MOTS-c is an exercise-induced mitochondrial-encoded regulator of age-dependent physical decline and muscle homeostasis

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Healthy aging can be promoted by enhanced metabolic fitness and physical capacity. Mitochondria are chief metabolic organelles with strong implications in aging that also coordinate broad physiological functions, in part, using peptides that are encoded within their independent genome. However, mitochondrial-encoded factors that actively regulate aging are unknown. Here, we report that mitochondrial-encoded MOTS-c can significantly enhance physical performance in young (2 mo.), middle-age (12 mo.), and old (22 mo.) mice. MOTS-c can regulate (i) nuclear genes, including those related to metabolism and proteostasis, (ii) skeletal muscle metabolism, and (iii) myoblast adaptation to metabolic stress. We provide evidence that late-life (23.5 mo.) initiated intermittent MOTS-c treatment (3x/week) can increase physical capacity and healthspan in mice. In humans, exercise induces endogenous MOTS-c expression in skeletal muscle and in circulation. Our data indicate that aging is regulated by genes encoded in both of our co-evolved mitochondrial and nuclear genomes.
The progressive loss of metabolic homeostasis is a hallmark of aging, which impedes parenchymal function and ultimately diminishes physical capacity\(^1\). In fact, aging is a leading risk factor for a myriad of non-communicable chronic diseases\(^2\)-\(^9\). Organismal fitness requires continuous adaptive cellular stress responses to the ever-shifting internal and external environment. Mitochondria not only produce the bulk of cellular energy, but also coordinate adaptive cellular homeostasis by dynamically communicating to the nucleus\(^10\) and other subcellular compartments\(^11\). Mitochondrial communication is mediated by multiple nuclear-encoded proteins, transient molecules, and mitochondrial metabolites\(^12\).

Mitochondria possess a distinct circular genome that has been traditionally known to host only 13 protein-coding genes. However, short open reading frames (sORFs) encoded in the mitochondrial genome have been recently identified. Such sORFs produce bioactive peptides, collectively referred to as mitochondrial-derived peptides (MDPs), with broad physiological functions\(^13\),\(^14\). MOTS-c (mitochondrial ORF of the 12S rRNA type-c) is an MDP that promotes metabolic homeostasis, in part, via AMPK\(^15\),\(^16\) and by directly regulating adaptive nuclear gene expression following nuclear translocation\(^17\),\(^18\). MOTS-c expression is age-dependent and detected in multiple tissues, including skeletal muscle, and in circulation\(^15\),\(^16\),\(^19\), thus it has been dubbed a “mitochondrial hormone”\(^16\) or “mitokine”\(^20\),\(^21\). In fact, we previously reported that systemic MOTS-c treatment reversed diet-induced obesity and insulin resistance\(^15\). We tested if MOTS-c functions as a mitochondrial-encoded regulator of physical capacity and performance\(^2\),\(^22\),\(^23\) in young (2 mo.), middle-aged (12 mo.), and old (22 mo.) mice.

Here, we show that exercise induces mtDNA-encoded MOTS-c expression in humans. MOTS-c treatment significantly (i) improves physical performance in young, middle-age, and old mice, (ii) regulates skeletal muscle metabolism and gene expression, and (iii) enhances adaptation to metabolic stress in C2C12 cells in an HSF1-dependent manner.

**Results**

**Endogenous MOTS-c levels increase upon exercise in humans.**

To determine if endogenous MOTS-c responds to physical exertion, and thus may be involved in driving adaptation to enhance physical capacity, we collected skeletal muscle and plasma from sedentary healthy young male volunteers (24.5 ± 3.7 years old and BMI 24.1 ± 2.1) that exercised on a stationary bicycle (Fig. 1a). Samples were collected before, during (plasma only), and after exercise and following a 4-h rest. Western blotting for endogenous MOTS-c in skeletal muscle revealed that relative levels (i.e. individual changes based on pre-exercise values) significantly increased after exercise (11.9-fold) and remained elevated after a 4-h rest, albeit exhibiting a trend to return to baseline (Fig. 1b, c); see Supplementary Fig. 1a for absolute quantification values. ELISA revealed that relative levels of circulating endogenous MOTS-c also significantly increased during (1.6-fold) and after (1.5-fold) exercise, which then returned to baseline after 4 h of resting (Fig. 1d and Supplementary Fig. 1b). These findings suggest that exercise induces the expression of mitochondrial-encoded regulatory peptides in humans.

**MOTS-c treatment improves physical performance in young mice.** We next probed if MOTS-c functions as an exercise-induced mitochondrial signal that improves physical capacity by treating young mice (CD-1; outbred) daily with MOTS-c [5 mg/kg/day; intraperitoneal injections (IP)] for 2 weeks; we previously reported that 7 days of MOTS-c treatment improved skeletal muscle insulin sensitivity in young and older C57BL/6J mice\(^15\). The rotarod performance test, whereby mice are placed on a rotating rod, revealed that daily MOTS-c significantly improved physical capacity and/or motor coordination (Supplementary Fig. 2a), but not grip strength (Supplementary Fig. 2b) in young mice. Because the rotarod test can also be affected by cognitive capacity, we assessed learning and memory using the Barnes maze and found no improvement (Supplementary Fig. 2c, d).

A treadmill running test confirmed that MOTS-c treatment can enhance physical performance. Because MOTS-c is a regulator of metabolic homeostasis that prevents high-fat diet (HFD)-induced obesity and insulin resistance\(^15\), we tested if MOTS-c also improved running performance under metabolic (dietary) stress. We fed young mice (CD-1) a HFD (60% calories from fat) and treated them with 2 doses of MOTS-c (5 and 15 mg/kg/day; IP; Supplementary Fig. 3a). Mice on the higher dose of MOTS-c showed significantly superior running capacity (Fig. 2a–c) and power output (joules; Supplementary Fig. 4a) following 10 days of treatment, but not following 7 days of treatment (Supplementary Fig. 5a). Notably, 7 days of MOTS-c treatment significantly improved skeletal muscle insulin sensitivity in young and older C57BL/6J mice also fed a high-fat diet\(^15\). We progressively increased the treadmill speed to test both endurance and speed. The final stage, which required mice to sprint (23 m/min), was reached by 100% of mice on the higher dose of MOTS-c, but only 16.6% in the lower dose and control (vehicle) groups (Fig. 2d). Body composition analysis using a time-domain NMR analyzer revealed that both doses of MOTS-c significantly retarded fat gain and that the high dose significantly increased lean mass in young mice (CD-1; Supplementary Fig. 6a–c), in accord with prior reports\(^15\). There was no correlation between body weight and running time (Supplementary Fig. 7a). Taken together, performance improvements in treadmill running and rotarod test, paired with lack of changes in the grip strength and Barnes Maze, indicate an enhancement in physical capacity.

In young CD-1 mice, we simultaneously initiated MOTS-c treatment and a HFD (Supplementary Fig. 3a). To test if MOTS-c can improve physical performance in mice that have been on a...
HFD, we fed young C57BL/6j mice a HFD, or a normal diet, for 2 weeks prior to initiating daily MOTS-c injections (15 mg/kg/day) for 2 weeks prior to a treadmill running test (Supplementary Fig. 3b). MOTS-c treatment significantly enhanced running performance and power output (joules; Supplementary Fig. 4b) on the treadmill regardless of the diet (Fig. 2e–h and Supplementary Fig. 5b). MOTS-c treatment enabled 25% of the young C57BL/6j mice to enter the final running stage (highest speed) on a normal diet, but none on a HFD (Fig. 2h). Consistent with our prior study,15 MOTS-c treatment curbed HFD-induced weight gain in C57BL/6j mice (Supplementary Fig. 6d), which was largely driven by reduced fat accumulation (Supplementary Fig. 6e), but not loss of lean mass (Supplementary Fig. 6f), as was largely driven by reduced fat accumulation (Supplementary Fig. 6e), but not loss of lean mass (Supplementary Fig. 6f), as determined by an NMR-based body composition analysis. However, there was no correlation between body weight and running time (Supplementary Fig. 7b). Also, consistent with our prior study,15, there were no differences in food intake between control diet or HFD groups (Supplementary Fig. 8). Further, targeted metabolomics revealed that MOTS-c treatment significantly regulated (i) glycolysis/PPP (pentose phosphate pathway) and (ii) amino acid metabolism (Fig. 2i; Supplementary Figs. 3b and 9a; and Supplementary Data 1 and 2) in skeletal muscle, but not in liver, consistent with our previous study.15 Together, these data indicate that MOTS-c treatment can improve overall physical performance, in part, by targeting skeletal muscle metabolism in young mice.

MOTS-c treatment enhances physical capacity in old mice. Aging is accompanied by a progressive decline in mitochondrial function1,23 and loss of metabolic homeostasis, in which MOTS-c may play a role.2,10 Aging is associated with reduced MOTS-c levels in certain tissues, including the skeletal muscle, and in circulation.15,19 We previously showed that an acute one-week MOTS-c treatment reversed age-dependent insulin resistance in mouse skeletal muscle.15 Thus, we investigated if promoting metabolic homeostasis by MOTS-c treatment could reverse age-dependent decline in physical capacity. Middle-aged (12 mo.) and old (22 mo.) C57BL/6N mice were treated daily with MOTS-c (15 mg/kg/day; IP) for 2 weeks, then subjected to a treadmill running test (Fig. 3a). Both middle-aged and old mice ran significantly longer with increased power output (joules) following MOTS-c treatment (Fig. 3b and Supplementary Fig. 4c). Old mice ran longer (2-fold; Fig. 3c) and farther (2.16-fold; Fig. 3d) when treated with MOTS-c. Further, MOTS-c enabled 17% of the old mice to enter the final running stage (highest speed), whereas none in the untreated group were successful (Fig. 3e). Notably, MOTS-c treatment enhanced old mice to outperform untreated middle-aged mice, suggesting a more pervasive physical reprogramming rather than just rejuvenation. Respiratory exchange ratio (RER), measured using a metabolic cage, indicates fuel preference (1.0: carbohydrates, 0.7: fat). “Metabolic flexibility”, which refers to the overall adaptive capacity to a shift in metabolic supply-demand equilibrium (e.g. exercise), declines with age.25,26

**Fig. 2 MOTS-c treatment increases physical capacity in young mice regardless of diet. a–c Treadmill performance of 12-week-old male CD-1 (outbred) mice fed a normal diet (n = 5, Control and 5 mg/kg MOTS-c; n = 6, 15 mg/kg MOTS-c; a) running curves (P = 0.0329), b) total time on treadmill (P = 0.0069, 0 vs. 15; P = 0.0081, 5 vs. 15), and c) total distance ran (P = 0.0418, 0 vs. 15; P = 0.0081, 5 vs.15), and d) percent capable of reaching the highest speed (sprint). e–h Treadmill performance of 12-week-old male C57BL/6j (inbred) mice fed a HFD (n = 8); e) running curves (P = 0.0003), f) total time on treadmill (P = 0.0237, normal diet control vs. normal diet + MOTS-c; P = 0.0084, HFD control vs. HFD + MOTS-c) g) total distance ran (P = 0.0326, normal diet control vs. normal diet + MOTS-c; P = 0.0087, HFD Control vs. HFD + MOTS-c) h) percent capable of reaching the highest speed (final stage). i PCA and MSEA on metabolomic data from skeletal muscle and liver of C57BL/6j mice that were fed a HFD, treated with MOTS-c, and exercised. Data expressed as mean ± SEM. Log-rank (Mantel-Cox) test was used for a, e. Otherwise, all statistics were performed using the two-sided Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001.
Acute MOTS-c treatment enhances physical capacity in old mice. (a) Schedule of MOTS-c treatment and assays in middle-aged and old C57BL/6N mice (n = 10, MA groups; n = 19, old control; n = 18, old MOTS-c), including (b) treadmill running curves (P = 0.0000000046), (c) total time on treadmill (P = 0.0458, MA control vs. old control; P = 0.0497, MA control vs. MA MOTS-c; P = 0.000002, old control vs. old MOTS-c), and (d) total distance ran on treadmill (n = 19, old control; n = 18, Old MOTS-c; P = 0.0464, MA control vs. old control; P = 0.0462, MA control vs. MA MOTS-c; P = 0.000002, old control vs. old MOTS-c), and (e) percent capable of reaching the highest speed on a treadmill (final stage). (f) Respiratory exchange ratio (RER) following 2 weeks of daily MOTS-c injection (n = 4; P = 0.0055). (g) Skeletal muscle from treadmill-exercised old mice (22.5 months) treated daily with MOTS-c (15 mg/kg/day) for 2 weeks (n = 10) were subject to (g) metabolomics and analyzed using PCA and MSEA and (h) GSEA analysis of muscle RNA-seq analysis. Balloon plots of select enriched terms using Gene Ontology Biological Process (GO_BP) database at false discovery rate (FDR) < 15%. NES Normalized Enrichment Score. Full GSEA results are available in Supplementary Data 5. Data expressed as mean ± SEM. Log-rank (Mantel-Cox) test was used for (b) and two-way ANOVA (repeated measures) was used for (f). GSEA statistics from R package ‘clusterProfiler’ were used for (h). Otherwise, all statistics were performed using the two-sided Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001. MA middle-age.
Indeed, we observed a shift in RER between daytime and nighttime, which trended more towards statistical significance for middle-aged mice \( (P = 0.076) \) compared to aged mice \( (P = 0.39; \text{Fig. 3f and Supplementary Fig. 10}) \), indicating reduced metabolic flexibility with age. RER during daytime, but not nighttime, was significantly different between middle-aged vs. old mice, whereby old mice preferred to continue to utilize carbohydrates more than their middle-aged counterparts (Fig. 3f and Supplementary Fig. 10). This phenomenon was fully reversed by MOTS-c treatment and the RER of old mice exhibited a similar circadian pattern to that of middle-aged mice (Fig. 3f and Supplementary Fig. 10).

Late-life MOTS-c treatment improves mouse healthspan. Anti-aging interventions that are applied later in life would be more translationally feasible compared to life-long treatments \(^{27–29}\). Building on the treadmill running tests, we tested if a late-life initiated (~24 mo.) intermittent (LLII) MOTS-c treatment \((3x/\text{week}; 15 \text{mg/kg/day})\) would improve healthy lifespan (Fig. 3a). To assess healthspan, towards the end-of-life (>30 mo.), we performed a battery of physical tests to further probe the effect of MOTS-c on reversing age-dependent physical decline (Fig. 3a). LLII MOTS-c improved (i) grip strength (Fig. 4a), (ii) gait, assessed by stride length (Fig. 4b), and (iii) physical performance, assessed by a 60-second walking test (running was not possible at this age; Fig. 4c). In humans, reduced stride length and walking capacity are strongly linked to mortality and morbidity \(^{30}\). Together, these data indicate that LLII MOTS-c treatment improves physical capacity in old mice.

Independent lines of research have shown that MOTS-c is a mitochondrial-encoded metabolic regulator at the cellular and organismal level \(^{1,15,17,19,31–35}\). We posited that LLII MOTS-c treatment would cause metabolic reprogramming in old mice. Consistent with our previous report \(^{12}\), non-fasting blood glucose was better maintained in LLII MOTS-c-treated old mice\((30 \text{mo.; Fig. 4d})\). Over course of their life, LLII MOTS-c-treated mice showed comparable body weight to their untreated counterparts (Fig. 4e, f). However, total food intake was significantly reduced (Fig. 4g, h and Supplementary Fig. 11), whereas total activity was significantly higher (Supplementary Fig. 15). Yet, the activity changes at 30 months of age was marginal and may not account for considerable increase in energy expenditure that could explain the effect of MOTS-c on body weight at different age groups.
Fig. 4 MOTS-c regulates aging metabolism and healthspan. Life-long measurements on male C57BL6/N mice treated intermittently (3x/week) with MOTS-c (15 mg/kg/day) starting at middle age and old age (13.5 and 23.5 mo.) as described in Fig. 2a. a grip strength test (n = 11; run in triplicate; P = 0.000078), b gait analysis (stride length; n = 5; run in triplicate; P = 0.0038), c 60-s walking test (n = 11; control; n = 12; MOTS-c; P = 0.0428), and d blood glucose levels (n = 11; P = 0.0397). e, f Body weight as a function of time and f the total sum (∑n = 19; old control; n = 18; old MOTS-c; P = 0.0013, MA control vs. MA MOTS-c; P = 0.001, old control vs. old MOTS-c; g, h Food intake g as a function of time and h the total sum (∑n = 19; old control; n = 18; old MOTS-c; P = 0.0276, MA control vs. MA MOTS-c; P = 0.00005, old control vs. old MOTS-c); i j Percent fat mass as a function of time and j the total sum (∑n = 19; old control; n = 18; old MOTS-c; P < 1E-50); k, l Percent lean mass as a function of time and l the total sum (∑; P = 0.026, MA control vs. MA MOTS-c; P = 0.00003, old control vs. old MOTS-c). m Lifespan curve; P = 0.05 until 31.8 months of age. Overall curve trended towards increased median and maximum lifespan (P = 0.23). Data expressed as mean ± SEM. Log-rank (Mantel-Cox) test was used for m. Otherwise, all statistics were performed using the two-sided Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001. MA middle-age.

( Supplementary Fig. 15). A likely possibility that could increase energy expenditure is, consistent with previous reports from our lab and others15,36, increased heat production. Body composition analysis using a time-domain NMR analyzer revealed significant reduction of fat mass (Fig. 4i, j) and a modest reduction in age-dependent loss of lean mass (Fig. 4k, l). The RER, measured using metabolic cages, at 30 mo. revealed increased fat utilization, consistent with that obtained at ~23.5 mo. (Fig. 3f), but with a circadian shift compared to the same mice measured 6 months before (Supplementary Fig. 16); this is also consistent with reduced total fat mass (Fig. 4i, j and Supplementary Fig. 6b, e) and increased lipid utilization15,35. Ultimately, LLII MOTS-c treatment showed a trend towards increased median (6.4%) and maximum (7.0%) lifespan and reduced hazard ratio (0.654); P = 0.05 until 31.8 months (Fig. 4m). Larger cohorts will be needed to confirm the broader significance of MOTS-c treatment on overall longevity. These data suggest that LLII MOTS-c treatment improves overall physical capacity in old mice and may compress morbidity and increase healthspan.

MOTS-c promotes metabolic adaptations in vitro. Skeletal muscle must adapt to various exercise-induced challenges37,38, including nutrient (e.g. metabolic supply-demand imbalance)38, oxidative39,40, and heat stress37,41, which share mitochondria as a common denominator. Because MOTS-c enhanced cellular resistance against metabolic/oxidative stress17, we tested if MOTS-c treatment improved skeletal muscle adaptation to metabolic stress using C2C12 mouse myoblast cells. Using crystal violet staining to determine cellular viability, we found that MOTS-c (10 µM) treatment significantly protected C2C12 cells (~2-fold) from 48 h of metabolic stress [glucose restriction (GR; 0.5 g/L) and serum deprivation (SD; 1% FBS)] (Fig. 5a). Next, we tested the replicative capacity of C2C12 cells following prolonged metabolic stress as a functional marker of protection. C2C12 cells were metabolically stressed (GR/SD) for one week with daily MOTS-c (10 µM) treatment, then replenished with complete medium for 2 days and stained with crystal violet. MOTS-c-treated C2C12 cells showed significantly enhanced proliferative capacity within 2 days (~6-fold; Fig. 5b). Because MOTS-c promotes fat utilization, which may underly its effect on “metabolic flexibility” (Figs. 3f and 4i, j and Supplementary Fig. 6)15,35,42, we tested if MOTS-c-treated C2C12 cells could survive on lipids without glucose (0 g/L). As expected, most control cells died without glucose even with lipid supplementation, whereas MOTS-c treatment provided significant protection (~2-fold; Fig. 5c). Real-time metabolic flux analysis revealed that MOTS-c treatment significantly increased lipid utilization capacity through increasing OCR, but not ECAR, in the presence of palmitate in C2C12 cells (Fig. 5d and Supplementary Fig. 17).

We previously reported that endogenous MOTS-c translocates to the nucleus to directly regulate adaptive nuclear gene expression in response to cellular stress17. Using fluorescently labeled MOTS-c peptide (MOTS-c-FITC), we confirmed that exogenously treated MOTS-c also dynamically translocated to the nucleus in a time-dependent manner (Fig. 5e)17, indicating a direct nuclear role. We performed RNA-seq on C2C12 cells treated with MOTS-c or vehicle control (10 µM) under GR/SD for 48 h and found (i) separation of groups in PC1 (first principle component) using a principal component analysis (PCA; Fig. 5f) and (ii) 69 genes that were differentially regulated at FDR < 5% (Fig. 5g). Further, using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database to assess putative changes in protein-protein interaction networks based on our RNA-seq results, we found that a cluster related to heat-shock responses, including Hsp40 (DNAJ) and Hsp70s (HSPA), were prominently regulated by MOTS-c in C2C12 cells under GR/SD; we also identified previously reported MOTS-c targets, including Atf3, Jun, Fosl1, and Mafg17 (Fig. 5h and Supplementary Data 5). Indeed, select GO_BP analysis revealed that protein homeostasis (i.e. proteinostasis) processes were significantly targeted with protein folding being the most significantly affected pathway in metabolically stressed myoblasts (Supplementary Fig. 18). Next, to identify common pathways in vitro and in vivo models, we overlaid RNA-seq data from MOTS-c-treated (i) old mouse skeletal muscle and (ii) metabolically stressed (GR/SD) C2C12. Consistently, select GO_BP analysis revealed several commonly targeted pathways by MOTS-c in both exercised skeletal muscle and metabolically stressed myoblasts that were related to proteostasis, including protein regulation and protein metabolism (select terms in Fig. 5i; full results in Supplementary Data 5 and 8); notably, commonly targeted pathways also provided a specific cellular context that reflects metabolic stress, including oxidative stress, mitochondria, cellular metabolism. To identify putative transcription factors involved in differential gene regulation upon MOTS-c treatment, we performed a statistical enrichment analysis inferred from the ChEA knowledge base43 (FDR < 5%). Consistent with the STRING and GSEA (GO_BP) analyses, we identified heat shock factor 1 (HSF1) as a putative transcription factor that was significantly enriched in both mouse skeletal muscle and myoblasts and could potentially regulate gene expression upon MOTS-c treatment (Fig. 5j). HSF1 is a master nutrient sensor and regulator of stress adaptation and proteostasis, in part, by inducing the expression of a myriad of heat shock proteins, including Hsp40 (DNAJ) and Hsp70s (HSPA), consistent with the effects of MOTS-c (Fig. 5h)44,45. Exercise induces HSF1 activation in skeletal muscle46 and cardiac muscle47 in rodents. Indeed, siRNA-mediated HSF1 knockdown reversed the protective effects of MOTS-c against GR/SD in myoblasts (Fig. 5k, l). Together, these data suggest that MOTS-c improves metabolic homeostasis/flexibility and protein homeostasis in skeletal muscle under exercise-induced stress conditions.

Discussion

We previously reported that MOTS-c prevented diet-induced obesity and insulin resistance and reversed age-dependent skeletal muscle insulin resistance in C57BL/6J mice, indicating a role in metabolic conditions such as diabetes15,48,49. MOTS-c functions
have been shown to be dependent on the master nutrient sensor AMPK15,17,34,36,50, including its nuclear translocation in response to metabolic imbalance55. Notably, MOTS-c treatment in mice activated AMPK in skeletal muscle and increased the expression of its downstream glucose transporter (i.e. GLUT4)15, suggesting improved muscle metabolism and physical capacity56. Notably, MOTS-c treatment improved running capacity independent of body weight, based on total work (joules) output and the lack of correlation between body weight and running time (Supplementary Figs. 4 and 7). This indicates that MOTS-c treatment enhanced physical performance by improving whole body energy metabolism, in part, by promoting adaptive cellular responses to exercise-related stress conditions (Fig. 2a–h and Supplementary Fig. 5), suggesting the need to reach a certain physiological state with an improved metabolic profile.

MOTS-c acts as an intracellular and endocrine factor, thus has been dubbed a mitochondrial-encoded hormone or a mitochondrial cytokine (mitokine)2,10,16,49,57, providing an excellent example of bioactive peptides that can enter a wide range of mechanisms in a concentration-dependent fashion, including energy-dependent transport via endocytosis61,62. Mitochondria are strongly implicated in aging at multiple levels1,2,24,63 and MOTS-c may contribute to longevity by providing an excellent example of bioactive peptides that can enter cells without being degraded and retain biological activity60; the cellular entry of peptides is still largely unclear and known to adopt a wide range of mechanisms in a concentration-dependent fashion, including energy-dependent transport via endocytosis61,62.

Mitochondria are strongly implicated in aging at multiple levels1,2,24,63-65 and MOTS-c may contribute to longevity by promoting cellular homeostasis2,10,57-59. Here, we present evidence that the mitochondrial genome encodes for instructions to maintain physical capacity (i.e. performance and metabolism) during aging and thereby increase healthspan. MOTS-c treatment initiated in late-life, proximal to the age at which the lifespan curve rapidly descends for C57BL/6N mice, significantly delayed the onset of age-related physical disabilities, suggesting "compression of morbidity" in later life66. Interestingly, an exceptionally long-lived Japanese population harbors a mitochondrial DNA (mtDNA) SNP (m.1382A>C) that yields a functional variant of MOTS-c67,68.

Our study shows that exogenously treated MOTS-c enters the nucleus and regulates nuclear gene expression, including those involved in heat shock response and metabolism. Thus, age-

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**Fig. 5 MOTS-c regulates myoblast gene expression and enhances adaptation to metabolic stress.** a-c Survival of MOTS-c-treated (10 μM; equal-volume vehicle control) C2C12 myoblasts assessed by crystal violet staining following a 48 h of glucose restriction (GR; 0.5 g/L) and serum deprivation (SD; 1% FBS) with MOTS-c treated only once initially (n = 12, duplicates; P = 2.57E-25), b 7 days of GR/SD with daily MOTS-c treatment, followed by a 2-day recovery in full media with MOTS-c (n = 10, duplicates; P = 8.95E-22), and c 48 h of complete GR (0 g/L) with chemically defined lipid supplementation and daily MOTS-c treatment (n = 6, duplicates; P = 1.17E-09). d Real-time oxygen consumption rate (OCR) in response to fatty acid (palmitate-BSA) in C2C12 myoblasts treated with MOTS-c (10 μM) for 48 h (n = 11, BSA baseline; n = 12, palmitate addition; P = 6.27E-07). e Time-dependent subcellular localization pattern of exogenously treated MOTS-c-FITC (10 μM) in C2C12 myoblasts. Scale bar: 10 μm. Images represent two biological replicates and three technical replicates. f-i RNA-seq was performed on C2C12 myoblasts following 48 h of GR/SD with/without a unique initial MOTS-c (10 μM) treatment (n = 6). f Principle Component Analysis (PCA) and g heatmap of significantly differentially regulated genes by MOTS-c at false discovery rate (FDR) < 5% by DESeq2 analysis. h Protein–protein interaction network analysis based on genes that were significantly differentially regulated by MOTS-c (FDR < 5%) using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database version 11.079. i Balloon plots of common biological processes derived from RNA-seq data between MOTS-c-treated (i) skeletal muscle from old mice (see Fig. 3) and (ii) C2C12 myoblasts, based on gene ontology biological process (GO_BP). j Balloon plots of common transcription factors derived from RNA-seq data between MOTS-c-treated (i) skeletal muscle from old mice (see Fig. 3) and (ii) C2C12 myoblasts, based on ChEA database. k Relative cell number at k 48 vs 72 h of GR/SD assessed by crys-

tal violet staining (n = 6; P = 0.00027, siCtrl + MOTS-c vs. siHsf1 + MOTS-c; P = 0.00017, siCtrl vs. siCtrl + MOTS-
c-) and l 72 h of GR/SD assessed by flow cytometry (n = 3, siCtrl; n = 6, siHsf1; P = 0.0053). Data expressed as mean ± SEM. Two-sided Student’s t test. *P < 0.05, **P < 0.01, ***P < 0.001.
related gene networks are comprised of integrated factors encoded by both genomes, which entails a bi-genomic basis for the evolution of aging. Although the detailed molecular mechanism(s) underlying the functions of MOTS-c is an active field of research we provide a proof-of-principles study that realizes the mitochondrial genome as a source for instructions that can regulate physical capacity and healthy aging.

Methods

Mouse care. All animal work was approved by and performed in accordance with the University of Southern California (USC) Institutional Animal Care and Use Committee. Mice were housed 3–4 per cage in a facility with a 12:12 h light–dark cycle (light period 0600–1800) at 24 °C. Food and water were available ad libitum. MOTS-c (New England Peptide, USA) was administered daily at 5 or 15 mg/kg via intraperitoneal injections. In all, 12-week-old male CD-1 (outbred) mice (Charles River, USA), 12-week old male C57BL/6J mice (Jackson Laboratory) and 8- and 18-month-old male C57BL/6N mice (National Institute on Aging; NIA) were obtained. All mice were fed either a HFD (60% calories from fat) or matching control diet (Research Diets, USA, #D12492 and D12450), respectively. NIA mice were sufficiently acclimated for 4 months in our vivarium until they were considered middle-aged (12 mo.) and old (22 mo.) at the start of MOTS-c injections. Body weight measurement were recorded daily, while on a horizontal bar connected to the treadmill. Mice were used over a 30 min. Finally, after 40 min of total run time, the running speed is increased to 21 m/min for 60 s. We recorded whether the mouse was able to walk, or not, on the treadmill. Mice resting on the platform were gently prodded to encourage re-engagement. Any mouse that resisted prodding and remained on the platform for 30 s was considered to be exhausted, and time was recorded.

Walking test. When the mice reached 30 months of age, they were no longer capable of performing the same treadmill routine. We developed a measure of mobility in the aged mice consisting of a 60-s walking test. The treadmill was set at 13 m/min for 60 s. We recorded whether the mouse was able to walk, or not, on the treadmill for 80 s, with gentle prodding as needed. Mice remaining on the treadmill were measured, and recorded time was recorded.

Rotorad. The Rotorad test was performed by placing the mice on the apparatus (TSE-Systems, USA) in all facing the opposite direction of rotation. The initial speed of rotation was 10 m/min and accelerated at 1 rpm every 10 s. Time to fall was recorded for each mouse, and three trials per mouse was run. Mice received no ≤5 min of recovery time between trials.

Grip strength. We measured grip strength using a horizontal bar connected to the grip strength meter (TSE-Systems) as a high precision force sensor for the forelimbs. After allowing the mouse to properly grip the bar they were firmly and quickly pulled in the opposite direction. Only trials where the mouse released its paws from the bar simultaneously were counted as successful. Mice underwent three trials, with at least 30 s recovery time between trials.

Gait analysis. To perform gait analysis, we applied a different color of non-toxic ink (BLICK®, USA) to the front and hind paw of the mice as a measure of cellular fitness. In this case, MOTS-c-containing (10 μM) media was changed once every 2 days; no additional MOTS-c supplementation was given between media changes. After 7 days of GR/SD, we returned the cells to full growth media (10% FBS and 4.5 g/L glucose) for 48 h with MOTS-c (10 μM) and stained them with crystal violet.

Biochemistry. All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Water was milliQ grade. Analytical grade (≥99.5%) chemicals were used. Fetal bovine serum (FBS) was obtained using the XP9 Bioanalyzer (Seahorse Bioscience) at the USC Leonard Davis School of Gerontology Seahorse Core. All values were normalized to relative

In vivo metabolism assessment

Metabolic cages. Metabolic activity in mice was measured using the Phenomaster system (TSE-Systems) equipped to detect indirect calorimetry, measure food and water consumption, and monitor activity. Prior to metabolic analysis, mice were housed 3–4 per cage in a facility with a 12:12 h light–dark cycle (light period 0600–1800) at 24 °C. Food and water were available ad libitum. For metabolic assessment, mice were moved into individual Phenomaster cages in an isolated room under the same environmental conditions. Mice were automatically monitored for 36 h to record oxygen consumption (VO2) and carbon dioxide production (VCO2). The respiratory exchange ratio (RER) was calculated as the ratio of carbon dioxide produced to oxygen consumption. The Phenomaster system allows for activity monitoring using a triple beam IR technology system. Breaking the IR beams through movement was considered a “count”. The three-beam system allows X1Y, monitoring that considers both ambulatory activity around the cage as well as rearing activity. All data are expressed as the mean of 3-hr acquisition cycles.

Barnes maze. Blood was collected via a single tail-nick and immediately analyzed using a glucometer (Freestyle, Abbott). Blood collection was performed by trained professionals and in accordance with the University of Southern California Institutional Animal Care and Use Committee.

Western blots. Protein samples were lysed in 1% Triton X-100 (Thermo Fisher Scientific, USA, #12568-2500) with 1 mM EDTA (Promega Life Sciences, USA, #V4231) and 100 mM Tris-HCl pH 7.5 (Quality Biological, USA, #351-006-101) and protease inhibitors (Roche, Germany, #11863617001) and sonicated using a Sonic Dismembrator (Fisher Scientific, USA). Samples were heated at 95 °C for 5 min. Samples were run on 4–20% gradient tris-glycine gels (TGX; Bio-Rad, USA, #161-1010) and transferred onto 0.2 μm PVDF membranes (Bio-Rad #162-0184) using a Transblot Turbo semi-dry transfer system (Bio-Rad) at 9 volts for 15 min. Membranes were blocked for 1 h using 5% BSA (Akrion Biotech, USA, #AK8905-0100) in tris-buffered saline containing 0.05% Tween-20 (Bio-Rad #161-0781) and incubated in primary antibodies against MOTS-c (1:250, rabbit polyclonal; YenZym, USA) and GAPDH (1:1,000, cat# 5174; Cell Signaling, USA) overnight at 4 °C. Secondary HRP-conjugated antibodies (#7074; Cell Signaling, USA) were then added (1:3,000) for one hour at room temperature. Chemiluminescence was detected and imaged using Clarity western ECL substrate (Bio-Rad #1705060) and Chemidoc XRS system (Bio-Rad). Western blots were quantified using ImageJ version 1.52k.

Cell studies

Cell culture. C2C12 cells were cultured in DMEM with 4.5 g/L glucose (Corning, USA #10-017-CV) and 10% FBS (Millipore-Sigma, USA, #F9026-500). All cells were stored at 37 °C and 5% CO2. Cells were passaged when they reached 75–80% confluence using TrypLE (Thermo Fisher Scientific #12605-010).

Cell survival assays. Protection against glucose restriction (GR) and serum deprivation (SD) was tested by culturing cells in DMEM (Thermo Fisher Scientific #11966-025) with 0.5 g/L glucose (Millipore-Sigma #G8769) and 1% FBS. MOTS-c (10 μM) or vehicle (PBS). MOTS-c (10 μM) was added to the media every 24 hr. After 48 h of GR/SD, we performed crystal violet (Thermo Fisher Scientific #CS81-25) staining as before to determine cell survival. We also tested cellular proliferation, following prolonged (7-day) GR/SD (DMEM with 1% FBS and 0.5 g/L glucose) as a measure of cellular fitness. In this case, MOTS-c-containing (10 μM) media was changed once every 2 days; no additional MOTS-c supplementation was given between media changes. After 7 days of GR/SD, we returned the cells to full growth media (10% FBS and 4.5 g/L glucose) for 48 h with MOTS-c (10 μM), then stained them with crystal violet. To determine the metabolic flexibility to utilize fatty acids, we cultured cells in DMEM with 1% FBS and 0.5 g/L glucose, and 1% chemically defined lipid mixture (Millipore-Sigma #L0288) for 48 h, then stained them with crystal violet.

Metabolic flux. Real-time oxygen consumption and extracellular acidification rates in C2C12 myoblasts treated with 16% palmitate-BSA (1 mM palmitate conjugated to BSA) or 16% oleate-BSA (1 mM oleate conjugated to BSA) were measured using the XF96 Bioanalyzer (Seahorse Bioscience) at the USC Leonard Davis School of Gerontology Seahorse Core. All values were normalized to relative
protein concentration using a BCA protein assay kit (Thermo Fisher Scientific #23227).

Confocal microscopy. Confocal images were obtained using a Zeiss Confocal Laser Scanning Microscope 700 (Zeiss, Germany). C2C12 myoblasts were cultured on glass coverslips (Chemglass, USA, aCLS-1760-015). Cells were treated with FITC-MOTS-c (New England Peptide) for either 0 h (immediate), 30 min, 4 h, or 24 h. All cells were treated with Hoechst (Biotum, USA, #40045) for 15 min and then washed three times with PBS. Cells were fixed in 100% formalin (Millipore-Sigma #EM-R04586-82) and washed an additional three times in PBS. Coverslips were affixed to glass slides (VWR, USA, #48300-025) using ProLong Gold antifade reagent (Life Technologies Corporation, USA, #P36934).

Human studies

Study outline. Participants gave written consent before the commencement of the study, which was approved by the Northern Health and Disability Ethics Committee, and carried out in accordance with guidelines for human research (New Zealand, 16/STH/A116/AM01). In total, 10 sedentary (<4 h aerobic exercise/week) healthy young males (24.5 ± 3.7 years old and BMI 24.1 ± 2.1) were recruited to take part in a two-visit exercise trial. Recruited participants were free of cardiovascular, metabolic and blood diseases and were not taking any medication or supplements. The trial was separated into two visits, each involving exercise bouts that were carried out on an electromagnetically braked cycle ergometer (VeloTron, RacerMate, USA).

Determination of peak oxygen uptake (VO2 peak) and maximal power output (visit 1). Peak oxygen uptake was determined using a ramped cycling exercise protocol. Prior to testing, participants warmed up for five minutes at a self-selected workload between 30 and 60 W. The ramp protocol began at 60 W, with the cycling power output set to increase by 1 W every 4 s (15 W/min) continuously until the participant was unable to maintain cycling workload (cycling cadence < minimum endurance speed) or maximal volitional fatigue was reached. Mean Peak oxygen uptake of participants was reported as 38.4 ± 7.3 ml.kg.min.

Acute high intensity cycling exercise session (visit 2). Prior to visit 2, participants were asked to fast overnight (from 10PM) and were instructed to abstain from physical activity for at least 48 h prior. Upon arrival to the laboratory, participants lay supine for 15-min and then had an intravenous cannula inserted into a forearm vein. A resting plasma sample was collected following a pre-exercise muscle biopsy taken from the vastus lateralis muscle (quadriceps muscle). Approximately 10-min later, participants completed 10, 60 s-cycling intervals at individually specified peak power workloads (determined from peak oxygen uptake test) followed by 75 seconds of rest/low intensity cycling (<30 W) per interval77. A mid-exercise blood sample was taken following the completion of the 5th exercise interval as well as immediately following the completion of the exercise bout. In addition, an immediately-post exercise muscle biopsy was taken (within ~5 min of completion of the exercise bout). Participants remained supine and resting in the procedure bed for a 4 h recovery period. Following 4 h of recovery, a final blood and muscle biopsy sample was collected.

Muscle biopsy and blood sampling. Muscle biopsies were extracted under local anesthesia (1% xylocaine) using the Bergstrom needle with manual suction technique80. Biopsies were snap-frozen in liquid nitrogen and stored at −80 °C until analyzed. Blood was drawn through a 20-gauge cannula, collected in 10-ml EDTA vacutainers and then centrifuged immediately upon collection at 4 °C at 2000 x g for 10 min. Plasma was extracted, and then stored at −80 °C until further analysis using an in-house ELISA as described before15. Human skeletal muscle was processed for downstream analyses.

Liquid chromatography-mass spectrometry metabolomics. Metabolites were extracted from randomly selected tissue samples by adding 1 ml of 80:20 metha-nol:water solution on dry ice. Samples were incubated at −80 °C for 4 h and centrifuged at 4 °C for 5 min at 21,000 × g. Supernatants were transferred into LoliBind Eppendorf microcentrifuge tubes and the cell pellets were re-extracted with 200 μl ice-cold 80% MeOH, spun down and the supernatants were combined. Metabolites were dried at room temperature under vacuum and resuspended in water for injection.

Samples were randomized and analyzed on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer coupled to an UltiMate 3000 UHPLC system (Thermo Scientific). The mass spectrometer was run in polarity switching mode (+3.00 kV/-2.25 kV) with an m/z window ranging from 65 to 975. Mobile phase A was 5 mM NH4AcO, pH 9.9, and mobile phase B was acetonitrile. The flowrate was 300 μl/min, and the gradient was from 15% A to 95% A in 18 min, followed by an isocratic step for 9 min and re-equilibration for 7 min. All samples were injected twice for technical duplicates. Metabolites were detected and quantified as area under the curve based on retention time and accurate mass (≤5 ppm) using the TraceFinder 3.3 (Thermo Scientific) software.

RNA-seq

RNA purification from tissue and cells. Total RNA extraction from skeletal muscle tissue or C2C12 mouse myoblasts was done using TRI Reagent (Millipore-Sigma #T9494). Muscle tissue samples were flash-frozen in liquid nitrogen until further processing. Tissues were resuspended in 600 μl of TRI Reagent, then homogenized on Lysing Matrix D 2 ml tubes (MP Biomekultur) on a BeadBug homogenizer (Benchmark Scientific). For both skeletal muscle and C2C12 cells, total RNA was purified using the Direct-zol RNA MiniPrep (Zymo Research #R2052).

RNA-seq library preparation. Total RNA was subjected to RNA depletion using the NEBNext RNA Depletion Kit (New England Biolabs), according to the manufacturer’s protocol. Strand specific RNA-seq libraries were then constructed using the SMARTer Strand DNA-Seq Kit (Clontech # 634839), according to the manufacturer’s protocol. Based on rRNA-depleted input amount, 13–15 cycles of amplification were performed to generate RNA-seq libraries. Paired-end 150 bp reads were sent for sequencing on the Illumina HiSeq Xten platform at the Novogene Corporation (USA). The raw sequencing data was deposited to the NCBI Sequence Read Archive (accession: PRJNA556045). The resulting data was then analyzed with a standardized RNA-seq data analysis pipeline (described below).

RNA-seq analysis pipeline. To avoid the mapping issues due to overlapping sequence segments in paired end reads, reads were hard trimmed to 75 bp using the Fastx toolkit v0.10.13. Reads were then further quality-trimmed using Trimgalore (https://github.com/FelixKrueger/Trimgalore) to retain high-quality reads with Phred score > 20. All reads were also trimmed by 6 bp from their 5’ end to avoid poor qualities or biases. cdsDNA sequences of protein coding and lincRNA genes were obtained through ENSEMBL Biomart for the GRCh38 build of the mouse genome (Ensemble release v94). Trimmed reads were mapped to this reference using kallisto 0.43.0-1 and the `-r-stranded option78. All subsequent analyses were performed in the R statistical software (https://cran.r-project.org/).

Read counts were imported into R, and summarized at the gene level, to estimate differential gene expression as a function of age.

Because of high sample variability, we used surrogate variable analysis to remove experimental noise from the muscle RNA-seq dataset76. R package ‘sva’ v3.24.277 was used to estimate surrogate variable, and the effects of surrogate variables were regressed out using 'limma'. Corrected read counts were then used for downstream analyses.

Deseq2 normalized fold-changes were then used to estimate differential gene expression between control and MOTS-c treated muscle or cell samples using the ‘DESeq2’ R package (DESeq2 1.16.1.78). The heatmap of expression across samples for significant genes (Fig. 5g) was plotted using the R package ‘pheatmap’ 1.0.10 (Raivo Kolde, 2015-12-11; https://CRAN.R-project.org/package=pheatmap).

Putative protein-protein interaction was derived using the STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database version 11.079 (https://string-db.org/).

Functional enrichment analysis. To perform functional enrichment analysis, we used the Gene Set Enrichment Analysis (GSEA) paradigm through its implementa-tion in the R package ‘ClusterProfiler’ v3.10.180, and Bioconductor annotation package ‘org.Mm.eg.db’ v3.7.0. Balloon plots representing the output were generated using R packages ‘ggplot2’ v3.1.0 and ‘scales’ 1.0.0. GSEA analysis was conducted using the Genome Ontology information from the R ‘org.Mm.eg.db’ package, as well as the GSEA knowledge base derived from Enrichr81, as formatted in our previous study81.

Principal component analysis

Metabolites. Principal component analysis (PCA) was performed using the mean-centered matrix of metabolite values per each mouse. Principal components that separated sample groups were identified with visual inspection. Loadings from principal components that stratify experimental samples versus controls were then superimposed against metabolic pathways using a KEGG-Orthology mapping against the expected distribution of metabolites. Metabolic pathway enrichment analysis (gene set enrichment analysis, GSEA)82 were performed using 28 metabolic pathways defined by the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using pathways with four or more measured metabolites.

RNA-seq. PCA was performed using the R base package function ‘prcomp’. The first 2 principal components were used.

Quantification and statistical analysis. Statistical significance was determined using the two-sided Student’s t test. Statistical tests were performed using GraphPad Prism version 8.1.2. Results of t-tests are indicated in all figures as P < 0.05, **P < 0.01, ***P < 0.001 and ns for not significant (P > 0.05).
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Author contributions

J.R., T.L.M., B.A.B., R.L., J.H.J., C.J.M., and D.C-S. performed experiments. J.R., T.L.M., J.S.T.W., B.A.B., R.L., P.C., N.A.G., J.H.J., and C.L. analyzed the data. J.R. and C.L. wrote the manuscript. All authors approved the manuscript.

Competing interests

P.C. and C.L. are consultants and shareholders of CohBar, Inc. All other authors declare no competing interests.

Additional information

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