Low abundance of the matrix arm of complex I in mitochondria predicts longevity in mice

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Mitochondrial function is an important determinant of the ageing process; however, the mitochondrial properties that enable longevity are not well understood. Here we show that optimal assembly of mitochondrial complex I predicts longevity in mice. Using an unbiased high-coverage high-confidence approach, we demonstrate that electron transport chain proteins, especially the matrix arm subunits of complex I, are decreased in young long-living mice, which is associated with improved complex I assembly, higher complex I-linked state 3 oxygen consumption rates and decreased superoxide production, whereas the opposite is seen in old mice. Disruption of complex I assembly reduces oxidative metabolism with concomitant increase in mitochondrial superoxide production. This is rescued by knockdown of the mitochondrial chaperone, prohibitin. Disrupted complex I assembly causes premature senescence in primary cells. We propose that lower abundance of free catalytic complex I components supports complex I assembly, efficacy of substrate utilization and minimal ROS production, enabling enhanced longevity.

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Mitochondria perform vital functions in life such as they provide energy (ATP), generate iron–sulphur clusters, participate in Ca\(^{2+}\) regulation and have major roles in fatty acid and amino-acid metabolism. They also produce reactive oxygen species (ROS) as metabolic by-products that can damage cellular components and participate in cellular signalling pathways. Mitochondria have long been regarded as crucial in the ageing process. This was originally associated with the (mitochondrial) oxidative stress theory of ageing. However, a recent research has revealed a more complex picture by uncoupling lifespan extension from a decrease in mitochondrial ROS generation or oxidative damage in multiple species\(^5\)–\(^3\), and other relevant aspects associated with mitochondrial function such as stress responses, apoptosis and cell senescence have also been attracting attention.

Mitochondrial biogenesis is an extremely complex process integrating the intra-mitochondrial synthesis of 13 electron transport chain (ETC) proteins coded by the mitochondrial DNA with far more than 1,000 additional proteins that are synthesized in the cytosol as pre-proteins and imported into mitochondria through specific transporters. There are several assembly factors for the ETC, and mitochondrial protein chaperones such as prohibitin (PhB) are thought to be involved in a dynamic balance in the composition of the mitochondriome that determines mitochondrial function\(^4\). The dynamics of the mitochondriome\(^5\) is further determined by targeted turnover\(^6\).

We aimed to identify in an unbiased approach the proteome composition of mitochondria that might predict longevity in mammals. We reasoned that such a ‘longevity-enabling phenotype’ of mitochondria should be (i) evident in mitochondria from long-living animals at young age (that is, before the death of the first member of the cohort); (ii) common in disparate longevity models and tissues; and (iii) counteracted by ageing. Reduced abundance of ETC proteins and of matrix arm subunits of complex I in particular was found in young mice from long-living mouse cohorts, which was associated with improved complex I assembly, higher state 3 oxygen consumption rates and decreased complex I-linked superoxide production, and was reversed in old mice. Moreover, suppression of complex I assembly decreased mitochondrial oxidative metabolism, increased mitochondrial ROS production and precipitated premature senescence. We suggest that partially assembled matrix arm subcomplexes of complex I, which contain the catalytic subunits, might be detrimental as they only contribute to ROS formation without facilitating electron transport activity and proton pumping. Minimizing the abundance of free catalytic complex I components might be an essential feature of a longevity-enabling mitochondrial phenotype in mammals.

Results

Low abundance of ETC complexes associates with longevity. To perform an unbiased analysis of mitochondrial protein composition as potential predictor of longevity, we analysed the mouse liver proteome at young age (100% cohort members alive) in two long-lived mice models: the ICRFa strain, a C57Bl/6 substrain\(^7\) with significantly extended longevity under standard ad libitum (AL)–fed condition (\(P = 0.017\); Fig. 1a); and C57Bl/6 subjected to dietary restriction (DR, 60% of AL food intake) from 3 months of age onwards. DR significantly extended lifespan beyond both AL C57Bl/6 (log-rank test, \(P = 0.0073\)) and AL ICRFa mice (\(P = 0.0085\); Fig. 1a). We addressed ageing-related changes by comparing young (7–8 months) and old (30–32 months) ICRFa mice. DR mice were significantly lighter than the AL C57Bl/6, AL ICRFa and old ICRFa mice, whereas there were no differences in body weights among the non-DR mice groups (analysis of variance, \(P = 0.05\); Supplementary Fig. 1). Food intake was not different between AL ICRFa and AL C7Bl/6 mice, suggesting that the extended lifespan in ICRFa mice is not caused by a concealed DR effect.

We studied liver mitochondrial function in C57Bl/6 during ageing in both AL- and DR-fed mice. Oxygen consumption rates under phosphorylating condition (state 3) using either a complex I-linked substrate (pyruvate plus malate) or a complex II-linked substrate (succinate; Fig. 1b) progressively declined with age in AL mice but were maintained under DR up to an age of 37 months, that is, beyond the maximum lifespan of the AL mice. Hydrogen peroxide release from complex I was higher under AL than under DR up to an age of 30 months. However, DR only postponed the age-dependent increase and did not prevent it: DR mice at 37 months of age (that is, at equivalent survival rate to 30-month-old AL mice) showed hydrogen peroxide release from complex I as high as AL mice at 30 months (Fig. 1c). There were only minor differences in hydrogen peroxide release from sites other than complex I (Fig. 1c). In a comparison between ICRFa and C57Bl/6 mice, longer lifespan in ICRFa was associated with lower hydrogen peroxide release from complex I in isolated liver and brain mitochondria (Supplementary Fig. 2).

Proteomic analysis was performed in two separate 6-plex tandem mass tag (TMT) labelling experiments using one-pooled sample in each for cross-standardization. After imposing a 1% false discovery rate (FDR) filter, we identified 7,047 and 6,704 peptides in the two experiments, respectively, and quantified a total of 631 proteins (531 and 497 proteins per experiment) (Supplementary Data 1–3). This represents about half of the mouse mitochondrial proteins in the MitoCarta database and is similar to published data\(^4\). The molecular weight distribution of the identified proteins mirrored that of all mitochondrial proteins according to the MitoCarta and MitoMiner\(^8\) databases (Supplementary Fig. 3). Our analysis quantified 57 out of the 96 known mouse ETC proteins, namely 67% of complex I proteins, 50% of complex II, 63% of complex III, 46% of complex IV and 76% of complex V. One-hundred seventy-seven proteins showed confidently different abundances in at least one of the three comparisons performed (Supplementary Data 1–3). The number of differentially abundant proteins was the largest (131 proteins) in the AL–DR comparison in C57Bl/6 mice, whereas there were only 57 and 52 differentially abundant proteins for the young C57Bl6–ICRFa and the young–old ICRFa comparisons, respectively (Fig. 1d). In general, proteins that were less abundant in the longevity models (and more abundant in old mice livers) tended to be smaller than those that were more frequent in long-living mice (Fig. 1e).

Out of the 13 proteins that were different in abundance in all three comparisons (Fig. 1d), 12 were possibly implicated with longevity because they were less abundant in both longevity models. Moreover, 11 of them were increased in old mice (Supplementary Table 1), together suggesting that low abundance of these proteins at a relatively young age by either genetic or environmental manipulation might predispose to longevity. These proteins included both PhB and PhB 2, and the majority (8 out of 13) were components of the ETC (complexes I, III and V; Supplementary Table 1).

To identify the functional traits of liver mitochondria associated with longevity, we performed mitochondria-specific KEGG pathway enrichment analysis based on the proteins that were more abundant in liver mitochondria from young C57Bl6/I mice under DR in comparison with AL. A wide range of diverse metabolic pathways, notably those associated with the Krebs cycle and fatty acid and amino-acid metabolism, was enriched under DR (Supplementary Table 2). However, few of these pathways were similarly enriched in young long-living ICRFa mice or were
As we were able to quantify the majority of ETC proteins, we examined the changes of the individual ETC subunits in all three comparisons in detail (Fig. 2a–c). The abundance of numerous subunits of complexes I, III, IV and V (but not of complex II) was reduced in both long-lived mouse models, which was generally reversed in old mice (Fig. 2a–c). There was no evidence for an induction of a mitochondrial unfolded protein response in the long-lived mice with low ETC abundance: several mitochondrial heat-shock proteins (HSPD1, HSPA9 and DSPE1) and the ClpP protease were not changed in any of comparisons, whereas heat-shock proteins HSPA5 and HSP90D1 were decreased under DR (Supplementary Data 1–3).

**Low abundance of CI matrix subunits in long-lived mice.** Strikingly there was a highly skewed pattern of protein abundance changes within complex I. Reduced abundance of complex I subunits in both long-lived mice models occurred almost exclusively in subunits localized in the matrix arm while membrane arm subunits were hardly affected (Fig. 2a,b). This pattern was partially reversed in the aged mice (Fig. 2c). The abundances of PhB and PhB 2, which function as chaperones for ETC proteins and/or as structural scaffolds, paralleled the changes in matrix arm subunits with strong statistical confidence and large fold differences (Supplementary Table 1).

We confirmed these observations in a series of SDS–PAGE western blot experiments, by measuring the ratios of selected matrix subunits (NDUFS3 and NDUVF2) and PhB to the membrane subunits NDUFB9 and NDUFA9, which is located at the junction between membrane and matrix arm and is involved in stabilizing this junction7 (Fig. 3a). In agreement with the proteomics results, we found a decreased ratio of matrix subunits and PhB to membrane subunits under DR (Fig. 3b) and in liver mitochondria from young long-lived ICRFa mice (Fig. 3c), which was reversed in aged liver mitochondria (Fig. 3d).

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**Figure 1 | Longevity and liver mitochondrial function in male long-living mice.** (a) Kaplan-Meier survival curves (right censored) of male C57Bl/6 (red) and ICRFa (green) mice under ad libitum (AL) feeding and of C57Bl/6 mice under 40% DR from an age of 3 months onwards (blue). Group sizes were (censored events in brackets): C57Bl/6 AL 310 (172), C57Bl/6 DR 241 (157) and ICRFa 2391 (1,061). (b) State 3 oxygen consumption rates of purified liver mitochondria from C57Bl/6 mice under AL and DR, respectively, with pyruvate/malate (top) and succinate (bottom) as substrate. Data are mean ± s.e.m., n = 4 animals per group. (c) H2O2 release from purified liver mitochondria from C57Bl/6 mice under AL and DR, respectively. Data are mean ± s.e.m., n = 4. Top: pyruvate/malate plus rotenone, indicating maximum ROS production from complex I (CI). Bottom: succinate plus rotenone, indicating ROS production from sites other than complex I. (d) Numbers of confidently changed proteins in all comparisons. (e) Molecular mass distributions for differentially enriched proteins under DR (blue), in young ICRFa (green) and in old ICRFa (pink).
The low relative abundance of matrix subunits of complex I in long-lived mice was not confined to liver. Ratios of matrix subunits NDUFS3 and NDUFV2 to membrane subunit NDUFA9 (as well as PhB/NDUFA9) were indeed lower in brain mitochondria from 15-month-old mice that were subjected to DR from 3 months of age (Fig. 3e,f). Similarly in skeletal muscle mitochondria, matrix subunit NDUFV2 showed a decreased abundance in relation to membrane subunit NDUFB9 in DR compared with controls (Fig. 3g,h).

To see whether the observations made so far are valid in further longevity models, we examined brain and liver mitochondria from mice fed with rapamycin for 4 months. Rapamycin, an inhibitor of the TOR (target of rapamycin) nutrient sensing pathway, is known to extend lifespan in mice. Liver (Fig. 4a) and brain (Fig. 4e) mitochondria from rapamycin-fed mice did not show altered state 3 oxygen consumption rates nor respiratory control ratio, but hydrogen peroxide release from complex I from both tissue mitochondria was lower (Fig. 4b,f). Importantly, ratios of matrix subunits (NDUFS3 and NDUFV2) as well as PhB to membrane subunits (NDUFA9 and NDUFB9) were generally lower in rapamycin-fed animals than in controls in both liver (Fig. 4c,d, see Supplementary Fig. 4 for uncropped scans) and brain (Fig. 4g,h) mitochondria. Thus, a shift to lower abundance of complex I matrix subunits is associated with lower

**Figure 2 | ETC proteins are less abundant in long-living mice and increase in old mice.** Heatmaps show changes in abundance of all ETC proteins in the comparisons young DR versus young AL C57Bl/6 (a), young ICRFa versus young C57Bl/6 (b) and old versus young ICRFa (c). CI and CV reach out from the inner membrane into the matrix space (upward). Grey symbols represent proteins not identified; and black/white symbols represent abundance that is not significantly changed. Colours indicate fold change.
Mitochondrial complex I is assembled in multiple stages. In Assembly of complex I is more complete in long-lived mice. Mitochondrial complex I is assembled in multiple stages. In HEK293 cells at steady state, complex I subunits exist in a dynamic equilibrium between subcomplexes, the fully assembled holocomplex and supercomplexes. We hypothesize that relatively lower abundance of matrix to membrane arm components might shift this equilibrium towards less abundant subcomplexes and more fully assembled holocomplex, thus be associated with tighter control of complex I assembly. To test this possibility, we examined the complex I assembly status in liver mitochondria under DR using blue native (BN) gel electrophoresis followed by western blotting (Fig. 5a). Complex I activity was preferentially found at the level of fully assembled complex I. Multiple weak activity bands were visible both at supercomplex level and at lower molecular weights (Fig. 5a,b); however, the sensitivity of the in-gel assay was not sufficient to quantify differences between cohorts. Western blotting of BN gels revealed differential fractions of the tested subunits in smaller subcomplexes (Fig. 5a). In DR mitochondria, relative amounts of all subunits in the partially assembled complexes tended to be lower than in AL mitochondria, and this was significant for both matrix arm subunits and for NDUFA9 (Fig. 5b). In contrast, complex II activity and the relative amount of the complex II enzyme SHDA was unchanged (Fig. 5a,b).

Similarly, in a comparison of ICRFa and C57Bl/6 liver mitochondria, relative amounts of NDUFS3 and NDUFB9 in the subcomplexes were lower in the long-lived strain (Fig. 5c,d). Longer exposure of the same blots showed incorporation of hydrogen peroxide release and a longevity phenotype in multiple tissues and multiple models of longevity.
NDUFS3, NDUFV2 and NDUFB9 into multiple subcomplexes that overlap with bands of weak complex I activity (Supplementary Fig. 5A). Even in these low-molecular-weight subcomplexes, less NDUFS3 and NDUFB9 was incorporated in the long-lived ICRFa strain (Supplementary Fig. 5B).

To assess the distribution of complex I subunits between the holocomplex and subcomplexes more comprehensively, we performed complexome profiling in mouse liver mitochondria. BN gels were cut into 12 slices covering the holocomplex (slice 1) and subcomplexes of different sizes (slices 2–12; Fig. 5e). The protein content in each slice was determined by spectral counting-based label-free semiquantitative proteomics (Supplementary Tables 4–7). We quantified 36 of the 44 complex I subunits (plus NDUFA4, which has now been identified as a complex IV subunit). We compared C57Bl/6 and ICRFa mice at 8 months of age as well as AL and DR C57Bl/6 mice at 15 months of age (following 12 months of DR) with three mice analysed separately per group. Peptide counts of the various proteins making up the holoenzyme were widely different, even after normalization for total peptide counts per mouse (Supplementary Tables 4–7). The detection efficiency in the major (holocomplex) slice in our proteomic approach is similar to other recent proteome profiling approaches. Importantly, our counts were highly reproducible between experiments and mice with an average standard error below 15% (Supplementary Tables 4–7).

Relative amounts of all complex I proteins that were detected in subcomplexes are shown in the heatmaps (Fig. 5f–i) assuming equimolar stoichiometry in the holocomplex. It should be noted that equimolar stoichiometry in the mammalian complex I holocomplex has so far been proven for only 7 out of 14 tested complex I subunits, therefore, absolute quantification should not be attempted from these data. Our results indicate that complex I subunits NDUFA7, 10 and 12 are exclusively found in smaller (below 200 kDa) subcomplexes (and in the holocomplex), whereas most other subunits are preferentially located in larger complexes (>500 kDa). Importantly, comparisons between the treatments show less matrix subunits in subcomplexes in mice treated with DR (compare Fig. 5g,d) and in ICRFa (Fig. 5i) versus C57Bl/6 (Fig. 5h) mice at the same age. In fact, hardly any complex I subcomplexes could be found in 8-month-old ICRFa mice (Fig. 5i). Moreover, a comparison between C57Bl/6 mice under AL feeding shows increased accumulation of subcomplexes from 8 months (Fig. 5h) to 15 months (Fig. 5f) of age. Taken together, these experiments suggest that complex I in liver mitochondria is more efficiently assembled in long-living mice, and this is counteracted by increasing age.

**Less efficient complex I assembly increases ROS and senescence.** The catalytic subunits of complex I are located in the matrix arm, but proton pumping occurs via the membrane arm; hence the electron transfer to the Q-pool is only possible with the fully assembled complex I. ‘Superfluous’ matrix subunits may produce superoxide on its own without involving electron transfer activity in the ETC. We hypothesized that less complete assembly of complex I should result in less efficient electron transfer and oxidative phosphorylation (OXPHOS)-dependent ATP production per unit of substrate used, and higher ROS production. To test this hypothesis, we disrupted the complex I assembly by small interfering RNA (siRNA)-mediated gene

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**Figure 4 | Rapamycin treatment reduces ROS production and complex I matrix arm components.** All data are mean ± s.e.m., 4–5 animals per group, *P < 0.05, **P < 0.01 (t-test). (a–d) Liver mitochondria from control (Cont) and rapamycin (Rapa)-treated mice. (a) Oxygen consumption rates with pyruvate/malate (PM) or succinate (Succ) as substrate. Left: state 3 respiration rates; and right: respiratory control ratios. (b) H2O2 release under native conditions using PM without inhibitors (native) and under PM + rotenone (CI max). (c) SDS-PAGE western blots of complex I matrix arm subunits NDUFS3 and NDUFV2, prohibitin (PhB), membrane arm subunits NDUFA9 and NDUFB9 and UQRC2 (UQ), a complex III subunit as complex I-independent reference. Please see Supplementary Fig. 4 for uncropped scans of the blots. (d) Relative abundance of matrix subunits NDUFS3 and NDUFV2 and PhB to membrane subunits NDUFA9 and NDUFB9. (e–h) Same as a–d, but from brain mitochondria.
Figure 5 | Efficient complex I assembly in long-living mice. (a) BN gels/western blots of the indicated subunits of complexes I and II in mouse liver mitochondria from 8-month-old mice under AL and DR. Large arrows mark positions of complex I and II holocomplexes. Small arrows indicate positive bands on the complex I activity gel. Arrowheads mark the positions of subcomplexes quantified in b. (b) Normalized ratios of subcomplex/holocomplex band intensity (complex I subunits) or relative intensity (SDHA) on BN gel western blots. Data are mean ± s.e.m. from four animals per group. *P<0.05, t-test. (c) BN gels/western blots of the indicated subunits of complex I in mouse liver mitochondria from 8-month-old ICRFa and C57Bl/6 mice. The large arrow marks the position of complex I holocomplex, small arrows indicate positive bands on the complex I activity gel. Arrowheads mark the positions of subcomplexes quantified in d. (d) Normalized ratios of subcomplex/holocomplex band intensity on BN gel western blots. Data are mean ± s.e.m. from four animals per group, *P<0.05, t-test. (e) Molecular weight marker positions (in kDa), activity gel and BN gel of isolated mouse liver mitochondria indicating the position of slices for proteomic profiling. (f-i) Heatmaps showing the abundance of all identified complex I subunits in slices 2-12 (normalized peptide counts relative to that in the holocomplex (slice 1)). Data are means from three animals per group. (f) C57Bl/6 mice at 15 months of age, AL; (g) C57Bl/6 mice at 15 months of age, DR; (h) C57Bl/6 mice at 8 months of age, and (i) ICRFa mice at 8 months of age. Matrix…complex I matrix arm subunits, Membr…complex I membrane arm subunits.

silencing of the complex I assembly factor NDUFAF1 in HeLa cells (Fig. 6a), which increases the relative abundance of partially assembled complex I16, decreasing protein abundance and activity in the holocomplex (Fig. 6f). Reduction of NDUFAF1 protein abundance to <50% was not associated with changes in the amount of the complex II protein SDHA, but with a potentially compensatory increase of PhB (Fig. 6a). This was associated with lower oligomycin-sensitive oxygen consumption and higher basal acidification rates in the medium (Supplementary Fig. 6), resulting in lower mitochondrial OXPHOS and higher glycolytic ATP production (Fig. 6b). Furthermore, despite lower basal oxygen consumption rates (Supplementary Fig. 6), mitochondrial superoxide production was increased as measured by fluorescence intensity of MitoSOX, a dihydroethidine (DHE) derivative directed into mitochondria (Fig. 6c). Fluorescence of the pan-cellular superoxide-sensitive dye DHE was slightly but significantly increased, and hydrogen peroxide release from complex I in isolated HeLa cell mitochondria under basal conditions was also increased following NDUFAF1 knockdown, with a tendency to increased H2O2 release under maximum stimulated respiration as well (Fig. 6d). However, dihydrorhodamine-123 (DHR-123) fluorescence indicating cellular peroxide levels was unchanged (Fig. 6c), which might indicate that under the given experimental conditions in HeLa cells hydrogen peroxide was still efficiently detoxified by cytoplasmic antioxidants. Together, these data indicate that mitochondrial superoxide production increased despite of lower OXPHOS activity as a result of NDUFAF1 knockdown.

PhBs may act as chaperones for incompletely assembled complex I subunits. To test this idea, we performed double knockdowns for NDUFAF1 and PhB in HeLa cells (Fig. 6e; see Supplementary Fig. 7 for uncropped scans). NDUFAF1 knockdown alone reduced activity of the fully assembled complex I (Fig. 6f) as described before16. It also decreased protein abundance in the holocomplex with a preference for matrix arm proteins (Fig. 6f). Importantly, both activity and protein abundance were restored by additional siRNA-mediated suppression of PhB (Fig. 6f). Moreover, knockdown of PhB completely rescued the increase in mitochondrial superoxide in...
Figure 6 | Knockdown of the complex I assembly factor NDUFAF1 increases mitochondrial ROS production and induces cellular senescence. (a) Western blots of the indicated proteins in HeLa cells treated with scrambled siRNA (scr si), siRNA against NDUFAF1 (FAF1 si) or untreated. (b) Relative ATP production by glycolysis (grey) and oxidative phosphorylation (black) in HeLa cells treated with the indicated siRNAs. Data are mean ± s.e.m., n = 3. **P < 0.001, t-test. (c) Relative fluorescence intensity of the ROS indicator dyes MitoSOX, dihydroethidine (DHE) and dihydrorhodamine-123 (DHR-123) in HeLa cells treated as indicated. Data are mean ± s.e.m., n = 6. **P < 0.001, t-test. (d) H₂O₂ release under pyruvate + malate (native conditions) and under PM + rotenone (CI max). Data are mean ± s.e.m., n = 3. *P < 0.05, t-test. (e) Western blots of the indicated proteins in HeLa cells treated with siRNAs in the indicated combinations. (f) Complex I in-gel activity (IGA) and BN/western for the complex I matrix arm subunits NDUFV2 and NDUFS3, the membrane arm subunits NDUFA9 and NDUFB9 and for SDHA as loading control. HeLa cells were treated with the indicated combinations of siRNAs. Bands at the level of the holocomplex I or complex II (SDHA), respectively, are shown. (g) Mitochondrial superoxide production (MitoSOX fluorescence activity) in HeLa cells treated with the indicated combinations of siRNAs. Data are mean ± s.e.m., n = 4. **P < 0.001, analysis of variance. (h) Western blots of NDUFAF1 and tubulin (control) in MRC5 human fibroblasts treated with siRNAs as indicated. The bar indicates 25 μm. (i) MRC5 fibroblasts treated with scr si (left) or NDUFAF1 siRNA (right) and stained for Sen-β-Gal (dark blue cytoplasmic stain). Nuclei are counterstained with DAPI. (j) Fractions of Sen-β-Gal-positive MRC5 cells following transfection with either scr si or anti-NDUFAF1 siRNA. Data are mean ± s.e.m., n = 6. **P < 0.001, Student’s t-test.

NDUFAF1 KD cells (Fig. 6g). It also tended to rescue hydrogen peroxide release from isolated HeLa mitochondria under conditions of maximum ROS production (Supplementary Fig. 8).

Finally, we asked the question whether disruption of complex I assembly would induce somatic cell ageing. When NDUFAF1 was reduced by siRNA-mediated knockdown in primary human MRC5 fibroblasts (Fig. 6h; see Supplementary Fig. 9 for uncropped scans), cell growth was decelerated and cells became positive for senescence (Sen)-associated β-galactosidase (Fig. 6i), an established marker for cell senescence. The increase in the frequency of Sen-β-Gal-positive cells was highly significant (Fig. 6i), indicating that disturbance of complex I assembly induced premature senescence of normal somatic cells.

Discussion

Few earlier studies examined changes in the liver mitochondrial proteome between young, old and dietary restricted animals. These identified a low number of ETC subunits, generally at lower abundance under DR and increased in old animals, which is consistent with our data. Similar results were found in other tissues including skeletal muscle, although...
Ghosh et al.\textsuperscript{24} reported that ageing reduced some ETC subunits along with decreased ROS production in human skeletal mitochondria. In-depth interpretation of many of these studies has been hampered by low coverage and/or sensitivity. We focused on changes that occur in the mitochondrial proteome of long-living animals at an early age and thus might be causally involved in longevity. We chose two models of longevity: DR, which leads to a robust extension of lifespan in our C57Bl/6 mice\textsuperscript{25}, and comparison between two strains (C57Bl/6 and ICRFa). We used a gel-free quantitative proteomics approach enabling detection of a large fraction of the mitochondrial proteins without recognizable bias against hydrophobic polypeptides. Use of a novel Bayesian model to evaluate the significance of differences in protein abundance from the measured peptide label distributions\textsuperscript{26} allowed high confidence even in relatively small fold-changes in protein abundance. Although we were aware of issues with the accuracy of relative quantification owing to signal compression effects when using isobaric approaches such as TMT\textsuperscript{27}, these do not affect our conclusions as they are based on high-confidence protein abundance changes, not on their magnitude.

Critically, what was common in long-lived animals was the low abundance of many of the ETC subunits proteins at a young age, which was reversed at old age. Downregulation of individual components of the ETC has been found to extend lifespan in \textit{Podospora anserina}\textsuperscript{28}, \textit{Caenorhabditis elegans}\textsuperscript{29,30}, and \textit{Drosophila melanogaster}\textsuperscript{31}, and resulted in lower acceleration of mortality with age in mice\textsuperscript{32}. In these models, successful lifespan extension was often ascribed to induction of stress–response pathways, specifically the mitochondrial unfolded protein response\textsuperscript{33,34}, although we did not see evidence for it in our long-living mice. Our data suggest that a coordinated decrease of ETC protein abundance, as found in the mitochondria from long-living mice, does not necessarily compromise mitochondrial function, so far as it supports efficient complex (and, possibly, ETC supercomplex) formation. In fact, emerging data indicate close interactions among supercomplex formation, ETC stability and ETC functionality, showing that supercomplex destabilization might contribute to increased ROS production during ageing\textsuperscript{35}.

Our study identified low abundance of complex I matrix arm subunits as the common feature in multiple tissues and multiple intervention models in long-living mice. Recent metabolite labelling study in mice\textsuperscript{36} showed that matrix arm proteins had shorter half-lives than membrane arm proteins in liver and heart, which is in agreement with the idea that matrix arm proteins might exist to a larger extent as free monomers or in less stable, smaller complexes. Furthermore, Lambert et al.\textsuperscript{36} identified low complex I content as the most probable explanation of the low hydrogen peroxide release in pigeon heart mitochondria as compared with the mouse. Our data specify this result by correlating the abundance of complex I matrix subunits with ROS production.

The structure of the mammalian complex I holocomplex is still far from clear\textsuperscript{37}. So far, stoichiometric relationships have only been established for 7 out of the 44 subunits in the holoenzyme\textsuperscript{1,2}. However, at least in HEK293 cells, the majority of complex I subunits exists in a steady-state equilibrium between supercomplexes, the holocomplex and subcomplexes\textsuperscript{1,2,13}. Our data confirm this finding for mitochondria in mouse liver. Importantly, we show that lower amounts of matrix arm subunits in mitochondria from long-lived mice are associated with a shift in the equilibrium from subcomplexes towards the holocomplex, suggesting that the relative abundance of the matrix subunits is a particularly important factor for functional optimization through more complete complex I assembly status.

Based on our data, we propose the following model (Fig. 7): relatively low abundance of matrix arm subunits favours more complete assembly of complex I. This enables efficient use of substrate for proton pumping and electron transport, resulting in high efficacy of ATP generation and only obligatory levels of superoxide production during normal electron transfer. It might be an adaptation to DR or other longevity-enabling intervention, which is independent from proton leak-mediated regulation of efficacy of ATP synthesis, and is associated with limited ROS production and long lifespan (Fig. 7, left). There are various possible mechanisms linking complex I assembly and activity with ROS production. For instance, an inverse relationship between complex I activity and ROS production was found in fibroblasts from patients with inherited complex I disorders\textsuperscript{38}. This might be because of an impact of complex I activity onto the local redox balance via oxidation of the NAD/NADH pool. Alternatively, severely compromised complex I activity with defective proton pumping might be linked to compensatory ATP hydrolysis by ATP synthase, resulting in high mitochondrial membrane potential causing high ROS production through reverse-electron flow at complex I\textsuperscript{39}. However, such an inverse correlation between complex I activity and ROS production is far from universal: In many cross-species comparisons and in multiple DR experiments, lower mitochondrial superoxide production in long-living cohorts was associated with a lower or unchanged complex I activity\textsuperscript{40–43}. Our model (Fig. 7) might explain this apparent paradox.

In old and/or shorter living mice, the relative amounts of complex I matrix arm subunits are increased, which might be caused by excessive production or insufficient degradation of unassembled matrix arm subunits, or by low efficacy of complex I assembly, although, complex I assembly factors NDUFAF1 and Acad9 were of equal abundance in all mice (Supplementary Data 1). The fact that abundance of the chaperone complex, PhB, changed in parallel to that of complex I matrix arm subunits (Supplementary Table 1), makes stabilization of unassembled subunits as its function an attractive possibility (Fig. 7, right). This was confirmed by our data showing that PhB knockdown rescued complex I formation and the enhanced complex I-dependent ROS production in cells with compromised complex I assembly. It is interesting in this context that diminished expression of PhBs is associated with pathological conditions such as chronic obstructive pulmonary disease\textsuperscript{44}, whereas increased expression of PhBs was associated with oxidative stress\textsuperscript{45}. Importantly, PhB depletion increased lifespan in \textit{C. elegans} mutants with compromised ETC\textsuperscript{46}, thereby suggesting possible long-term deleterious effects of chaperone-mediated stabilization of individual ETC components. Partially assembled subcomplexes of complex I can produce superoxide on
their own but cannot contribute to electron transfer and proton pumping, diminishing the efficiency of substrate utilization (Fig. 7, right). Such a state might be termed ‘mitochondrial cachexia’.

This model might be applicable to a number of mitochondrial diseases that are caused by mutations in complex I assembly factors. Cells from these patients accumulate partially assembled complex I subcomplexes and show impaired mitochondrial oxygen consumption. Moreover, aggressiveness of breast cancer cells in humans has been associated with a high ratio of mitochondrial DNA (NDUF53) to membrane subunits (NDUF9B) of complex I. According to our results, high NDUF53/NDUF9B ratios are indicative of suboptimal complex I assembly, which is in turn associated with a shift in ATP production from oxidative phosphorylation towards glycolysis (Fig. 6). Defective complex I assembly, or generally an imbalance between different subunits of the ETC, might thus be a mechanistic cause of shifts in the energy production that has been linked to fast growth and aggressiveness of tumour cells.

Moreover, unbalanced complex I assembly might cause premature cellular senescence by either increased ROS production, inducing DNA damage, including premature telomere shortening, by changed energy metabolism, or both. Cell senescence is mechanistically linked to accelerated ageing: Senescent cells increase with age in multiple tissues of mice and primates and decrease in frequency under DR. They secrete bioactive molecules including cytokines and ROS that can damage neighbouring cells. Targeted ablation of senescent cells rescued age-related functional deterioration in multiple tissues in at least one progeroid mouse model. Together, these cells rescued age-related functional deterioration in multiple tissues of mice, and 0.5 mM EDTA, pH 7.4, at 4°C. Mitochondria from HeLa cells were isolated using a mitochondrial isolation kit (MACS Miltenyi Biotec) according to the manufacturer’s instructions.

Mitochondrial proteomics by TMT labelling. Frozen mitochondrial pellets were disrupted by three cycles of thawing on ice and refreezing in liquid N2. Subsequently, 150 µg protein per sample was dissolved in 50 µl of 1.5 M urea and 90 mM triethylammonium bicarbonate, pH 8. Samples were reduced with 5 µl of 200 mM Triis(2-carboxyethyl)phosphine in 30 mM triethylammonium bicarbonate at 55°C for 1 h, followed by the addition of 5 µl of 375 mM iodoacetamide (ApplyChem) and incubation for 30 min at room temperature in the dark. Proteins were digested with trypsin (1:50 w/w) overnight. Peptides were labelled with 6-plex Tandem Mass Tags (TMT) (Pierce, Cranmerling, UK) according to manufacturer’s instructions. Samples were analysed in two sets of experiments (Exp1 and Exp2), each containing one-pooled sample for cross-standardization. In addition to the pool sample, Exp1 consisted of 3 × DR and 2 × AL C57/B6 and Exp2 included 2 × young ICRAF, 2 × old ICRAFs and 1 × AL C57/B6. Every experimental sample was from one separate animal.

Samples were separated in 11 (Exp1) or 10 (Exp2) fractions using a 3100 Offgator Fractionator (Agilent) following the manufacturer’s instructions. Peptides from each fraction were analysed on Dionex Ultimate 3000 nano-HPLC system with a Dionex PepMap column (75 µm × 250 mm) coupled to an Orbitrap LTQ XL mass spectrometer. Peptides were eluted with a gradient from 5% acetonitrile (MeCN), 0.08% formic acid to 60% MeCN, 0.08% formic acid over 132 min. The elution rate was set to 200 nl/min for the LC pump and the gradient was performed with the CID and the HCD spectra in the Orbitrap with a resolution of 30,000 at m/z 400 Da. Up to three sets of data-dependent MS/MS scans were acquired for the three most intense precursor ions, with a collision-induced dissociation (CID) spectrum (in the LTQ) and an higher energy collision dissociation (HCD) spectrum (in the Orbitrap) that was the same precursor ion. The CID spectra were acquired in the linear ion trap, collision energy of 35%, Q value of 0.25 and an activation time of 30 ms. HCD spectra were acquired with a resolution of 7,500 (at m/z 400) and a collision energy of 60%.

Raw data were processed using ProteomeDiscoverer v 1.1 (Thermo). HCD spectra were used for the ‘reporter ions quantizer’ node using an integration window tolerance of 20 p.p.m. and TMT batch-specific isotope impairment correction factors and raw quan values as an output variable. Using Proteome Discoverer as an interface, a concatenated database containing mouse proteins from swissprot (v 2010_04), trembl (v 2010_04) and cRAP (ftp://ftp.thepgm.org/fasta/cRAP), comprising a total of 55,359 sequences, was searched with the CID and the HCD spectra using Mascot v. 2.2 (Matrix Science) and the following parameters: 1 missed tryptic cleavage site, 1% FDR (determined using a reversed decoy database), 10 p.p.m. precursor tolerance, 0.8 Da fragment ion tolerance, carbamidomethyl (on Cys) and 6-plex TMT (on N-termini and on Lys) as fixed modifications, oxidation (Methylthiolation (M)) as variable modification (Lys) and 'Acetyl-lysine (T)' for the same precursor ion. The MS files were then converted into a format that was suitable for processing by the purpose-written Bayesian tool dpeaqms (deposited on R-forge, http://r-forge.r-project.org/projects/dpeaqms/). Dpeaqms uses a Bayesian approach and a Bayesian Chain Monte Carlo (MCMC) method. In this method, the posterior probability that the conducted experiments indicate a systematic change in protein concentration between groups. The statistical model takes into account (i) a sample specific normalization term (to account for loading differences); (ii) a mean reporter ion intensity (assuming no systematic differences for the same MS spectrum); (ii) a mean difference in ion intensity; and (iv) an independent and identically distributed noise terms following a normal distribution. A β < 0.5 was chosen as threshold for abundance differences, according a FDR in the order of 1% (ref. 26).

To perform group comparisons across both experiments, that is, for the DR–AL and the ICRAFs–C57/B6 comparisons, pooled samples were used as a standard (combined analysis). A DR–AL comparison was also performed within Exp1 only, and the young–old comparison was only made in Exp2. For these comparisons, the data were analysed within the set and the pooled sample was not used (solo analysis). For the DR–AL and the ICRAFs–C57/B6 analysis, the model (Supplementary Table 1A). This validation uses our pooled samples as a standard and indicates that the chosen number of animals are sufficient for a robust estimate of differences. For the final DR–AL comparison, we considered proteins as confidently...
different in abundance only when their $\beta$ values were $\geq 0.5$ in both sets of analysis. The results for all the comparisons are presented as Supplementary Data 1.

For mitochondria-specific KEGG pathway enrichment analysis, KEGG pathway finder was used to identify all KEGG pathways that contain at least one mitochondrial protein that has changed with a probability $p < 0.5$. Next, all mitochondrial proteins belonging to each identified KEGG pathway were identified using MitoMiner v2 (ref. 8). KEGG pathway terms enrichment analysis was performed in R with a hypergeometric distribution function separately for proteins that were either more or less abundant under DR. This was compared with enrichment of the same pathways based on proteins more or less abundant, respectively, in young or old ICRFa.

Heatmaps of the ETC subunits were drawn using Cytoscape. Based on proteins more or less abundant, respectively, in young or old ICRFa. Less abundant under DR. This was compared with enrichment of the same pathways hypergeometric distribution function separately for proteins that were either more or less abundant under DR. This was compared with enrichment of the same pathways based on proteins more or less abundant, respectively, in young or old ICRFa. Heatmaps of the ETC subunits were drawn using Cytoscape.

**Mitochondrial complex I profiling.** Mitochondria were solubilized in sample buffer (50 mM BisTris, 6 N HCl and 50 mM NaCl, 10% w/v glycerol, pH 7.2) with dithiothreitol (1:2) and destained using 3.2 g of detergent (decrease in detergent/protein ratio). The sample was then separated by BN gel electrophoresis according to manufacturer's instructions (Life Technologies), using a 4–16% gel. Gel lanes were cut into 12 slices (Fig. 4c, which were destained, reduced using 10 mM Dithiothreitol and alkylated in the dark using 50 mM iodoacetamide (Promega) before digestion with trypsin (Proteomic Technologies), using a 4–16% gel. Gel lanes were cut into 12 slices (Fig. 4e), which were desalted using stage tips concentrated in a speedvac and acidified using 1% trifluoroacetic acid.

Peptides were separated using a PepMap column (250 mm x 75 mm, Thermo) and a water/acetonitrile gradient from 4–40% acetonitrile over 100 min. A combination of data from all experiments and fragments (15 000 fragments) were used to generate the mass inclusion list (see Supplementary Data 1) in X!Tandem. Peptide retention times in the inclusion lists were either obtained from our own experimental data or estimated using the pyteomics mass inclusion list.

BioML files obtained from X!Tandem were parsed and the results were annotated using in-house written python scripts (available from the authors upon request). Peptide and protein sequences were extracted, annotated with band position, repeat number (1–3) and experimental treatment, peptide lists were filtered to include only complex I peptides, PhB and NDUFA4 and rolled up into protein lists. The number of peptides found for each protein in each band was normalized by dividing it by the number of all identified peptides for the corresponding lane (mitochondrial sample) in the BN gel and then multiplied with 10%. Averaged peptide lists from these three mice were per group analysed independently. Averaged counts were used to generate heatmaps following normalization to counts in slice 1 (the holocomplex).

**Functional experiments.** Mitochondrial oxygen consumption rates were measured using Seahorse XF 24 analyzer according to the manufacturer’s instructions (see also ref. 68 and comments therein). Five micrograms per well mitochondria were added to the 24-well plate containing 15% FCS and oxygen consumption rates were measured in the assay buffer (115 mM KCl, 10 mM KH2PO4, 2 mg/mL MgCl2, 3 mM HEPES, 1 mM EGTA and 0.2% fatty acid-free BSA, pH 7.2, at 37°C) with substrate (5 mM pyruvate and 5 mM malate for complex I-linked substrate or 4 mM succinate for complex II-linked substrate). State 3 oxygen consumption rates were obtained by adding 4 mM ADP. Mitochondrial hydrogen peroxide release was measured fluorometrically in the presence of 2 nitrophenyl substrates, 2,4-dinitrophenyl substrate (15 µM) and horseradish peroxidase (2 U·mL$^{-1}$) and Amplrex red (50 µM) at 37°C, and the fluorescence signals were calibrated against a hydrogen peroxide standard.

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**Additional information**

**Accession codes** The mass spectrometry proteomics data have been deposited in the Proteomics Identifications database (PRIDE) under the accession code PXD000272.

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

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