Mitonuclear protein imbalance as a conserved longevity mechanism

Riekelt H. Houtkooper1,2,*, Laurent Mouchiroud1,*, Dongryeol Ryu1, Norman Moullan1, Elena Katsyuba1, Graham Knott3, Robert W. Williams3 & Johan Auwerx1,2

Longevity is regulated by a network of closely linked metabolic systems. We used a combination of mouse population genetics and RNA interference in Caenorhabditis elegans to identify mitochondrial ribosomal protein S5 (Mrps5) and other mitochondrial ribosomal proteins as metabolic and longevity regulators. Mrp knockdown triggers mitonuclear protein imbalance, reducing mitochondrial respiration and activating the mitochondrial unfolded protein response. Specific antibiotics targeting mitochondrial translation and ethidium bromide (which impairs mitochondrial DNA transcription) pharmacologically mimic Mrp knockdown and extend worm lifespan by inducing mitonuclear protein imbalance, a stoichiometric imbalance between nuclear and mitochondrially encoded proteins. This mechanism was also conserved in mammalian cells. In addition, resveratrol and rapamycin, longevity compounds acting on different molecular targets, similarly induced mitonuclear protein imbalance, the mitochondrial unfolded protein response and lifespan extension in C. elegans. Collectively these data demonstrate that Mrps represent an evolutionarily conserved protein family that ties the mitochondrial ribosome and mitonuclear protein imbalance to the mitochondrial unfolded protein response, an overarching longevity pathway across many species.

Longevity is coordinated by intersecting pathways, often converging on metabolic networks1–4. A key player in lifespan regulation is the mitochondrion. Over a thousand proteins encoded by nuclear DNA (nDNA) translocate to and function in mitochondria5, in synchrony with mitochondrion. Over a thousand proteins encoded by nuclear DNA (nDNA) translocate to and function in mitochondria5, in synchrony with mitochondrial DNA (mtDNA) that require a separate translation machinery, including mitochondrial ribosomal proteins (MRPs)6,7. Many molecular studies of longevity have exploited simple organisms and loss- or gain-of-function mutations, but the complex connectedness of mitochondrial and metabolic longevity networks benefits from an integrative cross-species approach8.

Here we pioneered such a strategy and used the BXD reference population of mice9–11 to identify mitochondrial ribosomal protein S5 (Mrps5) and other members of the MRPs family as longevity genes. In C. elegans, we confirmed this role of MRPs and demonstrated that they induce a stoichiometric imbalance between nDNA- and mtDNA-encoded oxidative phosphorylation proteins, hereafter termed ‘mitonuclear protein imbalance’, which activates the mitochondrial unfolded protein response (UPRmt). Our conclusions were corroborated using specific antibiotics targeting bacterial/mitochondrial translation, and ethidium bromide, which inhibits mtDNA transcription. This mechanism is shared with pathways that induce mitochondrial protein imbalance from a nuclear perspective, such as the UPRmt and lifespan enhancing effects of rapamycin and resveratrol. Our data hence tie mitochondrial translation and metabolism to mitochondrial protein imbalance from a nuclear perspective, such as the UPRmt and lifespan enhancing effects of rapamycin and resveratrol.

A QTL for mouse longevity

The BXD family consists of fully inbred progeny of a cross between C57BL/6J and DBA/2J mice, with a complexity that matches many human populations11. Both parental strains have been sequenced, enabling analysis of sequence variants linked to phenotypes12. We used new genomic and genetic resources to re-analyse longevity data for BXD lines13 using forward and reverse genetic methods.

The forward strategy exploits longevity data and updated high-density single nucleotide polymorphism (SNP) genotypes14 archived in http://www.GeneNetwork.org. As reported13, lifespan of BXDs varies from ~365 days for the shortest lived strain to ~900 days for the longest lived strain (Fig. 1a). We remapped longevity using the new genotypes and detected one genome-wide significant locus on chromosome 2 with a peak at 124–129 Mb (Fig. 1b, log odds ratio (lod) = 4.0). Two additional loci, on chromosomes 4 and 7, were not significant, but suggestive (lod = 2.8 and 3.0, respectively). However, neither was suggestive after controlling for SNP rs6374387 on chromosome 2 using composite interval mapping.

The chromosome 2 locus contains ~70 genes (Supplementary Table 1), none of which were previously linked to longevity. To evaluate and rank candidates, we correlated lifespan with multiple gene expression data sets. Only three genes in the locus correlate strongly with lifespan (Fig. 1c, P < 0.01; Supplementary Fig. 1): solute carrier family 12 member 1 (Slc12a1), mitochondrial ribosomal protein S5 (Mrps5) and tubulin tyrosine ligase (Ttl). From the natural variation in expression of these genes, we deduced that 50% reduction of expression corresponds to a ~250 day lifespan difference.

Conservation of longevity in C. elegans

We identified Y37A1C.1/nkcc-1, E02A10.1/mrps-5, and F25C8.5/till9 as worm homologues of Slc12a1, Mrps5 and Ttl, respectively. RNA interference (RNAi)–mediated knockdown of nkcc-1 and mrps-5, but not of till-9, extended lifespan (Fig. 2a).

Next, we compared expression of Mrps5 and other Mrp family members in a muscle microarray of ageing and caloric restriction in C57BL/6J (ref. 15). Mrp expression decreased with age, an effect rescued by...
caloric restriction; in contrast, expression of Slc12a1 and Ttl was unaffected (Fig. 2b). Linkage of MRPs with lifespan is strengthened as many other Mrp family members also correlate with longevity (Fig. 2c). We extended our analyses to the DNA level using sequence data for Mrps5 in both parental strains and identified missense variants in exon 3 (rs29667217 and rs13471334; V60A and V67I, respectively). Other sequence variants in Mrps5 contribute to variation in transcript abundance; Mrps5 mRNA levels among the BXDs are associated with a strong quantitative trait loci (QTL) superimposed over the gene itself—a cis-expression QTL.

Using a reverse genetics approach, we studied the Mrps5-associated network. Mrps5 expression co-varies with genes involved in oxidative phosphorylation. Considering that oxidative metabolism is involved in known longevity pathways, the set of transcripts that co-vary with Mrps5 qualified as an appealing longevity network. Oxidative phosphorylation was the most enriched network of Mrps5 co-variates in both BXDs and a conventional F2 intercross (P = 1.53 × 10⁻²¹, P = 5.78 × 10⁻¹⁰, respectively). Finally, we generated an interaction network of oxidative phosphorylation genes with Mrps5 (Fig. 2d), in which Ndufb7 provides the hinge that links Mrps5 to oxidative phosphorylation. Knockdown of the worm homologues for the network components Ndufb7 and Ndufa6 robustly extended lifespan. Mrps5 hence emerged as a strong longevity candidate, integrating protein synthesis and mitochondrial metabolism—both important longevity modulators.

Figure 1 | Lifespan regulation in BXD recombinant inbred mice. a, Lifespan in different BXD strains. b, Interval mapping using the BXD lifespan data reveals a strong QTL on chromosome 2, between 124–129 Mb. The red line depicts the cut-off for statistical significance (P genome-wide < 0.05), the grey line represents the limit for suggestive QTLs. See also Supplementary Table 1. LRS, likelihood ratio statistics. c, Pearson’s r correlation coefficient with corresponding P values for the co-variation between BXD lifespan (x axis) and mRNA expression of the indicated gene in the BXD eye microarrays (y axis). Decreased expression of Slc12a1, Mrps5 and Ttl robustly correlates with longevity (P < 0.01). Correlation coefficient trend line is shown in green.

Figure 2 | Validation of Mrps5 as a candidate longevity gene. a, Knockdown of mrps-5, nck-1 or till-9 throughout the entire life of C. elegans increased lifespan by 60%, 23% or 3%, respectively. See also Supplementary Table 2. NS, not significant. b, Hierarchical clustering showing gene expression differences in gastrocnemius muscle between young (5 months), old (25 months) and caloric restricted C57BL/6J mice. Expression of mouse Mrp genes decreases upon ageing, and reverts with caloric restriction, whereas Slc12a1 and Ttl do not change. The colour-coded heat map represents relative gene expression differences (red, large gene expression values; blue, small gene expression values). c, Pearson’s r correlation coefficient with corresponding P values for co-variation between BXD lifespan (x axis) and mRNA expression of eleven other Mrp genes (y axis) indicates robust correlation. Principle component analysis (PCA) reveals a highly significant correlation between the Mrp gene family and BXD lifespan. d, Mrps5 strongly correlates with genes involved in oxidative phosphorylation. Red lines indicate a positive Pearson correlation coefficient of 0.7–1.0, and blue lines indicate a correlation coefficient of 0.5–0.7.

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Mitonuclear protein imbalance and ageing

To define causality of the MRPs in determining lifespan, we knocked down mrp genes during the entire life of the worm and robustly increased lifespan (Fig. 3a and Supplementary Table 2). Similar to well-characterized mitochondrial mutants that live longer, larval development was delayed (Supplementary Fig. 2a). Knockdown during development proved crucial and sufficient to extend lifespan, whereas RNAi during adulthood alone did not (Fig. 3b, Supplementary Fig. 2b and Supplementary Table 2), as reported in other long-lived mitochondrial mutants. Increased lifespan was not due to effects on feeding, as pharyngeal pumping rates were normal (Supplementary Fig. 2c). mrps-5 RNAi also delayed physiological decline with age. Even though they moved slightly less in early adulthood (day 3), mrps-5 RNAi worms move twice as much as controls at day 13, and this effect becomes more pronounced at day 20 (Supplementary Fig. 2d, e and Supplementary Videos 1–4). This difference was accompanied by a delay in decline of pharyngeal pumping (Supplementary Fig. 2c) and in muscle fibre disorganization (Fig. 3c), hallmarks of fitness of aged mrps-5 RNAi worms.

In line with the mitochondrial connection of Mrps5, basal respiration was reduced upon mrp knockdown and unresponsive to the mitochondrial uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Fig. 3d). As a consequence, mrps-5 RNAi worms displayed reduced ATP levels and citrate synthase activity (Fig. 3e, f), indicative of reduced mitochondrial abundance or activity. Consistent with its role in mitochondrial translation, mrps-5 RNAi induced a stoichiometric imbalance between nDNA- and mtDNA-encoded oxidative phosphorylation subunits, termed mitonuclear protein imbalance, visualized by selective reduction in MTCE.26 (MTCO-1 homologue; from mtDNA) relative to H2O16.1 (ATP5A homologue; from nDNA) expression (Fig. 3g). The mitonuclear protein imbalance and consequences for mitochondrial function was similar to the long-lived cco-1 mutant—deficient for the nDNA-encoded worm homologue of complex IV, subunit Vb/COX4—but not observed in the short-lived complex II SDHC mutant mec-1 (Fig. 3g). Furthermore, mitochondria had a more punctuate globular pattern instead of the regular reticular or tubular appearance in both muscle (Fig. 3h) and intestine, a finding confirmed by electron microscopy (Supplementary Fig. 3a, b).

To identify which longevity pathways—insulin/IGF-1 signalling,23 caloric restriction26 or mitochondrial dysfunction22—are required for the lifespan phenotype, we reduced mrps-5 expression in worms carrying mutations in these pathways. mrps-5 RNAi increases lifespan by ~40% in wild type (Fig. 3i), similar to the effect in daf-2, daf-16, eat-2, sir-2.1 and aak-2 mutants (Fig. 3j–l and Supplementary Fig. 4a–e) indicating that mrps-5 regulates longevity independently of insulin/IGF-1 (daf-16/daf-2) and caloric restriction (eat-2/sir-2.1) and acts downstream of mitochondrial regulator aak-2.

We focused on the mitochondrial pathway, because (1) it robustly affects longevity23; (2) MRPs function in the translation of mtDNA-encoded oxidative phosphorylation subunits;21 and (3) in the BXDs, Mrps5 networked with several oxidative phosphorylation components (Fig. 2d). mrps-5 RNAi reverts the short-lived phenotype of mev-1 mutants, with a dramatic 112% lifespan extension (Fig. 3m and Supplementary Fig. 4f). mrps-5 RNAi in the cco-1 mutants did not extend lifespan compared to mrps-5 RNAi alone, indicating that cco-1 and mrps-5 act in a similar fashion (Fig. 3n and Supplementary Fig. 4g). The same is true for mrps-5 RNAi in the mitochondrial clk-1(e2519)

**Figure 3** | mrps-5 RNAi prevents ageing-associated functional decline and alters mitochondrial function. a, Knockdown of mrpl-1, mrpl-2 or mrpl-37 increased lifespan by 57%, 54%, or 41%, respectively. b, When RNAi of mrps-5 was performed during the larval stages only, lifespan increased by 48%, whereas RNAi started from the L4 stage had no effect. P ≤ 0.001 is for larval-only versus either vector control or adult-only. c, mrps-5 or coo-1 RNAi prevented age-related changes in muscle morphology as evidenced by a worm pmyo-3::MYO-3-GFP reporter marking myosin heavy chain. d, mrp RNAi in C. elegans decreased respiration. Respiration per worm is shown, respiration was similarly decreased when corrected for protein. FCCP was added at the indicated time. Values are mean ± s.e.m. (n = 10). **P ≤ 0.001. e–g, mrps-5 RNAi decreased ATP levels (e, n = 3), citrate synthase activity (f, n = 3), and altered the ratio between nDNA (ATP5A) versus mtDNA-encoded (MTCO1) oxidative phosphorylation proteins, similar to cco-1, but not mev-1 (g, n = 4). *P ≤ 0.05, A, attendance. h, mrps-5 RNAi resulted in fragmented mitochondria, as visualized in body wall muscle (day 2, adult worms) using the pmyo-3::mito::GFP reporter, which expresses mitochondrial-targeted GFP driven by the musclespecific myo-3 promoter. i, mrps-5 RNAi increased mean lifespan by 40%. j–m, mrps-5 RNAi extends lifespan of daf-16(mu86) (j), sir-2.1(ok3434) (k), aak-2(ok524) (l), mev-1(kn1) (m) mutants by 37%, 40%, 69% and 112%, respectively. n, Knockdown of cco-1 does not extend lifespan of mrps-5 RNAi worms. See Supplementary Table 2 and Fig. 4.
mutant, confirming the link with mitochondrial longevity pathways (Supplementary Fig. 4h).

Mitochondrial unfolded protein response

The mitochondrial unfolded protein response accounts for longevity upon cco-1 loss-of-function and is selective for the mitochondrial pathway and not involved in the caloric restriction or insulin/IGF-1 pathways. UPRmt is induced by mitochondrial stress, subsequently activating a nuclear transcriptional response, inducing the chaperones HSP-6 (HSP-70 in mammals) and HSP-60 to restore mitochondrial proteostasis. We monitored UPRmt using hsp-6::GFP (green fluorescent protein) and hsp-60::GFP reporter worms with reduced mrp expression. Similar to the cco-1 mutant, hsp-6 and hsp-60 were induced in worms with reduced mrp (Fig. 4a–c and Supplementary Fig. 5a, b). This was specific for UPRmt, as mrps-5 RNAi did not affect UPR in the endoplasmic reticulum (UPRER) and cytoplasmic heat shock response (Supplementary Fig. 5c). As for lifespan, UPRmt was not induced when mrp expression was only inhibited during adulthood (Supplementary Fig. 5d). We measured UPRmt upon combined mrps-5 and mev-1 inactivation. Whereas mev-1 RNAi alone did not induce UPRmt (Fig. 4d, c), combined inactivation induced mitochondrial protein imbalance (Supplementary Fig. 5e) and synergistically induced UPRmt (Fig. 4d), accounting for the extended lifespan. Double inactivation of mrps-5 and cco-1 did not further enhance UPRmt compared to mrps-5 alone (Fig. 4d), in line with the similar lifespan.

There are individual differences in the degree of UPRmt within the mrps-5 RNAi worm population, which tightly correlate with lifespan extension (Supplementary Fig. 5f, h). GFP expression stayed similar with RNAi against genes that HSP-6 (HSP-70 in mammals) and HSP-60 to restore mitochondrial function, activating a nuclear transcriptional response, inducing the chaperones HSP-6 (HSP-70 in mammals) and HSP-60 to restore mitochondrial proteostasis. We monitored UPRmt using hsp-6::GFP (green fluorescent protein) and hsp-60::GFP reporter worms with reduced mrp expression. Similar to the cco-1 mutant, hsp-6 and hsp-60 were induced in worms with reduced mrp (Fig. 4a–c and Supplementary Fig. 5a, b). This was specific for UPRmt, as mrps-5 RNAi did not affect UPR in the endoplasmic reticulum (UPRER) and cytoplasmic heat shock response (Supplementary Fig. 5c). As for lifespan, UPRmt was not induced when mrp expression was only inhibited during adulthood (Supplementary Fig. 5d). We measured UPRmt upon combined mrps-5 and mev-1 inactivation. Whereas mev-1 RNAi alone did not induce UPRmt (Fig. 4d, c), combined inactivation induced mitochondrial protein imbalance (Supplementary Fig. 5e) and synergistically induced UPRmt (Fig. 4d), accounting for the extended lifespan. Double inactivation of mrps-5 and cco-1 did not further enhance UPRmt compared to mrps-5 alone (Fig. 4d), in line with the similar lifespan.

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Two downstream effectors of UPRmt are HAF1, a mitochondrial peptide transporter, and UBL5, a ubiquitin-like protein that regulates the transcriptional activation of mitochondrial chaperones. Knockdown of haf-1 along with mrps-5 RNAi reduced lifespan extension, UPRmt and increased respiration (Fig. 4f–h). Similarly, when both ubl-5 and mrps-5 were knocked down, lifespan extension, the respiration phenotype and UPRmt were partially lost, in line with the double cco-1 and ubl-5 RNAi treatment (Supplementary Fig. 7a–f).

This network could be traced back to mice, as Ubl5 and the most likely mouse haf-1 homologue—Abcb10—correlated tightly with Mrp genes, for instance in the hippocampus of the BXDs and in adipose tissue of F2-intercrossed mice (Fig. 4i and Supplementary Fig. 7g). Additionally, Hsp61 (also known as Hsp60) correlated with several Mrp genes (Fig. 4i and data not shown). Gene ontology analysis showed strong connectivity between Ubl5 and oxidative phosphorylation genes (P = 9 × 10^−4 in eye; P = 8.62 × 10^−10 in hippocampus), the translation process or ribosome (P = 6 × 10^−4 eye; P = 6.03 × 10^−15 hippocampus) and the mitochondrial inner membrane (P = 1 × 10^−4 eye; P = 3.31 × 10^−27 hippocampus) in the BXDs. Finally, we tied Hsp61 in a close correlation network with various Mrp and oxidative phosphorylation genes (Fig. 4j).

Pharmacological mitonuclear protein imbalance

Many mitochondrial functions can be traced back to their endosymbiotic ‘bacterial’ origin. Consequently, antibiotics that target bacterial translation also inhibit mitochondrial translation. We therefore used doxycycline to confirm the role of mitochondrial translation in longevity, using carbenicillin—targeting the bacterial cell wall—as a control. We used heat-killed OP50 or live HT115 bacteria—the latter insensitive to low concentrations of doxycycline (data not shown)—to feed worms, to prevent antibiotic effects on bacteria. Doxycycline, given throughout life, dose-dependently extended lifespan, induced UPRmt not UPRer, and reduced oxygen consumption, without affecting ATP levels or citrate synthase activity (Fig. 5a–e and Supplementary Fig. 8a, b). Doxycycline at 60 μg ml^−1 caused developmental delays in hippocampus and adipose tissue (data not shown). UPRer correlated with several oxidative phosphorylation genes (P = 9 × 10^−4 in eye; P = 8.62 × 10^−10 in hippocampus), the translation process or ribosome (P = 6 × 10^−4 eye; P = 6.03 × 10^−15 hippocampus) and the mitochondrial inner membrane (P = 1 × 10^−4 eye; P = 3.31 × 10^−27 hippocampus) in the BXDs. Finally, we tied Hsp61 in a close correlation network with various Mrp and oxidative phosphorylation genes (Fig. 4j).

Figure 4 mrp genes confer longevity effects through UPRmt. a, RNAi of mrp genes induced UPRmt (hsp-6::GFP reporter), similar to cco-1 knockdown. Worms were synchronized at day 1 of adulthood. DIC, differential interference contrast. b, Quantification of UPRmt upon knockdown of mrp or cco-1 (n = 4). c, mrps-5 and cco-1, but not mev-1. RNAi induce UPRmt as reflected by the induction of HSP-6::GFP protein. d, Combined RNAi of mrps-5 and mev-1 synergistically increased UPRmt, whereas combined cco-1 and mrps-5 RNAi did not further increase UPRmt (n = 6). e, Knockdown of different mrp genes results in different levels of UPRmt, which correlates with mean lifespan (n = 33–61 worms for lifespan, n = 3 for GFP). f–h, Epistasis with UPRmt regulator haf-1. Double RNAi of mrps-5 and haf-1 partially prevented lifespan extension (f), UPRmt (g, n = 5), and reduction in respiration (h, n = 10), compared to mrps-5 RNAI alone. i–j, In various tissues of mouse crosses, Ubl5, Abcb10 and Hsp61 expression correlated with Mrp expression. j, Hsp61 (also known as Hsp60) ties in a correlation network with Mrp and oxidative phosphorylation genes. Connecting lines indicate a Pearson correlation coefficient of 0.75–1.0. Bar graphs show mean ± s.e.m., *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001. See also Supplementary Figs 5–7 and Supplementary Table 3.
Delay, like mrps-5 RNAi, but no abnormalities were apparent at lower concentrations (data not shown). A low concentration of doxycycline (6 μg ml^{-1}), given only during development, also increased lifespan and UPR^mt and attenuated respiration (Fig. 5i–h). Chloramphenicol—belonging to a different class of antibiotics targeting translation—also increased lifespan and UPR^mt, at the same time as decreasing respiration (Fig. 5i–k), when administered during development. Similar to mrps-5 RNAi, doxycycline increased the ratio of nDNA- (ATP5A) over mtDNA-encoded (MTCO1) oxidative phosphorylation proteins (Fig. 5i).

Linking back to mammals, doxycycline decreased respiration in a cultured hepatocyte cell line (Fig. 5m). Doxycycline also induced UPR^mt, as evidenced by induction of Hsp60 (Fig. 5n) and the UPR^mt protease ClpP (Supplementary Fig. 6c), and increased HSP60 protein expression in hepatocyte cell lines and primary murine hepatocytes (Fig. 5o, p). Doxycycline induced a striking mitonuclear protein imbalance in hepatocytes (Fig. 5o, p). Finally, feeding mice with doxycycline for 10 days lowered oxygen consumption in vivo, indicative of attenuated mitochondrial function (Fig. 5q).

Similar effects on mitonuclear protein imbalance, UPR^mt, respiration and lifespan, without affecting mitochondrial morphology, were also observed in worms exposed to low concentrations of ethidium bromide, which inhibits mtDNA transcription specifically\(^32\) (Supplementary Fig. 8d–h). This indicates that mitonuclear protein imbalance is the common underlying mechanism that links basic mitochondrial function to lifespan regulation.

**A conserved longevity mechanism**

To define how intricately mitonuclear protein imbalance and UPR^mt are involved in longevity, we analysed its activation in worms exposed to rapamycin\(^33,34\). Rapamycin inhibits TOR signalling to alter nDNA translation, inducing mitonuclear protein imbalance\(^35\), and increases lifespan in various species, including mice\(^31\). Rapamycin also increased mean worm lifespan (by 16%)\(^34\) in a \(ubl-5\)-dependent manner, induced UPR^mt, but not UPR^ER or heat shock response, and increased respiration (Fig. 6a, c and Supplementary Fig. 9a). This was associated with increased ATP levels, equal citrate synthase activity and altered nDNA/mtDNA oxidative phosphorylation protein ratio (Fig. 6d, e). Additionally, rapamycin changed the balance between nDNA- and mtDNA-encoded oxidative phosphorylation subunits in mouse hepatocytes in a dose dependent manner (Fig. 6f, g). This mitonuclear protein imbalance induced HSP60 and ClpP (Fig. 6f–h). Similarly, the lifespan enhancer resveratrol induced mitonuclear protein imbalance in hepatocytes (Fig. 6i) and \(ubl-5\)-dependently increased worm lifespan and UPR^mt but not UPR^ER or heat shock response, at the same time as increasing respiration and maintaining ATP levels and citrate synthase activity (Supplementary Fig. 9b–f). Mitonuclear protein imbalance and UPR^mt hence represent an overarching mechanism of longevity that also can be engaged by pathways that signal mainly through the nucleus.

Finally, we tested whether reactive oxygen species (ROS) and mitohormesis, a theory which posits that an initial ROS burst (after 24 h) induces adaptive long-term protection\(^36\), could explain our worm phenotypes. However, no ROS was produced after 24 h of mrps-5 RNAi or doxycycline, rapamycin or ethidium bromide treatment (Supplementary Fig. 10a). In addition, the mitohormesis regulators \(daf-16\) and \(aak-2\) (ref. 36, 37) were not involved in UPR^mt induction (Supplementary Fig. 10b) or lifespan extension (Fig. 3j, l) following mrps-5 RNAi. Finally, the ROS scavenger N-acetylcysteine (NAC) did...
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rapamycin induces mitonuclear protein imbalance (induced mitonuclear protein imbalance (ubl-5)), not abrogate the mrps-5 RNAi- or rapamycin-induced UPR\textsuperscript{mt}, nor did it suppress longevity (Supplementary Fig. 10c–f), similar to NAC-ageing\textsuperscript{37}, UPR\textsuperscript{mt}-mediated longevity is independent of ROS.

Discussion

Using the BXD inbred wild type mouse panel, we identified a chromosome 2 QTL that is responsible for longevity, with Slc12a1, Mrps5 and Tll showing strong correlation with lifespan. A holistic approach, involving bioinformatics, genetics and pharmacological strategies in both worms and mammals, established that Mrps5 and the Mrp protein family are the main actors in metabolic lifespan regulation. The MRPs constitute the mitoribosome that regulates translation of 13 mtDNA-encoded proteins, underscoring the vital importance of mitochondrial protein production\textsuperscript{67}.

Inhibiting mitochondrial translation reduced respiration and extended lifespan. There is an apparent dichotomy, however, as rapamycin (this study) as well as NAD\textsuperscript{+} boosters—resveratrol (this study), nicotinamide riboside, nicotinamide and PARP inhibitors (L.M., R.H.H. and J.A., unpublished observations)—couple longevity to increased respiration. Abnormal mitochondrial proteostasis could reconcile these disparate observations. Knockdown of mrps-5 or cco-1 affect proteostasis and activate UPR\textsuperscript{mt} (refs 25, 28). From the cco-1 study\textsuperscript{25} it was, however, unclear if there was a direct connection between the level of UPR\textsuperscript{mt} and the lifespan extension. Our data demonstrate a positive correlation between the level of UPR\textsuperscript{mt} and lifespan. Moreover, UPR\textsuperscript{mt} seems to result from an imbalance between mtDNA- and nDNA-encoded proteins and is a common feature linking mitochondrial longevity pathways. Genetic defects in mrp or respiratory chain genes, antibiotics that inhibit translation, or moderate mtDNA transcription inhibition, induce such a mitonuclear protein imbalance from within mitochondria. Conversely, shown at the protein (f, g, n = 3), and transcriptional (h, n = 8) level.

resveratrol and rapamycin change the production of nDNA-encoded mitochondrial proteins and if this is not matched with the levels of mtDNA-encoded proteins, mitonuclear protein imbalance and UPR\textsuperscript{mt} will also ensue, which favours longevity (Fig. 6j). The reason why mev-1 mutants do not display UPR\textsuperscript{mt} is consistent with the fact that complex II is entirely nDNA-encoded and therefore does not require a balanced production of proteins from the nDNA and mtDNA. Additionally, complex II can be bypassed for mitochondrial ATP generation and is not part of oxidative phosphorylation supercomplexes\textsuperscript{38}. Although further work to validate this hypothesis is warranted, this could explain apparent contradictions such as why mutations that either decrease or increase respiration can both induce longevity.

Our data identify MRPs as a novel longevity protein family, conserved from worms to mammals. The identification of these genes was triggered by analysis of murine reference populations. Hence, it is natural variation in Mrp expression, not artificial loss- or gain-of-function, that translates to longevity. In worms, longevity involves enhanced fitness and UPR\textsuperscript{mt}, and correlates tightly with levels of mrp knockdown. Our data suggest that stoichiometric imbalance between nDNA- and mtDNA-encoded oxidative phosphorylation proteins, or mitonuclear protein imbalance, is at the core of UPR\textsuperscript{mt} activation, both in worms and mammals. The apparent conservation of mitonuclear protein imbalance and UPR\textsuperscript{mt} as a general longevity mechanism should invite further studies to explore whether targeting UPR\textsuperscript{mt} can prevent ageing-associated functional decline and treat diseases linked with ageing.

METHODS SUMMARY

For the identification of novel longevity genes we used publicly available longevity data of the BXD genetic reference population. We used interval mapping in GeneNetwork (http://www.genenetwork.org; Trait ID 10112) for QTL analysis. Pearson’s r genetic correlation was performed to establish genetic correlations with longevity. Candidate longevity genes were knocked down in wild type C. elegans strain Bristol N2, which were tested for lifespan at 20°C. Worm UPR\textsuperscript{mt} was measured in the hsp-6::GFP or hsp-60::GFP reporter strains, and additional
mechanistic mechanism was obtained after knockdown in other worm strains, as described in the online Methods. Treatments with carbenicillin, doxycycline, chloramphenicol, ethidium bromide, N-acetylcysteine, resveratrol and rapamycin (all from Sigma) were performed with heat-killed bacteria or with HT115 bacteria. Oxygen consumption in worms was measured using the Seahorse XF24 (Seahorse Bioscience) using 50 worms per well.

Mouse hepatocyte cell lines AML-12 or Hepa-6, or primary mouse hepatocytes were used for mammalian conservation assays. UPR\textsuperscript{med} was measured using luciferase reporters containing human Hsp60 and ClpP promoter fragments and cellular oxygen consumption was measured using the Seahorse XF24 equipment. Mouse indirect calorimetry was performed in wild type C57BL/6N mice treated for 10 days with 50 mg kg\textsuperscript{-1} day\textsuperscript{-1} doxycycline as a food admix.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions D.R., N.M. and E.K. contributed equally to this work. R.H.H., L.M. and J.A. conceived and designed the project. R.H.H. and R.W.W. performed QTL mapping and sequence analyses. R.H.H., L.M., E.K., D.R., N.M., G.K. performed experiments. R.H.H. and J.A. wrote the manuscript with contributions from all other authors.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.A. (admin.auwerx@epfl.ch).
METHODS

Forward genetics. For the in silico search for longevity genes, we have used publicly available longevity data of the BXD genetic reference population\(^6\). We used interval mapping in GeneNetwork (http://www.genenetwork.org), Trait ID 10112 for QTL analysis. Pearson’s r genetic correlation was performed to establish genetic correlations with longevity (Eye M430v Data Set (Sep08) RMA)\(^6\). Principle component analysis (PCA) was performed using tools implemented in GeneNetwork.

Reverse genetics. We used BXD mice (Trait ID 14484888, at Eye M430v2 (Sep08) RMA) and an F2 cross (Trait ID 10024407239 from UCLA BHF Adipose (Jane05) mlratio)\(^6\) to analyse Mrps5 correlates. We primarily used microarray data from the eye of BXD mice, as this organ contains multiple tissues and cell types—for example, neuronal and muscle—and because this microarray is very well annotated. We analysed correlates as follows: we selected the top 250 Mrps5 correlates in both databases and performed Kyoto encyclopedia of genes and genomes (KEGG) and gene ontology analysis. For the network approach, we selected the oxidative phosphorylation genes within the top 250 traits associated with longevity (Eye M430v2 Data Set (Sept08) RMA)\(^40\).

Construction of ttll-9 RNAi clones. To establish genetic correlations with longevity (Eye M430v2 Data Set (Sept08) RMA)\(^40\), and an F2 cross (Trait ID 10024407239 from UCLA BHF Adipose (Jane05) mlratio)\(^6\), we sequenced N2, CB1370 and resveratrol and rapamycin (all from Sigma) were performed with heat-killed N. Construction of ttll-9 RNAi clones.

Hierarchical clustering. Unsupervised hierarchical clustering was performed using complete linkage and Pearson rank correlation distance on the z-score normalized gene expression data using software implemented in GenePattern\(^6,25\).

RESULTS

C. elegans strains and RNAi experiments. Strains used were wild-type Bristol N2, CB1370 daf-2(e1370) III, CB1378 ekt-1(c951) III, CF1038 daf-16(mu86) I, DA465 eat-2(ad465) II, RB754 aak-2(ad524) X, VC199 sir-2i(ok3434) IV, TK22 mec-1(1k11) III, SJ1400 (zcs1l[+36-GFP]), SJ405 (zcs16[+36-GFP]), SJ4005 (zcs4k[+36-GFP]), SJ103 (zcs14mo-3:GFP[mut]), SJ1419 (zcs17gates:1:GFP[mut]), RW1596 stEx30[myo-3p:GFP + rol-6su1006]), and CJ2070 (dvl[+36-1.2-GFP]). Male RNAi experiments were carried out as described\(^4\). Clones used were mrps-5 (E02A10.1), mrpl-1 (F33D4.5), mrpl-2 (F56B3.8), mrpl-37 (Y48E1B.5), cco-1 (F26E4.9), nck-1 (T37A1C.1), ubl-5 (F46F1.14), haf-1 (C30H6.6), mev-1 (T07CA7.4), mev-17 (R12E12.12), mev-47 (B26DA.1), mev-23 (T08B8.2), mrp-2 (F56B5.6), mrp-30 (B0511.8), mrp-27 (K11B4.1), mrp-15 (Y29H12.8R). Clones were purchased from GeneService and sequenced and RNAi clone information is shown in Supplemental Table 4. Double RNAi experiments were carried out by mixing the bacterial cultures directly before seeding on NGM plates. Controls were RNAi clones 50% diluted with control empty vector RNAi bacteria.

Lifespan tests were performed at 20° C as described\(^8\). Treatments with carbicillin, doxycycline, chloramphenicol, ethidium bromide, N-acetylcysteine, resveratrol and rapamycin (all from Sigma) were performed with heat-killed bacteria\(^9\) or with HT115 bacteria.

Construction of ttll-9 RNAi clones. The RNAi clone was cloned by reverse transcription PCR amplification of the corresponding cDNA from total RNA with following primers: ttll-9bX (X01-960): 5′-GGGCGTCTGAATCAGGCC ATACACGTGGAACAGTGGTTACT-3′, ttll-9cKpnI (X01-960): 5′-GGGTAACACGGTATCTGCAAGTGTTTT-3′. PCR products were digested with KpnI/XbaI and ligated into appropriately digested plasmid pCRII-TOPO vector.

Microscopy and GFP analysis. GFP expression and quantification were carried out as described\(^9\). Briefly, eighty worms (day 1 adults) were picked (20 per well of a black-walled 96-well plate) and GFP was monitored on a Victor X4 plate reader (Perkin Elmer). Each experiment was repeated at least twice. For picture acquisition of hsp-60::GFP expression, animals were mounted on 2% agarose pads in 10 mM tetramisole (Sigma) and examined using a Zeiss microscope (Carl Zeiss). Time lapse was performed by recording 10 pictures of mobile worms every 4 h. In each experiment, several worms were imaged. Image processing was performed with Fiji software. Tracing of the mitochondrial network contour was done by the use of Leica Micosystems, HPM100) in low melting point agarose, freeze substituted to 80% ethanol and 30% acetone in 1% uranyl acetate for 45 min. Serial sections were cut at 100 nm thick and stained with 10% aqueous uranyl acetate for 1 h. Image processing was performed with Fiji software. Tracing of the mitochondrial network contour was done by the use of MitoSox staining. MitoSox staining was performed as previously described with slight modification\(^9\). Briefly, a population of 100 worms was recovered in 1 ml of M9 buffer, washed three times to remove residual bacteria, and resuspended in 200 μl of 1:200 MitoSox stock solution (initial stock solution was dissolved at 1 mM in dimethylsulfoxide (DMSO)). After 20 min of treatment, worms were washed five times in 1 ml of M9 buffer to eliminate the MitoSox reagent and then transferred in a black-walled 96-well plate for reading.

 Quantification of ATP levels and citrate synthase enzymatic activity. Total ATP content was measured by the CellTiter-Glo luminescent cell viability assay (Promega). The luminescence was recorded with a Victor X4 plate reader (PerkinElmer) and values normalized by the total protein concentration determined using a Bradford assay.

Citrate synthase enzymatic activity was determined using the CS assay kit (Sigma). Absorbance at 412 nm was recorded on a Victor X4 (PerkinElmer) with 10 readings over the 0.15 min timespan. These readings were in the linear range of enzymatic activity. The difference between baseline and oxaloacetate-treated samples was obtained and used to calculate total citrate synthase activity according to the formula provided in the manual.

Worm electron microscopy. Worms were fixed using high pressure freezing (Leica Microsystems, HPM100) in low melting point agarose, freeze substituted in 0.5% osmium tetroxide, and 0.5% uranyl acetate in acetone at −90 °C, and then slowly warmed to −10 °C and transferred to pure acetone. Worms were embedded in increasing concentrations of epon resin at 20 °C, transferred to flat embedding moulds in pure resin, and cured at 65 °C for 48 h. Serial sections were cut at 50 nm, and placed onto formvar support films on single slot copper grids. These were imaged at 80 kV filament tension in a transmission electron microscope with a charge coupled device (CCD) camera (Tecnai Spirit, FEI Company, with Eagle eye 4k CCD camera).

Hsp60 and ClpP reporter assays. Human HSPD1 (also known as Hsp60) and ClpP promoter fragments (−603 to +735 for Hsp60, and −1272 to +337 for CLP) were amplified and ligated into the pGL3 basic vector (Promega). Primers used were Hsp60-forward (5′-GACACCGGTAAACAAAGAAGGGGCTC3′) and Hsp60-reverse (5′-GACATCTAGGCTAAGCGCAAACAAACTGCG3′), and ClpP-forward (5′-GACACCGGTGTTCTCGTACCCG3′) and ClpP-reverse (5′-GACATCTAGGGTATCGTCCACCAAC3′). The primers were tagged with MluI site (forward) and XhoI site (reverse). The mouse hepatocyte cell line AML-12 (alpha mouse liver 12) was obtained from ATCC (Manassas). Cells were grown according to the supplier guidelines but in the absence of antibiotics unless specified.

Western blotting. Western blotting was performed with antibodies against Hsp60 (N-20), HSP90 (BD Transduction Laboratories), β-actin (Santa Cruz Biotechnology), MitoProfile Total OXPHOS RDent WB Antibody Cocktail against ATP5A (H28O16.1 in worms), MTCO1/COX1 (MTCE.26 in worms) and UQCR2 (Abcam), green fluorescent protein (Cell Signaling) HRP-labelled anti-goat and anti-mouse secondary antibodies.

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