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Long term rapamycin treatment improves mitochondrial DNA quality in aging mice

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Abstract

Age-induced mitochondrial DNA deletion mutations may underlie cell loss and tissue aging. Rapamycin extends mouse lifespan and modulates mitochondrial quality control. We hypothesized that reduced deletion mutation abundance may contribute to rapamycin's life extension effects. To test this hypothesis, genetically heterogeneous male and female mice were treated with rapamycin, compounded in chow at 14 or 42 ppm, from 9 months to 22 months of age. Mice under a 40% dietary restriction were included as a control known to protect mtDNA quality. To determine if chronic rapamycin treatment affects mitochondrial DNA quality, we assayed mtDNA deletion frequency and electron transport chain deficient fiber abundances in mouse quadriceps muscle.

At 42 ppm rapamycin, we observed a 57% decrease in deletion frequency, a 2.8-fold decrease in ETC deficient fibers, and a 3.4-fold increase in the number of mice without electron transport chain deficient fibers. We observed a similar trend with the 14 ppm dose. DR significantly decreased ETC deficient fiber abundances with a trend toward lower mtDNA deletion frequency. The effects of rapamycin treatment on mitochondrial DNA quality were greatest in females at the highest dose. Rapamycin treatment at 14 ppm did not affect muscle mass or function. Dietary

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Competing Interests

The authors report no conflicts of interest.

restriction also reduced deletion frequency and ETC deficient fibers. These data support the concept that the lifespan extending effects of rapamycin treatment result from enhanced mitochondrial DNA quality.

Keywords

aging; mitochondrial DNA deletion mutations; rapamycin; skeletal muscle

Introduction

Mitochondrial dysfunction contributes to mammalian aging.(Sun, Youle et al. 2016) With age, somatically derived mtDNA deletions clonally accumulate within a subset of individual cells. These deletion mutations are large, centered in the mitochondrial major arc, and disrupt multiple genes that encode the protein subunits necessary for oxidative phosphorylation. For example, a previously described murine major arc deletion ablates subunit 3 of cytochrome c oxidase, four subunits of NADH dehydrogenase, and five transfer RNAs.(Brossas, Barreau et al. 1994, Tanhauser and Laipis 1995, Taylor, Ericson et al. 2014) When the intracellular deletion abundance exceeds 90%, the electron transport chain (ETC) function is disrupted.(Herbst, Pak et al. 2007) These cells lack cytochrome c oxidase (COX) activity. COX negative, ETC deficient cells, have been detected in the brain, heart, kidney, and skeletal muscle of aged mammals and in the mitochondrial mutator mice.(Wanagat, Cao et al. 2001,Ekstrand, Terzioglu et al. 2007, McKiernan, Tuen et al. 2007, Baris, Ederer et al. 2015)

In skeletal muscle, electron transport chain deficiency is localized to muscle fiber segments where the deletion mutations have accumulated to high levels (Fig 1).(Herbst, Pak et al. 2007, Lushaj, Johnson et al. 2008) ETC deficient regions atrophy, split, and undergo cell death and contribute to the age-induced loss of skeletal muscle fibers.(Wanagat, Cao et al. 2001, Cheema, Herbst et al. 2015) In humans, quadriceps muscle fiber number decreases by ~40% between 50 and 80 years of age.(Lexell, Taylor et al. 1988) In rodents, fiber loss, mtDNA deletions, and ETC deficient fibers are retarded by dietary restriction (Bua, McKiernan et al. 2004), but the mechanisms are unclear and this intervention has not seen broad clinical application.

Like dietary restriction, rapamycin treatment broadly decelerates age-related pathologies and extends lifespan in mammals.(Wilkinson, Burmeister et al. 2012) Rapamycin inhibits mTOR, which directly controls autophagy and leads to selective degradation of organelles including dysfunctional mitochondria.(Twig, Hyde et al. 2008) Activation of mitophagy has been suggested to control mtDNA quality through elimination of dysfunctional mitochondria.(de Grey 1997, Lemasters 2005, Suen, Narendra et al. 2010) In mtDNA mutation containing cybrid cells, rapamycin treatment robustly induced mitophagy (Gilkerson, De Vries et al. 2012) and decreased *in vitro* mitochondrial mutation frequency (Dai, Zheng et al. 2014). In a *Drosophila* model of mtDNA heteroplasmy, rapamycin treatment decreased the abundance of deletion bearing mtDNA and improved mtDNA quality.(Kandul, Zhang et al. 2016) In a mitochondrial helicase mutant mouse model,

rapamycin treatment for 70 days ameliorated myopathic progression. [Khan & Suomalainen, 2017] The *in vivo* effects of rapamycin on age-induced mammalian mtDNA deletions or ETC deficient cells are unknown.

We hypothesized that long term rapamycin treatment in mice would reduce mtDNA deletion mutation frequency and ETC deficient fiber abundance. To test this hypothesis, we measured mtDNA deletion abundances (Brossas, Barreau et al. 1994, Tanhauser and Laipis 1995, Taylor, Ericson et al. 2014) and ETC deficient fibers in 22 month old mice treated with rapamycin for 15 months (Drake, Peelor et al. 2013, Miller, Harrison et al. 2014). We found that rapamycin treatment decreased deletion frequency and ETC deficient fiber abundance.

Methods

Mice and experimental treatments

The mice used in this study were experimentally manipulated as part of the National Institute on Aging Intervention Testing Program. Details of breeding and husbandry of the genetically heterogenous UM-HET3 mice and experimental treatments with rapamycin have been previously described.(Drake, Peelor et al. 2013, Miller, Harrison et al. 2014) Briefly, at 9 months of age, mice were fed a diet containing encapsulated rapamycin at 14 or 42 ppm (mg of drug per kg of food). We denote these as R14 or R42, respectively. Other mice were placed on a 40% dietary restriction (DR) diet. Muscle samples from DR mice were included as controls as DR decreased muscle ETC deficient fibers in rats.(Bua, McKiernan et al. 2004) Predetermined cross-sectional cohorts were euthanized at 12 or 22 months following physiological measurements. At 22-months of age, in this strain, represents a population from which only about 5% of females to 20% of males have died spontaneously, minimizing selection bias effects.

Muscle physiological measurements

Mouse muscle physiological measurements including maximal isometric force (mN) and maximal specific force (N/cm^2) were done as previously described.(Sataranatarajan, Qaisar et al. 2015) *In situ* gastrocnemius muscle contractile properties were measured, as described by Larkin et al.(Larkin, Davis et al. 2011) Because sarcopenia was not yet evident in the control, R14 or DR mice, muscle physiological studies were not performed on the R42 mice.

Histochemical and immunohistochemical staining

Mice were euthanized, quadriceps muscles dissected from the animals, bisected at the mid belly and frozen in liquid nitrogen. A portion of each frozen muscle was embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), flash frozen in liquid nitrogen and stored at -80°C. A minimum of one hundred 10μm thick consecutive transverse cross-sections were cut with a cryostat at -20°C and placed on Probe-On-Plus slides or PEN membrane glass slides (Life Technologies) for laser microdissection. Slides were stored at -80°C until needed.

At 100um intervals, sections were stained for cytochrome c oxidase (COX, brown) or succinate dehydrogenase (SDH, blue) as previously described.(Wanagat, Cao et al. 2001) A

third slide was dual stained, first for COX and then for SDH. ETC abnormal fibers appear blue on a brown background following dual staining (Figure 1). Gömöri trichrome and H&E staining were performed according to standard protocols.(Sheehan and Hrapchak 1980)

Image analysis, counts and quantitation

ETC deficient fiber counts and cross-sectional area of the quadriceps were obtained from digital images. The absolute number of ETC deficient fibers (COX-/SDH++) were identified and annotated throughout 1000um of tissue in both control and treated mice. ETC deficient fiber numbers were normalized to the volume of muscle tissue examined. Observers were blinded to the treatment groups.

Digital PCR quantitation of murine mtDNA deletion mutations

MtDNA deletion mutation frequency was measured by droplet or chip-based digital PCR as previously described.(Taylor, Ericson et al. 2014) This assay interrogates the portion of the mouse major arc between the primer sets exclusive of the portion encompassed by the TaqI restriction enzyme sites (Figure 1C). Briefly, total DNA was isolated and purified by proteinase K digestion and phenol:chloroform extraction. Ten micrograms of total DNA was digested with TaqI (New England Biolabs) and subsequently extracted with phenol:chloroform. The digested total DNA was diluted until the concentration was within the detection range for digital PCR according to the manufacturer's instructions. For droplet-based digital PCR, reaction mixtures containing the digested DNA were prepared and droplets generated on a DG8 cartridge (Bio-Rad) before thermocycling using the following protocol: initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, and 63.5 °C for 4 min. The thermally cycled droplets were then analyzed by flow cytometry on a QX100 Droplet Digital PCR system (Bio-Rad). The number of mtDNA deletion mutation genomes per droplet was calculated by QuantaSoft software (Bio-Rad).

For chip-based digital PCR, diluted samples were loaded onto Quantstudio 3D digital PCR 20K Chip (Version 2, ThermoFisher; Waltham, MA). Final primer and probe concentrations were 900 nM and 250 nM, respectively. Chip-based digital PCR cycling conditions were Taq-polymerase activation at 96°C for ten minutes, 40 cycles of denaturation at 98°C for 30 seconds and annealing/extension at 63.5°C for 4 min. Reaction threshold fluorescence and target copy numbers per microliter were determined using QuantStudio 3D Analysis Suite Cloud Software (Version 3, ThermoFisher; Waltham, MA).

The following primer/probe sets were used to determine deletion frequency. Control site: 5'-GAC ACA AAC TAA AAA GCT CA-3' (forward primer), 5'- TAA GTG TCC TGC AGT AAT GT-3' (reverse primer), 5'- 6FAM-CCA ATG GCA TTA GCA GTC CGG C-MGB-3' (probe). MtDNA deletion: 5'- AGG CCA CCA CAC TCC TAT TG-3' (forward primer), 5'-AAT GCT AGG CGT TTG ATT GG-3' (reverse primer), 5'-6FAM-AAG GAC TAC GAT ATG GTA TAA-MGB-3' (probe).

Long Extension PCR amplification of mtDNA deletions from single muscle fibers

Mouse mtDNA deletions were amplified from single laser microdissected ETC deficient muscle fibers as previously described.(Herbst, Widjaja et al. 2017) Long extension PCR

reactions were assembled according to the manufacturer's instructions, Go-Taq Long PCR master mix (Promega, Madison, WI). PCR cycling conditions were polymerase activation at 95°C for two minutes, denaturation at 94°C for twenty seconds, and annealing at 68°C for ten minutes, repeated for 40 cycles. PCR products were fractionated on 1% agarose gels, stained with ethidium bromide, and visualized under UV light.

Statistical analysis

Data were analyzed using GraphPad Prism (Version 6, GraphPad Software, San Diego, California, USA). Data are presented as mean ± SEM. Data in Table 1 were analyzed using Student's t-tests to compare treatment to controls within sex. Non-normal data in Figures 2 and 3 were analyzed using Mann Whitney U-tests to determine significance. Contingency data were analyzed using Fisher's exact test. Alpha was determined by Bonferroni's method to control for multiple comparisons. Two-way ANOVA was used to identify sex and treatment effects on log transformed data. In datasets with zero values, the average was added prior to log transformation.

Results

We examined the effects of age, chronic rapamycin treatment and DR on gastrocnemius mass and function in the genetically heterogeneous mice. Between 12- and 22- months of age gastrocnemius mass did not change in control mice regardless of sex (Table 1). Similarly, long-term rapamycin administration did not diminish muscle mass or contractile function in either sex. Although after three months of DR, body masses, muscle masses and the resultant force generation were smaller than controls, specific force normalized for muscle size was not different from control values and no declines with aging were observed. A 21% increase in body mass was observed following 3-months of rapamycin treatment in male mice, but this increase did not persist (Table 1).

We examined mtDNA quality in quadricep muscles from 22-month old male and female mice following 13 months of rapamycin treatment at two doses, 14- and 42-ppm (denoted R14 and R42, respectively). Serial, histochemically stained cryosections demonstrated muscle fibers with age-induced ETC deficient segments (Figure 1A, **top row**). ETC deficient fibers lack cytochrome c oxidase activity with concomitant increases in succinate dehydrogenase activity as shown by histochemistry. These ETC deficient fibers were typically atrophied. Dual stained sections (combined COX and SDH) were used to determine the abundance of ETC deficient fibers. ETC deficient fibers were observed in all sexes and treatment groups at 22 mo (Figure 1A, **bottom row**). The phenotype of dual stained fibers was confirmed as COX-negative in serial sections. Laser microdissection and long extension PCR were used to determine the mtDNA genotype of ETC deficient fibers. Each isolated ETC deficient fiber contained a unique mtDNA deletion (Figure 1B) when amplified by primers flanking the entire major arc of the mitochondrial genome, whereas ETC normal muscle fibers did not. In the four fibers examined, we did not observe the murine common deletion as described by others. (Brossas, Barreau et al. 1994, Tanhauser and Laipis 1995, Taylor, Ericson et al. 2014)

We used digital PCR to measure the frequency of mouse mtDNA deletions. Rapamycin at 42ppm significantly reduced deletion frequency (Figure 2A; Cohen's d = 0.54). DR also reduced deletion frequency, but this trend was not significant when corrected for multiple comparisons (p=0.0266, Figure 2A). R42 and DR significantly decreased ETC deficient fiber abundances (Figure 2B; Cohen's d of 0.63 and 0.82, respectively). A higher fraction of mice in the R42 and DR treatment groups lacked ETC deficient fibers within the 1,000 microns of quadriceps sampled which was significant for DR ($p<0.0001$) and a trend is noted for R42 (p=0.02666) (Figure 2C). None of the treatments significantly affected ETC deficient fiber segment length (Figure 2D).

Two-way ANOVA of the log transformed data identified significant sex and treatment effects on deletion mutation frequency (sex effect $p<0.001$, treatment effect $p<0.001$, interaction $p<0.001$) and ETC deficient fiber abundance (sex effect p=0.0193, treatment effect $p<0.001$, interaction p=0.0107). The analysis did not identify sex or treatment effects on ETC deficient segment length. To explore sex effects, we analyzed male and female data separately by non-parametric Mann-Whitney tests (Figure 3A, 3B). R42 treatment and DR of females resulted in a trend toward lower deletion frequency p=0.024 (Cohen's d = 1.00) and 0.054 (Cohen's d = 0.83) respectively, and significant decreases in ETC deficient fiber abundance. Deletion frequency and ETC deficient fiber abundances also decreased in males treated with rapamycin (Cohen's d = 0.91), but this change was not statistically significant (Figure 3A, 3B). Across the treatments, the fraction of females free of ETC deficient fibers within the examined muscle sample was always greater than in males (Figure 3C) with significant effects observed in R42 females and DR males.

Discussion

We found that 13 months of rapamycin treatment of genetically heterogenous mice reduces mtDNA mutation frequency and ETC deficient fiber number, with the greatest effects in the female 22-month-old mice. A reduction in ETC deficient fiber abundance by DR has also been observed in aged hybrid rats.(Bua, McKiernan et al. 2004) In cybrid cells and a transgene-based *Drosophila* model of mtDNA deletion heteroplasmy, rapamycin treatment induced mitophagy (Gilkerson, De Vries et al. 2012) and decreased mtDNA mutation frequency *in vitro* and *in vivo* (Dai, Zheng et al. 2014, Kandul, Zhang et al. 2016) The cybrid and *Drosophila* studies are consistent with our findings and, taken together, indicate a beneficial effect of enhanced mitochondrial quality with age.

In the current study of aging mice, the effects of rapamycin treatment on mtDNA deletion frequency and ETC deficient fiber abundance were dose dependent. Rapamycin treatment was effective at the higher dose, 42ppm, with no statistically significant effect observed at 14ppm. The dose effect on mtDNA deletions mirrors the greater benefit observed with higher rapamycin doses in lifespan studies. In these studies, female mouse median lifespan was extended by 16, 21 and 26% at 4.7, 14 and 42ppm of rapamycin treatment respectively. (Miller, Harrison et al. 2014) Since rapamycin had greater protective effects at the higher dose, further dose increases may confer additional benefit.(Johnson and Kaeberlein 2016)

Our data show a greater benefit of rapamycin treatment in females. Miller et al. (Harrison, Strong et al. 2009, Miller, Harrison et al. 2014) previously identified sex-specific effects of rapamycin treatment. These effects included a 14% increase of lifespan in females versus males at 42ppm rapamycin, alterations of xenobiotic metabolism, differing sensitivity to mTORC1 inhibition (Drake, Peelor et al. 2013) and improvements in late life spontaneous activity (Miller, Harrison et al. 2011, Wilkinson, Burmeister et al. 2012). Sexual dimorphism in rapamycin pharmacokinetics contributes to the sex-specific effects of rapamycin in these genetically heterogeneous mice. Female mice attain higher serum rapamycin concentrations than males at equivalent dosage. (Miller, Harrison et al. 2014) Our data add to the growing evidence of sex-specific differences in mTOR signaling and mitochondrial quality control. (Lamming, Mihaylova et al. 2014, Ribas, Drew et al. 2016)

Chronic rapamycin treatment showed no adverse effects on muscle mass or force generation. At 22 months of age, we did not observe declines in muscle mass or function in the gastrocnemius of genetically heterogeneous control mice of either sex. This result is unexpected because AKT-mTORC1 regulates muscle hypertrophy and rapamycin inhibition of mTORC1 block muscle hypertrophy under chronic loading.(Bodine, Stitt et al. 2001) Since sarcopenia was not yet evident at this age in this mouse strain, the abundance of ETC deficient fibers was also low. MtDNA deletions and ETC deficient fibers increase exponentially with advancing age in skeletal muscle.(Lee, Chung et al. 1993, Eimon, Chung et al. 1996, Aspnes, Lee et al. 1997, Herbst, Widjaja et al. 2017) Rapamycin might have greater effect at older ages when the control mice have higher deletion loads and accelerating muscle mass loss.

Conceptually, mitophagy should clear damaged mitochondria including those with mtDNA deletions. This concept conflicts, however, with single cell data showing that age-induced mtDNA deletions accumulate, disrupt homeostasis, trigger apoptosis and necrosis, and result in cell death and loss. (Cheema, Herbst et al. 2015, Herbst, 2016 #1565). One general explanation may be an age-specific loss of the balance between mitophagy and mitochondrial biogenesis. Mitophagy and mitochondrial biogenesis are known to decline with age (Lopez-Lluch, Irusta et al. 2008, Diot, Morten et al. 2016) and perturbations of this balance in old animals affects mtDNA deletion mutation abundance.(Herbst, Wanagat et al. 2016, Lin, Schulz et al. 2016) Rapamycin, by shifting the balance towards mitophagy, may improve mtDNA quality. Another possible explanation more specific to mtDNA deletions may be the intracellular level of heteroplasmy. At low mtDNA deletion burden, mitochondrial membrane potential is maintained and mitophagy is not activated.(Gilkerson, De Vries et al. 2012) Conversely, as deletion mutation abundance exceeds the phenotypic threshold and membrane potential is compromised, damaged mitochondria may be able to escape recognition by the energy-dependent mitochondrial quality control machinery involving PNK1 and Parkin.(Mishra and Chan 2014) Furthermore, even if damaged mitochondria are correctly recognized and targeted for degradation, the ability to acidify the lysosome may be compromised in muscles of aged mice due to ATP production failure commensurate with ETC deficiency.(Hughes and Gottschling 2012) Chronic rapamycin treatment may overcome these limitations by promoting mitophagy at a lower level of heteroplasmy.

Future studies to address translation of these finding to mtDNA deletions and sarcopenia in humans should involve higher rapamycin doses, older ages of onset, and different species, as well as concomitant assays of mitophagy and biogenesis. Rapamycin treatment joins a small but growing list of interventions that decrease mtDNA deletions and ETC deficient fiber abundances and that also extend lifespan.

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Highlights

- Age-induced mitochondrial DNA deletions may cause cell loss and tissue aging.
- Long term rapamycin treatment reduces mtDNA deletions and ETC deficient fibers.
- Dietary restriction reduces deletions and ETC deficient fibers as previously shown in rats.
- Enhanced mtDNA quality may contribute to the lifespan extending effects of rapamycin.

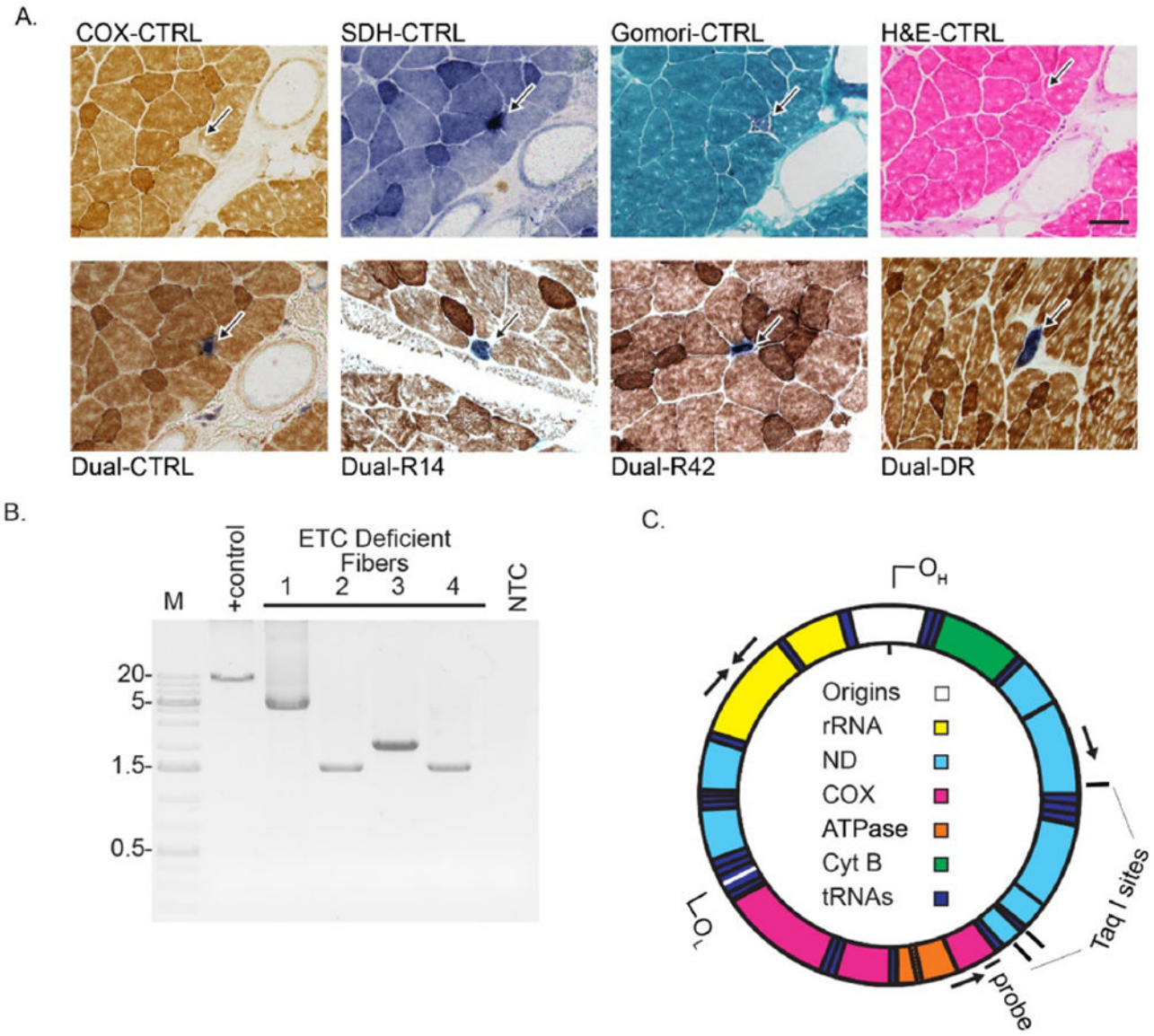

Figure 1.

A. Electron transport chain deficient fibers from 22-month old mice. Top micrographs: Serial skeletal muscle histological sections from a control mouse. A single ETC deficient fiber is denoted by the black arrow. Bottom micrographs: Dual COX and SDH staining was used to identify ETC deficient fibers (black arrow) from control, 14 ppm rapamycin (R14), 42 ppm rapamycin (R42), and dietary restricted (DR) mice. The black bar indicates 100 microns. B. Detection of mtDNA deletion mutations in laser microdissected ETC deficient fibers. C. Primer and Taq I restriction site locations used to detect deletions in the major arc of mouse mitochondrial DNA.

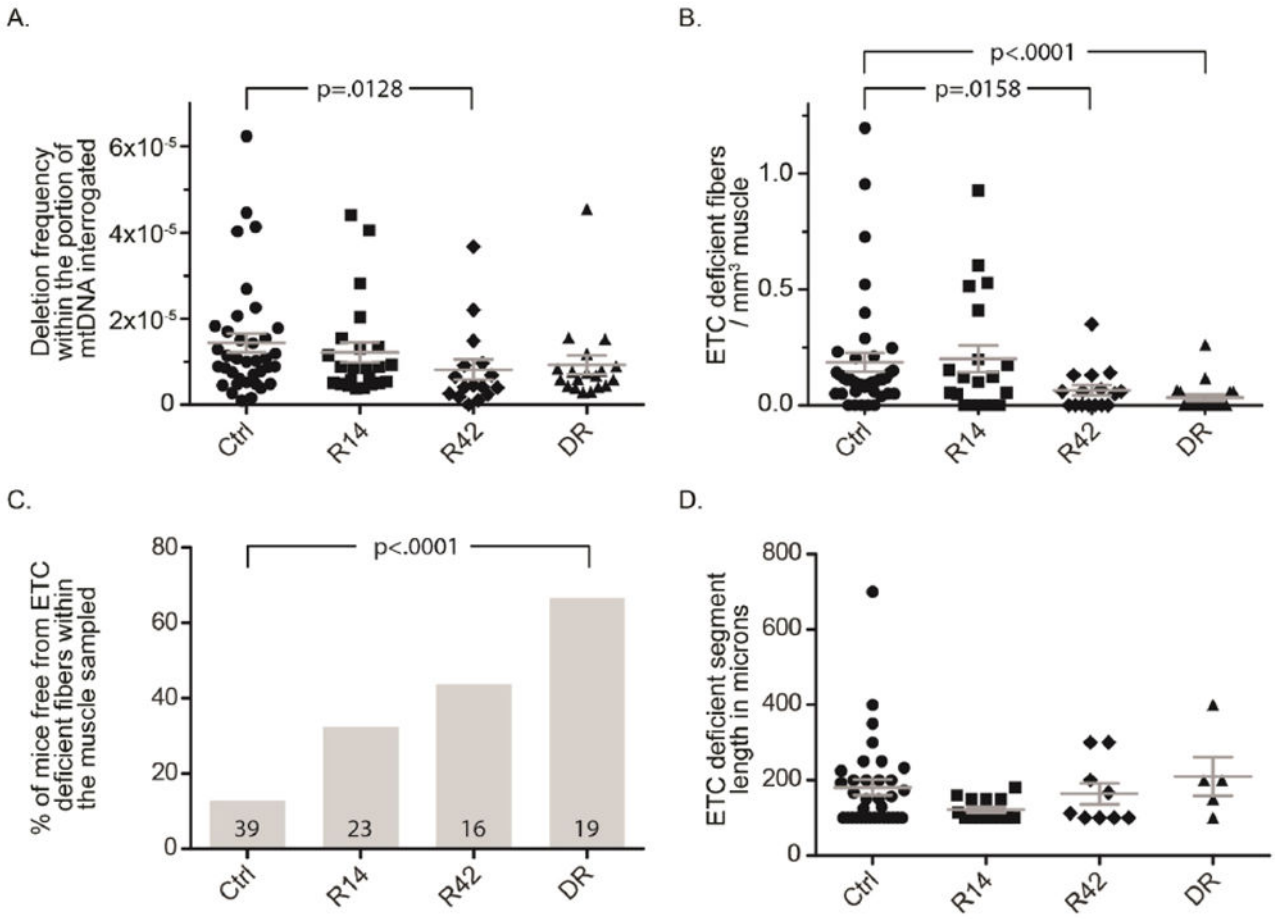

Figure 2.

Impact of chronic rapamycin treatment and dietary restriction (DR) on mtDNA deletion mutations in 22mo mouse quadriceps. Each point indicates an individual mouse with animal numbers of control = 37 in panel A and 39 in panels B, C, and D, R14 = 23, R42 = 16 and DR = 19. A. Mouse mtDNA deletions/total mtDNA copy number. B. ETC deficient fiber abundance. In A and B, bars denote mean ± standard error and p-values are determined using Mann-Whitney U tests. C. Percentage of mice from panel 2B that are free of ETC deficient fibers within the sampled muscle. p-values are determined using Fisher's exact test. Numbers within the columns denote the total number of mice sampled. D. ETC deficient segment lengths. Bars denote the median and quartiles. For all panels, connecting bars are significant, alpha=0.0167.

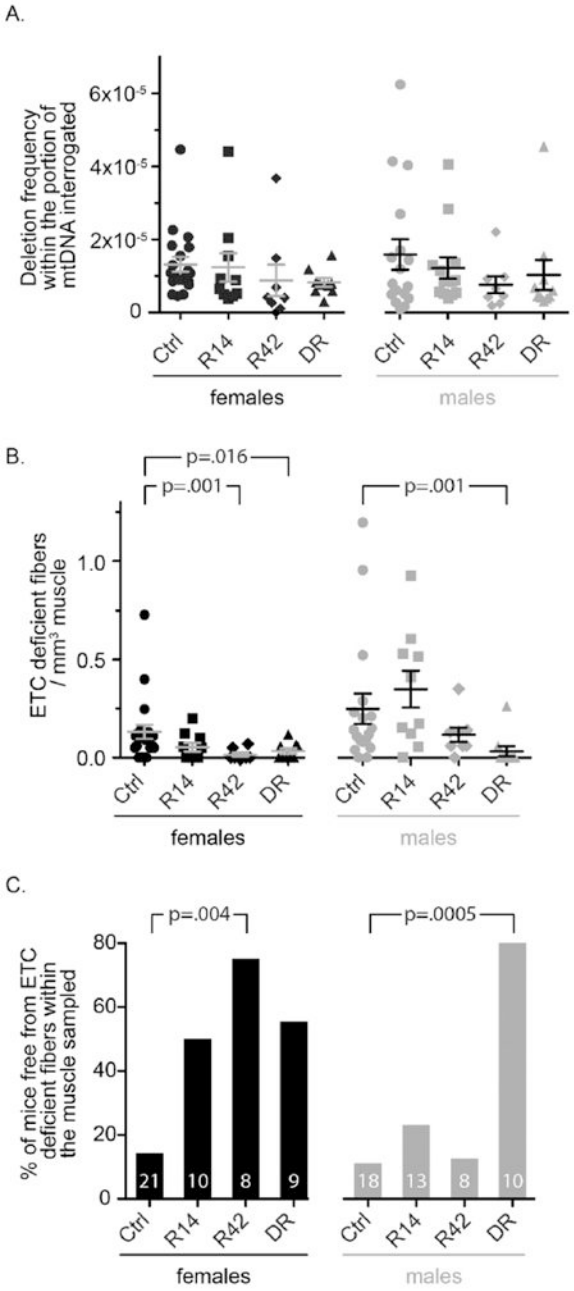

Figure 3.

Rapamycin treatment and dietary restriction differentially affect mtDNA deletion abundances in the quadriceps muscles of 22month old female and male mice. Each point indicates an individual mouse muscle sample. For female mice, animal numbers are control = 20 in panel A and 21 in panels B and C, R14 = 10, R42 = 8, and DR = 9. For male mice, animal numbers are control = 17 in panel A and 18 in panels B and C, R14 = 13, R42 = 8 and DR = 10. A. Mouse mtDNA deletions/total mtDNA copy number. B. ETC deficient fiber abundance. In A and B, bars denote mean ± standard error and p-values ares determined using Mann-Whitney U tests. C. Percentage of mice from panel 3B that are free

of ETC deficient fibers within the sampled muscle. p-values are determined using Fisher's exact test. Numbers within the columns denote the total number of mice sampled. For all panels, connecting bars are significant, alpha=0.0167.

Table 1

Gastrocnemius muscle mass, body weight, and muscle force characteristics.

Treatment	Age (months)	Sex	Gastrocnemius Mass (mg)	Body Mass (g)	Gc mass/Body Mass (%)	Max Isometric Force (mN)	Max Specific Force (N/cm^2)
Control	12	F	147.3 ± 4.4	35.2 ± 1.2	0.42 ± 0.02	4566 ± 118	25.7 ± 1.0
R14	12	F	136.6 ± 3.0	34.3 ± 3.0	0.40 ± 0.01	4458 ± 202	26.5 ± 0.9
DR	12	F	120.3 ± 4.2 *	25.4 ± 0.5 *	0.47 ± 0.01	3828 ± 189 *	24.4 ± 1.2
Control	12	M	174.1 ± 3.8	36.2 ± 1.8	0.49 ± 0.03	5173 ± 79	24.5 ± 0.6
R14	12	M	163.8 ± 5.0	43.8 ± 2.2 *	0.38 ± 0.02 *	4519 ± 133 *	22.8 ± 0.9
DR	12	M	156.0 ± 4.9	32.0 ± 1.1	0.49 ± 0.01	4635 ± 122 *	23.7 ± 0.9
Control	22	F	142.6 ± 2.6	33.2 ± 1.6	0.44 ± 0.02	4162 ± 177	24.3 ± 1.2
R14	22	F	134.7 ± 4.3	38.3 ± 2.1	0.36 ± 0.01 *	4264 ± 241	25.0 ± 1.1
DR	22	F	118.6 ± 3.8 *	25.9 ± 0.5 *	0.46 ± 0.02	4049 ± 84	27.8 ± 0.9
Control	22	M	168.7 ± 4.3	39.4 ± 1.7	0.44 ± 0.02	4635 ± 301	23.3 ± 0.8
R14	22	M	164.7 ± 4.5	36.9 ± 1.1	0.45 ± 0.01 $	4245 ± 149	22.0 ± 0.9
DR	22	M	156.6 ± 4.1	32.5 ± 1.0 *	0.48 ± 0.01	4435 ± 124	23.6 ± 0.6

Data is the average ± S.E.M,

* indicates a treatment effect versus age-matched control.

$ indicates an age-specific effect vs 12 month old. alpha=0.0167.