybrid 2 metaphase spreads were of insufficient quality for adequate chromosome analysis, we will restrict our telomere fusion comments exclusively to Cybrid 1 and include the quantifications in our revised manuscript.




**Author response image 1.** **The number of fusions and total chromosomes analyzed are indicated on each bar.**

> *Do the increased fusions correlate with an increase in telomere signal-free ends? These should be apparent in the telomere FISH images of metaphase chromosomes.*

While this is a strong argument, the telomeres within these cybrid models are critically short. Consequently, the FISH signal intensity falls below the threshold required for reliable quantification of telomere-free ends.

> *(5) Lines 168-169. What is the evidence that the "in vitro metabolic shift" causes acute oxidative stress?*

The reviewer is correct that we have not formally demonstrated this in our experimental system. Instead, our assumption was based on the fact that the initial phase of Rho0 cell repopulation involves a temporary ROS burst, partly driven by incompletely assembled ETC supercomplexes that are known to elevate ROS levels (Maranzana et al, 2013). This is supported by our experimental observation that the NAC antioxidant, combined with NR,

potently inhibits telomere shortening in cybrids with low CI activity. We propose to add this explanation in the revised manuscript.

> *(6) Why did the elevated ROS in cybrid #3 (Figure 4C) not translate to shorter telomeres in the cybrid (Figure 2A)? Perhaps there is a difference between factors that determine TL in the cybrid vs the donor's lymphocytes?*

We cannot fully explain the discrepancy, but we indeed suspect *in vivo* oxidative stress differs significantly from cell cultures (21% $O_2$). Additionally, early cybrid replenishment involves an unknown telomere elongation step that may offset mitochondrial ROS-induced shortening.

To further investigate this question, we measured telomere length across varying population doublings (PDs) and observed the following shortening after 66-67 PDs:

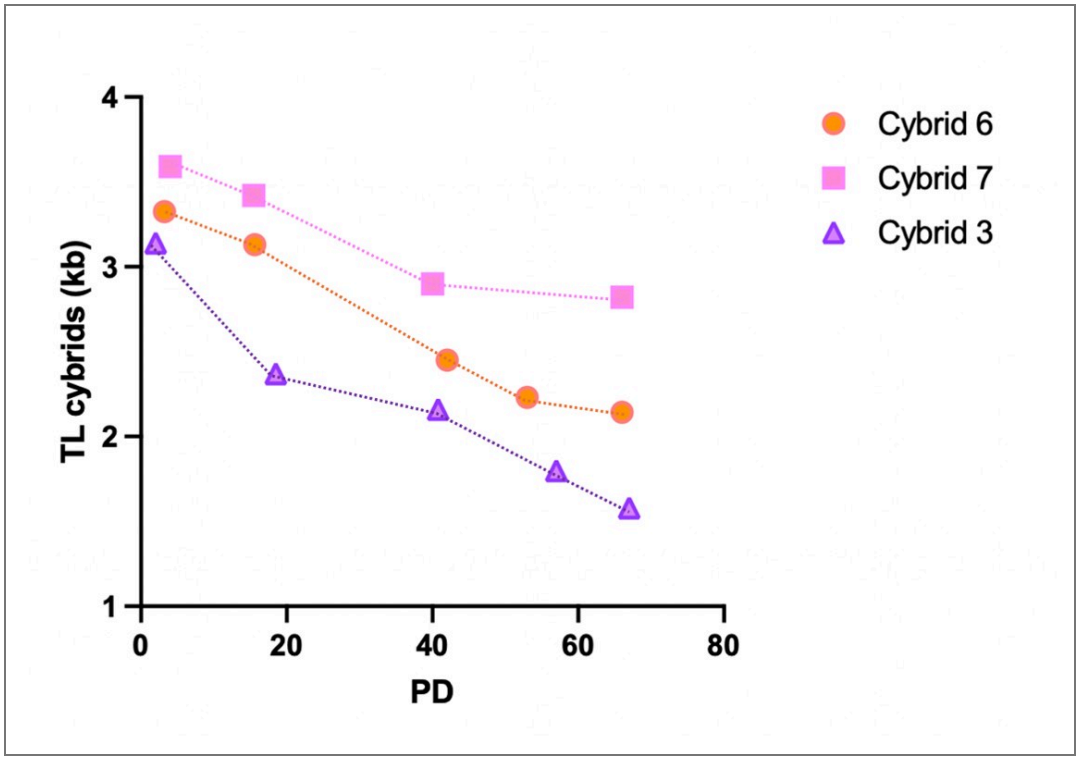
- Cybrid 3: about 1.6 kb reduction

- Cybrid 6: about 1.2 kb reduction

- Cybrid 7: about 0.8 kb reduction

These results suggest that the rate of telomere shortening in culture may be higher in Cybrid 3 cells, possibly due to increased ROS levels.

We propose to include (as Supplementary figure) and discuss these data in the revised manuscript.




**Author response image 2.**

> *In Figure 4B, it appears that the statistical comparisons for mitochondrial superoxide are all relative to Cyb3. If so, why are the comparisons not with the parental 143B rho0 cell line? Please clarify.*

We excluded Rho0 cells from our superoxide measurements because these cells were grown in a different culture medium. Instead, we focused on comparing mitochondrial ROS across cybrids to accurately correlate these values with the telomere length of the corresponding donors' lymphocytes. Consequently, Rho0 cell measurements would not have contributed to this correlation analysis.

We propose to discuss this in the revised manuscript.

> *(7) Given the heterogeneity in TL and mtDNA variants in the human population, the conclusions could be further strengthened by increasing the number of donors and cybrids analyzed. However, there are admittedly practical factors. Overall, these findings are compelling and provide a solid foundation for expanding this analysis in the future. This is more of a comment than a weakness.*

We thank the reviewer for this constructive feedback and entirely agree that including more donors would have added depth to our findings. While we acknowledge this limitation, pursuing this further is currently impossible without acquiring new ethical approvals and establishing fresh collaborations with clinicians.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The authors aim to determine whether mitochondrial genotype influences telomere length. By generating cybrids harboring different mitochondrial backgrounds, the authors seek to establish a mechanistic link between mitochondrial status and telomere biology.*
>
> *Strengths:*
>
> *A major strength of the study is the use of cybrid technology, which provides a great approach to investigate the role of mitochondrial DNA independently of the nuclear genome. The authors also employ multiple complementary assays to assess telomere-related phenotypes associated with mitochondrial dysfunction. Together, these experiments generate an interesting dataset that will be of value to researchers interested in the intersection between mitochondrial biology, genome stability, aging, and development. These results also build on previous work supporting roles for ROS/mitochondria in driving telomere shortening.*

We thank the reviewer for this very positive evaluation of our work.

> *Weaknesses:*
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> *The data support the conclusion that mitochondrial background is associated with differences in telomere length and telomere-related phenotypes. However, some of the mechanistic interpretations would benefit from additional evidence. In particular, the manuscript discusses mitochondrial influences on telomere shortening, yet telomere length in some experiments is assessed at a single time point. Consequently, the current data do not directly address the rate of telomere attrition. Differences observed between cybrid lines could potentially arise from events occurring during cybrid formation, clonal selection, or subsequent cell expansion. Longitudinal analyses across multiple passages, ideally beginning immediately after cybrid generation and controlling for population doublings, would help establish whether mitochondrial function directly affects telomere shortening dynamics. Some experimental results would also benefit from additional quantification, clarification, and some biological replicates are missing.*

We limited our TL measurements to the earliest viable time point after cybrid formation to avoid the confounding effects of cellular adaptation in culture. For instance, the early telomere shortening observed in Cybrid 1 and Cybrid 2 was later alleviated—likely due to the upregulation of the NAD+ salvage pathway genes *NAMPT* and *NAPRT1*.

To accurately capture the effects specific to cybrid formation, we isolated 4 independent clones per donor, all of which showed highly consistent TL values, as shown in Figure 2A and S3D.

While we acknowledge the reviewer's point about multi-passage longitudinal analyses, we did, in fact, measure telomere length across varying population doublings (PDs) in Cybrid 3 (high mito ROS levels), 6 and 7 (low mito ROS levels) and observed the following shortening after 66-67 PDs:

- Cybrid 3: about 1.6 kb reduction

- Cybrid 6: about 1.2 kb reduction

- Cybrid 7: about 0.8 kb reduction

These results suggest that the rate of telomere shortening in culture may be higher in Cybrid 3 cells, possibly due to increased ROS levels.

We propose to include a Supplementary figure and discuss these data in the revised manuscript.

We further propose to carefully edit the manuscript so as to clarify the text and, whenever possible, add quantifications. Among others, as suggested by Reviewer #1, we will add the quantification of chromosome end fusions in the parental 143B Rho0, Cybrid 1 and Cybrid 6 cells in our revised manuscript (See Author response image 1).

> *Overall, this study provides interesting evidence linking mitochondrial background to telomere biology. The cybrid models represent a useful resource for the field, and the work raises important questions regarding mitochondria-telomere communication.*
>
> ***Reviewer #3 (Public review):***
>
> *Strengths:*
>
> *Mahieu and colleagues address an interesting and underexplored question: whether non-pathogenic variation in the mitochondrial genome contributes to the inter-individual variability of human telomere length (TL). Using a Belgian Flow-FISH reference cohort (n=491) to identify donors at TL extremes, they generate transmitochondrial cybrids from platelets of seven donors of distinct mtDNA subhaplogroups and characterize the resulting cells with a broad and well-executed toolkit (TRF, TeSLA, ddTRAP, EPR-based mitoROS, Seahorse with permeabilized-cell ETC dissection, LC-MS metabolomics, telomeric PAR-FISH). The most compelling finding is that cybrids derived from donors with low complex I (CI) activity undergo rapid telomere shortening during the glycolysis-to-OXPHOS transition of cybrid formation, and that this is largely prevented by co-treatment with NAC and the NAD⁺ precursor nicotinamide riboside, supporting a model in which CI sustains the NAD⁺ pool required for PARP1-mediated repair of oxidative damage at telomeres. The authors further report an inverse correlation between donor lymphocyte TL and mitoROS in the corresponding cybrids, and provide preliminary evidence that the K1a-defining ATP6 A177T variant (m.G9055>A) may be enriched in long-telomere individuals.*

We thank the reviewer for this very positive evaluation of our work.

> *Weaknesses:*
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> *(1) Statistical support and donor sampling for the central in vivo correlation (Figure 4C).*
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> *The inverse correlation between donor lymphocyte TL and cybrid mitoROS ($R^2$=0.794, p=0.007) is the principal in vivo claim of the paper, but it is built on seven donors deliberately selected from the extremes of the Flow-FISH distribution. Sampling at the tails of the outcome variable can substantially inflate apparent correlation strength and significance. I would encourage the authors to (i) explicitly state this sampling structure where the correlation is introduced, (ii) report a leave-one-out sensitivity analysis to confirm the relationship is not driven by one or two donors (Cyb3 and Cyb6 appear to anchor the line), and (iii) where feasible, extend the analysis to additional donors with intermediate TL to test whether the relationship holds across the full distribution. Even a modest expansion (e.g., 4 to 5 additional donors at P25 to P75) would substantially strengthen this central claim.*

We thank the reviewer for this insightful comment. As suggested, we will explicitly describe the sampling structure upon introducing the correlation analysis. Furthermore, we have conducted the requested leave-one-out analysis:

- removing Cyb3: $R^2$=0.730; p=0.0302

- removing Cyb6: $R^2$=0.782; p=0.0194

- removing Cyb1: $R^2$=0.740; p=0.0278

While we agree that additional donors would enhance the study, further experiments are however currently impossible without new ethical clearances and additional clinical partnerships.

> *(2) Reconciling the cybrid CI / TL relationship (Fig 3B) with the absence of a CI / TL relationship in donor lymphocytes (Figure 4A).*
>
> *Figure 3B shows a strong correlation between CI activity and TL in cybrids ($R^2$=0.87), while Figure 4A shows no correlation between donor CI activity (measured in the same cybrids) and donor lymphocyte TL. The authors acknowledge this, but the manuscript subsequently builds toward a CI-centric model of in vivo TL regulation, which seems to outrun the data. The most internally consistent interpretation is that the cybrid CI phenotype reports a sensitized in vitro response to the acute oxidative stress of the metabolic shift, rather than a steady-state determinant of leukocyte TL. I would suggest reframing the abstract, significance statement, and Discussion to make this distinction clearer. The in vitro CI / $NAD^+$ / PARP1 axis is a strong finding on its own, while the in vivo role of CI activity (as opposed to ROS more broadly) is not yet established here. Donor #1's profile (very long lymphocyte TL, low CI activity, severe shortening in cybrids, no telomere inheritance in offspring) is informative in this regard and could be discussed more directly as a case that helps delineate where the cybrid model does and does not recapitulate in vivo biology.*

We acknowledge that our study does not establish the *in vivo* role of CI activity in TL regulation. Our abstract specifically highlights an *in vitro* phenomenon: “Under the specific conditions of cybrid formation, which involve a metabolic shift from glycolysis to oxidative phosphorylation, mtDNA variants associated with reduced CI activity induced rapid telomere shortening, …”.

We are nevertheless happy to revise the text to clearly separate our *in vitro* results from *in vivo* biology as requested.

> *(3) The K1a / ATP6 A177T inheritance claim.*
>
> *The proposal that K1a (and specifically ATP6 A177T) contributes to maternal inheritance of long telomeres is intriguing but currently rests on three pedigrees (one of which, donor #1, does not support the hypothesis) and a chi-square test that does not reach significance (p=0.153, Figure 4F). The supporting evidence is also limited by the fact that platelet-mediated mitochondrial transfer delivers donor mitochondrial proteins, lipids, and residual mtRNA in addition to mtDNA, making it difficult to attribute the cybrid phenotype of donor #6 specifically to the ATP6 A177T variant. I would recommend either: (a) extending the genotyping screen to additional unrelated donors and, if feasible, confirming the effect of ATP6 A177T through an isogenic approach (e.g., mtDNA base editing in a clean background), or (b) softening the relevant statements to "suggestive trend warranting larger studies," and presenting the K1a observation as hypothesis-generating rather than supportive. The Ashkenazi-centenarian connection raised in the Discussion is an excellent direction for follow-up and could be framed accordingly.*

We agree that the evidence for the AT6 A177T inheritance claim remains inconclusive. To clarify, we do not argue that this mitochondrial variant is solely responsible for longer telomeres; indeed, the mtDNA genome of donor #1 suggests otherwise. Furthermore, the phenotypic impact of such mtDNA variants likely depends on nuclear variants in other telomere-related genes (e.g., *hTERT* or *hTR*), meaning AT6 A177T may not consistently result in elongated telomeres. Unfortunately, our ethical protocol precludes screening additional unrelated donors. We will revise the text to soften our statements accordingly.

New references:

DeBoy EA et al. Familial clonal hematopoiesis in a long telomere syndrome. 2023. N Engl J Med 388, 2422-2433.

Davidson-Swinton HR et al. Lymphoid malignancy and clonality in the POT1-mediated long telomere syndrome. 2026. Blood 147, 2226-2237.

Maranzana E et al. Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. 2013. Antioxid Redox Signal 19, 1469-1480.

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