Mouse Skeletal Muscle Decrease in the MICOS Complex and Altered Mitochondrial Networks with age

Zer Vue*, Edgar Garza-Lopez*, Kit Neikirk*, Larry Vang, Heather Beasley, Andrea G. Marshall, Sandra A. Murray, Antentor Hinton, Jr

Affiliations:
Z.V., K.N., L.V., H.B., A.G.M., J.A.G., A.H.J.: Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN, 37232, USA.
E.G.,: Department of Internal Medicine, University of Iowa, Iowa City, IA, 52242, USA.
S.M.: Department of Cell Biology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, 15260, USA.

*These authors share co-first authorship.

Corresponding Author:
Antentor Hinton
Department of Molecular Physiology and Biophysics
Vanderbilt University
antentor.o.hinton.jr@Vanderbilt.Edu 319-383-3095

Graphical Abstract:

ABSTRACT
Skeletal muscle gradually loses mass, strength, endurance, and oxidative capacity during aging. While mitochondrial aging is associated with endoplasmic reticulum stress, fragmented mitochondria, and decreased mitochondrial capacity, the genes associated with morphological changes in mitochondria during aging still requires further elucidation. Further, it is not completely understood how 3D mitochondrial networks and the specialization of mitochondria are altered during aging. We measured changes in mitochondrial morphology and mitochondrial connectivity during the aging of the mouse gastrocnemius muscle through serial block facing-scanning electron microscopy and 3D reconstruction. We found changes in mitochondrial network configuration, nanotunneling, size, shape, number, contact sites, cristae organizing system (MICOS) dynamics and gene expression in skeletal muscle during aging. We also found an association of OPA-1 and the MICOS complex in the gastrocnemius with mitochondrial aging. Further, the loss of the MICOS complex was linked with decreased oxidative capacity and altered mitochondrial metabolism. MICOS proteins decreased with age and mitochondrial morphology was similar between aged skeletal muscle and that of young mice with MICOS protein loss. In tandem, our data suggest a relationship between the MICOS complex and aging, which could be potentially linked to disease states with additional 3D reconstruction.

Keywords: MICOS, aging, mitochondria, 3D morphometry; mitochondrial disease; mitochondrion; nanotunnel; reconstruction; reticulum; serial block-face SEM; skeletal muscle.

45
46

INTRODUCTION

The loss of muscle mass associated with aging, known as sarcopenia, impacts the quality of life affecting both type I and type II fibers, but more so for type II fibers. The mitochondrial free radical theory of aging (MRFTA) which proposes that the accumulation of reactive oxygen species (ROS) byproducts causes damage that leads to aging [1–3]. The critical role of mitochondrial oxidative stress in aging has been shown using mitochondrial-targeted antioxidants, such as catalase targeted to mitochondria, in cardiac aging [4] and sarcopenia [5]. In addition to the main function of generating cellular ATP by oxidative phosphorylation [6], the mitochondria play critical role in ROS signaling, calcium homeostasis, programmed cell death, and regulation of innate immunity and stem cells. To carry out the tremendous variety of tasks, mitochondria maintain finely regulated ultrastructures, through various mechanisms, one of which is mitochondrial dynamic machinery through fission and fusion. Since their structure changes dynamically as mitochondria shift from fission to fusion, many important mitochondrial functions depend on their ultrastructure[7–9], therefore, it is important to understand mitochondrial structural changes over time. Essential mitochondrial functions are associated with the mitochondrial inner membrane folds known as cristae, which house the oxidative phosphorylation machinery[8] and various transporters. Our objective was to determine how mitochondrial networks and individual mitochondrial structures change during aging. We hypothesized that age-related oxidative stress causes increased mitochondrial mitophagy and fragmentation over time with concomitant losses in mitochondrial cristae integrity.

Disruption of optic atrophy 1 (OPA-1), an inner membrane protein that regulates mitochondrial fusion, causes mitochondrial fragmentation and affects the dimensions, shapes, and sizes of the cristae [8]. Disruption of Drp1, a protein associated with mitochondrial fission, causes elongated mitochondria and resistance to remodeling of the cristae [10,11]. Nanotunnels or "mitochondria-on-a-string" are thin, double-membrane protrusions lacking cristae that allow mitochondria to communicate across distances. Nanotunnels may increase in mitochondrial disease [12,13] and may be associated with mitochondrial dysfunction during aging. Mutations in genes that regulate the morphology of cristae have been associated with aging cardiomyocytes [14]. These gene products are located at the crista junctions in the inner membrane and are a part of the mitochondrial contact site and cristae organizing system (MICOS), which are important for maintaining mitochondrial shape and size [15]. DRP1 or OPA-1 loss can similarly affect mitochondria morphology [16,17]. Cristae membranes contain the electron transport chain complexes and ATP synthase for ATP synthesis via oxidative phosphorylation [18–20]. Since mitochondrial morphology affects function, altering the structure by knocking out MICOS-associated genes or the GTPase of OPA-1 could alter the metabolism and function of mitochondria during aging [18–20]. Critically, we predicted that MICOS-associated genes were lost over age, therefore, loss of MICOS-associated genes would mimic loss in mitochondria morphology observed across age.

To better understand the alterations of mitochondrial specialization ultrastructure in aging, we compared size, shape, quantity, complexity, and branching of mitochondria using 3D reconstructions of aged gastrocnemius, and we measured nano tunneling in mouse muscles at these three ages. Multivariate analysis was used to identify changes in metabolites, the MICOS complex, OPA-1, nano tunneling, mitochondrial complexity, and morphological changes to better understand how mitochondrial specialization and structure change during aging.

RESULTS

Aging Results in Smaller, Shorter, Poorly Connected Mitochondria with Decreased Branching in Mouse Skeletal Muscle

The gastrocnemius is a mixed muscle with both oxidative fibers containing many mitochondria and glycolytic fibers with few mitochondria. This heterogeneity makes the gastrocnemius ideal to study changes in mitochondrial dynamics. Since many mitochondrial functions depend on structure [7–9], it is important to examine mitochondrial structural changes over time. We hypothesized that increasing mitochondrial mitophagy and fragmentation over time is concomitant with losses in mitochondrial crista integrity.

Our objective was to determine how aging alters mitochondrial networks and individual mitochondrial structure. We imaged gastrocnemius biopsies from young (3-month-old), mature (1-year-old) and aged (2-year-old) mice by serial block-face scanning electron microscopy (SBF-SEM) with resolutions of 10 µm for the x- and y- planes and 50 µm for the z-plane, which allows visualization of the electron connectome. Approximately 50 intermyofibrillar (IMF) mitochondria were segmented from each image stack (Figure 1A-C) and a 3D surface view was generated (Figure 1A''-C''). We analyzed mitochondria sub-network volumes from four ROIs with an average of 175 mitochondria for each mouse (n = 3), for a total of 500 mitochondria. Mitochondrial networks in aged mice showed largely interconnected mitochondria that were not composed of a single reticulum (Figures 1A'''–C'''). We found that mitochondrial volume decreased with age (Figures 1 A'''–C'') as did mitochondrial network volumes (Figure 1D). The area and perimeter of mitochondria of samples from 2-year-old mice were significantly lower than those from 3-month-old mice; however, there was no significant change for 1-year-old mice compared to 3-month-old mice (Figures 1E-F). While there was some variability across the three mice for each age cohort (SFigure 1), in general the trend showed a downwards trajectory emblematic of increased fragmentation and smaller mitochondria. This showed that the size and length of mitochondria change with age, but the specific complexity of mitochondria, which is implicated with mitochondria communication, needed to be further elucidated.

We next measured mitochondrial complexity to better understand changes in mitochondrial shape during aged mitochondrial complexity, quantification of the 2D form factor as a 3D shape, as above. We hypothesize that less networks and simpler shape would occur as aging and dysfunction continued. Previously established methods [21,22] were used to determine three dimensional mitochondrial complexity from two dimensional form factor measurements [21,22]. First, we first examined transverse (Figure 2A-C) and longitudinal (Figure 2A'-C') mitochondrial dimensions across ages in skeletal muscle. These measures showed networking and broad mitochondria. Mitochondrial branching index (MBI) is the ratio of transverse (Figures 2A–C) to longitudinal (Figures 2A'-C') branching, which better quantifies the orientation of the branching, and MCI and MBI together measure mitochondria morphology [21]. We found that MBI decreased across the age cohorts (Figure 2D). Since we observed in 3D reconstructions that mitochondrial populations are heterogeneous and diverse, we utilized mito-typing, which is the karyotyping-like method for arranging mitochondria [21], to capture the diversity of IMF mitochondria (Figure 2E). This analysis suggested at every volumetric measurement, mitochondria were smaller and less complex with age. We also measured sphericity to further understand changes in complexity. There was a progressive increase in sphericity with aging (Figure 2F). When examining the specific three male mice sampled for each age cohort, some variation exists for both metrics; however, the trends again clearly show sphericity stays consistent while MBI decreases across age (SFigure 2). Together, these data suggest that complexity decreases across all mitotypes with age.

139
140 **Mitochondrial Nanotunnels Change in Size and Amount with Aging in Skeletal Muscle**
141 To quantify nanotunnels in skeletal muscle, we performed 3D reconstruction in mice at
142 three ages, measuring 550 mitochondria per mouse (n=3) using four different regions of interests
143 (ROIs). Although nanotunnels are reported to exist primarily in mitochondrial disease states [12],
144 we found them in all three ages of mice (Figures 3A–C). The diversity and heterogeneity in the
145 organization, sizing, and branching of nanotunnels necessitated mito-otyping (Figure 3D). We
146 found nanotunnels in a greater proportion of mitochondria in 1-year-old mice than in 2-year-old
147 mice (Figure 3E). However, the number of nanotunnels in each mitochondrion was significantly
148 less in the 2-year-old mice than the 3-month-old and 1-year-old mice (Figure 3F). The length and
149 diameter of nanotunnels increased in the older mice compared to the 3-month-old mice (Figures
150 3G–H), but the increase was significantly greater in the 1-year-old mice than the 2-year-old mice
151 (Figures 3G–J). These data show that nanotunnel structure, frequency, and length change during
152 aging.
153 Since we observed mitochondrial dynamics and cristae change with aging , we measured
154 transcripts for *Opa-1*, the gene associated with mitochondrial fusion, and *CHCHD3* (*Mic19*),
155 *CHCHD6* (*Mic25*), and *Mitofilin* (*Mic60*), the genes for the principal MICOS complex subunits.
156 We used reverse-transcriptase qPCR (RT-qPCR) to determine the effect of aging on
157 transcription. Since aging causes fragmentation of mitochondria, a hallmark for this process is
158 loss of *Opa-1* and MICOS complex proteins. We hypothesized that aging would also lead to
159 decreases in the associated MICOS complex proteins, confirming prior studies which have
160 found Opa-1 loss across aging [19,23–25]. We found progressive loss of both *Opa-1* and MICOS
161 complex subunits, as measured by loss of transcripts for the proteins (SFigure 3A–D). In 1- and
162 2-year-old mice, the amount of mRNA made for the MICOS complex subunit genes or the *Opa-*
163 *1* gene was less than half of that made in 3-month-old mice (SFigure 3A–C). Furthermore, for
164 all MICOS genes, there was a statistically significant decrease in the amount of mRNA over
165 time with a higher level of significance in 2-year-old mice. These data suggest a correlation
166 between mitochondrial morphologic changes and decreases in expression of genes for *Opa-1*
167 and MICOS subunits *CHCHD3*, *CHCHD6*, and *Mitofilin* during aging; however, they are not
168 sufficient to demonstrate causation, therefore we did additional measurements to show that
169 reduced protein expression levels may be responsible for the morphologic changes.
170
171 **Changes in Cristae and Mitochondria in Myotubes and Oxygen Respiration Rate Upon**
172 **Knockout of MICOS complex and *Opa-1***
173 The MICOS complex and OPA-1 are key players in mitochondrial biogenesis [15,19,26], but
174 how their interactions regulate aging and mitochondrial ultrastructures is poorly understood. To
175 determine the role of OPA-1 and the MICOS complex in mitochondrial structure and
176 networking, we ablated the genes for *Opa-1* and the MICOS complex in isolated primary skeletal
177 muscle cells from 3-month-old mice. We isolated primary satellite cells, then differentiated
178 myoblasts to myotubes. Using CRISPR/Cas9 method (Table 2), and a control plasmid, we
179 knocked out the genes for MICOS complex components and *Opa-1* from skeletal muscle cells.
180 As observed previously, in vitro deletion of OPA-1 altered mitochondrial morphology [16,27,28].
181 We measured 1250 mitochondria across 10 cells, with loss of *Opa-1* as a positive control for
182 mitochondrial morphological changes. Although *Opa-1* expression decreases with age [29], the
183 effect of the MICOS complex being lost in mitochondria morphology is unknown. Using
184 transmission electron microscopy (TEM) images, we compared mitochondria and cristae in

185 myotubes from wild type (WT) and knockouts of *Opa-1* and *Mitofilin* genes, which are essential
186 for the organization of mitochondrial cristae [30,31] (Figures 4A–C). Mitochondrial average area
187 decreased for both *Opa-1* and *Mitofilin* knockout myotubes (Figure 4D), while mitochondrial
188 circularity (the roundness and symmetry of mitochondria) and number increased for both *Opa-1*
189 and *Mitofilin* knockout myotubes (Figures 4E–F). For both *Opa-1* and *Mitofilin* knockouts, the
190 number of cristae per mitochondrion decreased, as did the cristae score and cristae surface areas
191 compared to the WT (Figures 4G–I). Cristae score measures the uniformity and regularity of
192 cristae compared to typical cristae, with a lower number representing abnormality and
193 dysfunction.
194 To further elucidate these changes in event of loss of other MICOS proteins, we also
195 compared WT with *Opa-1*, *CHCHD3*, and *CHCHD6* knockout myotubes (Figures 5A–D).
196 Knockout of MICOS subunit *CHCHD3* results in fragmented mitochondria with less *Opa-1* as
197 the cristae lose their normal structure [32]. Similarly, *CHCHD6* is important in maintaining crista
198 structure and its downregulation results in hollow cristae lacking an electron-dense matrix,
199 thereby inhibiting ATP production and cell growth [31,33,34]. Mitochondrial average area decreased
200 for *Opa-1*, *CHCHD3*, and *CHCHD6* knockout myotubes (Figure 5E), while the circularity index
201 and the number of mitochondria, once normalized, increased (Figures 5F–G). For *Opa-1*,
202 *CHCHD3*, and *CHCHD6* knockouts, the number of cristae per mitochondrion decreased, as did
203 the cristae score and cristae surface area compared to the WT (Figures 5H–J). The least
204 significant change was seen in the *CHCHD3* knockout. Together, these data show quantitative
205 and structural changes in both mitochondria and cristae upon loss of MICOS proteins.
206 Loss of OPA-1 induces bioenergetic stress and decreased electron transport chain
207 function [28] and ablation of the MICOS complex alters mitochondrial capacity [35,36]. We found that
208 loss of OPA-1 or *Mitofilin* in myotubes decreased basal oxygen consumption rate (OCR)
209 (Figures 4J–K) and decreased ATP-linked, maximum, and reserve capacity OCR (Figures 4L–
210 N). Although OPA-1 knockout myotubes exhibited a decrease in proton leak, which represents
211 protons that go from the mitochondria to matrix without producing ATP, *Mitofilin* knockouts
212 showed no significant difference (Figure 4O). In *Opa-1*, *CHCHD3*, and *CHCHD6* knockouts,
213 there was a decrease in basal, ATP-linked, maximum, and reserve capacity OCR compared to the
214 control (Figures 5K-O). Although proton leak OCR decreased in *Opa-1* and *CHCHD3* knockout
215 myotubes (Figure 5P), there was no significant difference between the control and *CHCHD6*.
216 The decrease in OCR may be attributed to smaller and fragmented mitochondria; mitochondrial
217 density decreases as fragmentation targets them for autophagy [29,37]. Together, these results show
218 that MICOS and *Opa-1* are essential for normal respiration of muscle tissue.
219 TEM provides mitochondrial detail, but not 3D morphology; therefore, we used SBF-
220 SEM to look at the 3D structure of the mitochondria. Using myotubes with ablated genes for
221 *Opa-1* and MICOS complex subunits, as described above, we measured a total of 200
222 mitochondria across 10 cells. We compared mitochondria in WT, *Opa-1*, *Mitofilin, CHCHD3*,
223 and *CHCHD6* knockout myotubes (Figures 6A-E).
224 We found that compared to the elongated mitochondria in the WT, the 3D length was
225 much shorter in *Opa-1* and MICOS protein knockouts (Figure 6F). Similarly, the volume of
226 mitochondria was also less in *CHCHD3*, *CHCHD6*, *Mitofilin*, and *Opa-1* knockouts compared to
227 the control (Figure 6G). The 3D reconstruction data, in combination with the prior TEM results,
228 show how mitochondrial dynamics change with the loss of MICOS subunits.
229

Metabolomics Show Key Metabolite Changes in Knockout of MICOS Complex Proteins and *Opa-1* in Myotubes.

To determine the global effects of loss of *Opa-1* or the MICOS complex in skeletal muscle myotubes, we analyzed the metabolome to identify changes in metabolites that occurred with changes in mitochondria and cristae. Principal component analysis (PCA) revealed distinct populations in the control and the *Mitofilin* knockout strains, which suggested that their genotypes contributed to the clustering (SFigure 4A). To identify metabolites with the most robust ability to discriminate between true positives and false positives, we constructed a model using analysis of variance (ANOVA) to determine several key metabolites that were statistically significant (SFigure 4B). This unique metabolite signature revealed that *Mitofilin* plays a critical role in regulating amino acid metabolism and steroidogenesis (SFigure 4C–D). Upregulation of steroidogenesis pathways may result from the increased fluidity of membranes caused by *Mitofilin* [38,39]. We also measured the effect of ablation of genes for *CHCHD3* and *CHCHD6* in skeletal muscle myotubes on bioenergetic metabolism. PCA revealed distinct populations in the control and the *CHCHD3* and CHCHD6 knockouts, which showed a similar profile (SFigure 5A). We constructed a model using ANOVA to determine which metabolite changes in *CHCHD3* and *CHCHD6* knockouts were statistically significant (SFigure 5B) There was a loss of protein synthesis and changes in carbohydrate metabolism (SFigure 5C–D). Loss of *Opa-1* typically favors fatty acid synthesis, so these results showing increased carbohydrate metabolism differ from previous *Opa-1* knockout responses [40–42]. This atypical response was evident in the increase in lactose and starch synthesis, but there was poor protein turnover, as seen in methionine metabolism (SFigure 5D).

DISCUSSION

We demonstrated that either aging or loss of MICOS proteins in skeletal muscle resulted in suboptimal mitochondria morphology, suggesting a correlation between aging and MICOS protein expression. Previous studies used 3D focused ion beam-scanning electron microscopy (FIB-SEM) to characterize the networking of the mitochondria in human[43] and mouse skeletal muscle[44]. Another report did quantitative 3D reconstructions using SBF-SEM to define the morphological differences in the skeletal muscles of humans versus mice, and they compared patients with primary mitochondrial DNA diseases with healthy controls[21]. To the best of our knowledge, our current study is the first to use 3D reconstruction method as a novel approach to study the connections between mitochondria and to determine how disconnections and reduced mitochondrial communication altered in skeletal muscle aging.

Skeletal muscle highly mitochondrial dependent as well as mitochondria enriched and is highly enriched in , which comprise ~6% of the cell volume and is well known to change with aging[45]. Gastrocnemius muscle has both type I slow-twitch muscle fibers and type II fast-twitch muscle fibers; type I fibers are more effective for endurance, while type II fibers better support short bursts of muscle activity [45–47]. Our 3D morphologic data, however, did not permit discrimination of the two fiber types, although we observed many variable muscle fibers within a sample and between all differently aged cohorts. Futures studies may develop novel methods that are able to distinguish fiber types to determine the changes in mitochondria. Past studies in human skeletal muscle did this by taking two portion of samples and assessing fiber type via SDS-PAGE [48].

275 Using 3D reconstructions, we found that all parameters of the mitochondria changed
276 during aging including smaller volume, area, and perimeter (Figure 1D-F), and the mitochondria
277 became less interconnected. This increased fragmentation suggests a decreased mitochondrial
278 fusion, in association with age-dependent decreases of OPA-1, which is a regulator of
279 mitochondrial fusion. We also saw a decrease in the MBI during aging, suggesting that
280 mitochondria decrease in networking, but their shape may not radically change as they increase
281 in sphericity as they age (Figure 2).
282 The change in morphology in aging mitochondria could be explained by the MFRTA,
283 which proposes that ROS alter other functions of mitochondria, including ATP production, as
284 they induce aging [49]. Since oxidative stress has been linked to many diseases of aging (diabetes,
285 heart disease, sarcopenia, arthritis, kidney disease)[50], understanding the association between
286 aging and mitochondria is important.
287 We found nanotunnels, which are thin, double-membrane mitochondrial connections that
288 can transport proteins and nutrients, in all skeletal muscle samples. While healthy humans have
289 almost no nanotunnels [21], we unexpectedly found a moderate number of nanotunnels in young 3-
290 month-old mice (Figures 3E–H), and a consistent increase of nanotunnels in the skeletal muscle
291 of 1-year-old mice. These structures may have an unclear beneficial role in skeletal muscle of
292 mice. Given the association of nanotunnels with human diseases, we predicted that the 2-year-
293 old "aged" mouse skeletal muscle would have the most nanotunnels, however the nanotunnel
294 number was quite heterogeneous in 2-year-old mice and less were observed [12,51]. The 2-year-old
295 samples may have shown fewer nanotunnels due to the high rate of fragmentation (Figure 1) and
296 lack of connectedness (Figure 2) preventing nanotunnel formation. In contrast, perhaps the
297 remaining networks permitted the increased number of nanotunnels in 1-year moderately aged
298 samples. The presence of nanotunnels in the healthy 3-month-old mouse skeletal muscle
299 suggests significant differences in mouse versus human muscle [21].
300 MICOS proteins play key regulatory roles in mitochondrial structure and function [19,26,37].
301 We determined by TEM 3D reconstructions that the loss of *Mitofilin*, *CHCHD3*, and *CHCHD6*
302 (Figures 4-5) resulted in fragmentation, disrupted cristae, and smaller mitochondria (Figures 4A–
303 I, Figures 4P–Y; Figure 5), similar to the loss of OPA-1, which is known to cause changes in
304 oxidative phosphorylation (Figures 4Z–AE) [19,23,28]. Overall, mitochondria lacking the MICOS
305 genes had characteristics similar to those of aged mouse skeletal muscle (Figures 1−2). This
306 similarity in phenotype suggests there may be an association. Thus, changes in mitochondrial
307 morphology due to aging may be caused by a lack of MICOS gene expression. This is supported
308 by the RT-qPCR data that showed decreased *CHCHD3*, *CHCHD6*, *Mitofilin*, and *Opa-1*
309 transcription with aging (SFigure 3). Although there is a link between aging and loss of *Opa-1*
310 [24,29], little is known about the role of the MICOS complex in aging. Changes in mitochondrial
311 architecture and loss of integrity may be caused by decreased MICOS proteins; thus, it will be
312 important to develop methods to restore MICOS proteins and *Opa-1* lost during aging to
313 decrease the deleterious effects of mitochondrial dysfunction. Although studies have examined
314 the role of knockouts on mitochondrial dynamics, few studies have examined how loss of
315 MICOS proteins may be restored to mitochondria [23,52].
316 Many studies have analyzed the mitochondrial metabolome using mouse skeletal muscles
317 [45,53–57]. We found that loss of *Mitofilin* affected cristae morphology (Figures 4G–H), decreased
318 oxidative phosphorylation (Figure 4J), and may have increased lipid and steroid synthesis, which
319 may be important for mitochondrial endoplasmic reticulum contact (MERC) regulation and
320 cristae formation. We found an increase in tryptophan and methyl histidine metabolism (SFigure

321 4D) and an increase in taurine metabolism and hypotaurine, a key sulfur-containing amino acid
322 for the metabolism of fats. Loss of *Opa-1* also changes amino acid and lipid metabolism, similar
323 to the loss of *Mitofilin* [40–42]. Steroidogenesis, which makes the membrane less rigid, was
324 increased. Since the loss of *Mitofilin*, *CHCHD6*, or *CHCHD3* caused a loss of oxidative capacity
325 (Figure 4J-O; 5K-P), increased steroid synthesis may allow the cell to recover bioenergetic
326 functions, as steroid compounds decrease membrane viscosity, with the incorporation of estrogen
327 [38]. In the absence of *Mitofilin*, the cristae junctions and contact sites fall apart [58]; thus, *Mitofilin* is
328 critical for maintaining cristae [59,60]. Cells lacking *Mitofilin* may make steroids to help the
329 membrane to reconfigure broken cristae. Since the loss of *Opa-1* causes more MERCs [3], loss of
330 *Mitofilin* may increase phospholipids (SFigure 4D) because of increased smooth MERCs, which
331 are associated with lipid changes [61]. This is supported by the fact that biosynthesis of
332 phosphatidylethanolamine and phosphatidylcholine and metabolism of arachidonic acid and
333 sphingolipid increased with loss of *Mitofilin* (SFigure 4D). Since these phospholipids aggregate
334 around MERCs and may shuffle into the ER, *Mitofilin* may be a critical gene for regulating
335 cristae morphology and with a key role in regulating mitochondrial metabolism, which is novel.
336 *Mitofilin* may be an important target in the future to restore energy production. Loss of
337 *Mitofilin* may lead to ER stress, which, via ATF4, activates amino acid transporters [62] that further
338 activate mTORC1. ER stress activates mTORC as a result of a decrease in glucose [57]. Critically,
339 mTORC1 affects glucose homeostasis [63], and this may lead to inefficiency in energy use. This
340 can result in changes in autophagy. Therefore, as a downstream effect of *Mitofilin* loss increasing
341 mTORC1, this may explain why deletion of MICOS in *Drosophila* increases autophagy [37].
342 Similarly, previously loss of *Opa-1* increases ER stress [28], and loss of *Mitofilin* may cause a
343 similar increase in amino acid catabolism. If ER stress activates amino acid transporters,
344 branched-chain amino acids could increase ER stress, resulting in a positive feedback loop that
345 affects the health of the cell, cellular energy, metabolism, and antioxidants. ER stress may also
346 be responsible for the poor performance and fragmentation of mitochondria (Figure 4-5). Loss of
347 *Mitofilin* may result in the breakdown of protein pathways that regulate ER stress. Other amino
348 acid pathways, such as homocysteine (SFigure 4D), are involved in triglyceride uptake and
349 increased intracellular cholesterol, suggesting that proteins like ATF4 [57] and the MICOS
350 complex [15,26] are important for aging. In particular, the MICOS components may prevent
351 mitochondrial fragmentation by blocking ER stress pathways in aging. Further studies are
352 needed to better understand the role of MICOS in MERC formation and the relation between
353 smooth MERC and lipid synthesis.
354 Although *Mitofilin* is a key component of the MICOS complex, other components are
355 likely also important. The loss of *CHCHD3* or *CHCHD6* leads to a decrease in and disassembly
356 of all *Mitofilin* subcomplex components in mammals, with abnormal cristae morphology and
357 growth defects [33,64–69]. Downregulation of *CHCHD3* is linked to type 2 diabetes [70]. In our
358 metabolomics enrichment dataset (SFigure 5D), loss of *CHCHD3* or *CHCHD6* in mouse
359 myotubes resulted in a preference for alternative fuel sources, such as lactate, lactose, and
360 starches. Supplementation of healthy myotubes with galactose leads to a 30% increase in
361 oxidative capacity (i.e., OCR) due to an increase in AMPK phosphorylation and cytochrome c
362 oxidase (COX) activity, thereby forcing cells to become more oxidative to maintain ATP levels
363 [71]. In our samples, as oxidative metabolism decreased, anaerobic metabolism and lactate levels
364 increased, forcing cells to produce ATP by anaerobic glycolysis. However, long and high-level
365 exposure to D-galactose generates free radicals, which alter MERCs, cause mitochondrial

366 dysfunction, and induce aging [2,72]. This is the likely explanation for mitochondrial fragmentation
367 in aged samples and loss of the MICOS complex, which should be investigated further.
368 In conclusion, we present a quantitative evaluation of mitochondrial and cristae
369 morphology in mouse skeletal muscle using 3D reconstructions and TEM analysis. We found
370 oxidative differences upon loss of MICOS proteins. 3D reconstructions of nanotunnels in
371 mitochondria showed novel patterns with a moderate number of nanotunnels in young skeletal
372 mouse tissue and relatively low numbers in the most aged mouse skeletal muscles. Similar
373 changes in mitochondrial morphology were observed in aging muscles and for loss of MICOS
374 proteins in mouse skeletal muscle, and MICOS proteins decreased with age. This suggests a
375 relationship between the MICOS complex and aging, and further studies through 3D
376 reconstruction could elucidate the linkage between age-related muscle dysfunction, the MICOS
377 complex, and disease states in mitochondria.
378
379 **EXPERIMENTAL PROCEDURES**
380 *Animal Care and Maintenance*
381 All procedures for the care of mice were in accordance with humane and ethical protocols
382 approved by the University of Iowa Animal Care and Use Committee (IACUC) following the
383 National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals as
384 described previously [28]. All experiments used WT male C57Bl/6J mice housed at 22°C on a 12-
385 hour light, 12-hour dark cycle with free access to water and standard chow. Mice were
386 anesthetized with 5% isoflurane/95% oxygen.
387
388 *RNA Extraction and RT-qPCR*
389 Total RNA was extracted from tissue using TRIzol reagent (Invitrogen, cat #), purified with the
390 RNeasy kit (Qiagen Inc, cat #), and quantitated by the absorbance at 260 nm and 280 nm using a
391 NanoDrop 1000 (NanoDrop products, Wilmington, DE, USA) spectrophotometer. Total RNA
392 (~1 µg) was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit
393 (Applied Biosciences, Carlsbad CA, cat #) followed by real-time quantitative PCR (qPCR)
394 reactions using SYBR Green (Life Technologies, Carlsbad, CA, cat #) [73]. Triplicate samples for
395 qPCR (~50 ng) in a 384-well plate were placed into ABI Prism 7900HT instrument (Applied
396 Biosystems) programmed as follows: 1 cycle at 95°C for 10 min; 40 cycles of 95°C for 15 s;
397 59°C for 15 s, 72°C for 30 s, and 78°C for 10 s; 1 cycle of 95°C for 15 s; 1 cycle of 60°C for
398 15 s; and one cycle of 95°C for 15 s. Data were normalized to glyceraldehyde-3-phosphate
399 dehydrogenase (*Gapdh*), and results are shown as fold changes. qPCR primers were designed
400 using Primer-Blast or were previously published sequences [28] as shown in Table 1.
401 Table 1: qPCR Primers Used

Gene	Primers	
Opa-1	Forward	5'-ACCAGGAGACTGTGTCAA-3'
	Reverse	5'-TCTTCAAATAAACGCAGAGGTG-3'
CHCHD3	Forward	5'-GAAAAGAATCCAGGCCCTTCCACGCGC-3'
	Reverse	5'-CAGTGCCTAGCACTTGGCACAACCAGGAA-3'
CHCHD6	Forward	5'-CTCAGCATGGACCTGGTAGGCACTGGGC-3'
	Reverse	5'-GCCTCAATTCCCACATGGAGAAAGTGGC-3'
Mitofilin	Forward	5'-CCTCCGGCAGTGTTCACCTAGTAACCCCTT-3'
	Reverse	5'-TCGCCCGTCGACCTTCAGCACTGAAAACCTAT-3'

402

403 *Isolation of Satellite Cells*
404 Satellite cell differentiation was performed as previously described [16,28]. Cells were cultured at
405 37°C, 5% CO_2 Dulbecco's modified Eagle's medium (DMEM; GIBCO, cat #) supplemented
406 with 10% FBS (Atlanta Bio selected, cat #), and 1% penicillin-streptomycin (Gibco, Waltham,
407 MA, USA, cat #).
408
409 *CRISPR-Cas9 Knockouts*
410 After three days, myotubes were infected with adenovirus to produce the following knockouts—
411 control CRISPR/Cas9 (sc-418922), *CHCHD6* (Mic25) CRISPR (sc-425817), *CHCHD3* (Mic19)
412 CRISPR (sc-425804), and *mitofilin* (Mic60) CRISPR (sc-429376) (Santa Cruz Biotechnology,
413 California, US), with the use of guide RNA (Table 2). We incubated 2.5% relevant CRISPR,
414 2.5% RNAiMax (ThermoFisher Scientific; cat # 13778075), and 95% Opti-MEM (Gibco; cat
415 #31985070) in a tube for 20 minutes. Cells were washed twice with PBS after removal of the
416 medium and 800 µL of OPT-MEM and 200 µL of the CRISPR mixture were added to each well,
417 ran in triplicates. Cells were incubated for 4 hours at 37 C, 1.0 mL of DMEM medium was
418 added, cells were incubated overnight. The myotubes were then washed with PBS and the
419 medium was replaced. Experiments were performed between 3 and 7 days after infection for a
420 total of 6 days of differentiation.
421 Table 2: Guide RNA and Plasmids Used

Gene Name	Type of Plasmid	CAS Number
Mitofilin	CRISPR/Cas9 KO (m)	sc-429376
CHCHD6	CRISPR/Cas9 KO (m)	sc-425817
CHCHD3	CRISPR/Cas9 KO (m)	sc-425804
Control	CRISPR/Cas9 KO (m)	sc-418922

422
423 *Serial Block-Face Scanning Electron Microscope (SBF-SEM) Processing of Mouse Muscle*
424 *Fibers*
425 SBF-SEM preparation was performed as described previously [17,27,74]. Running on a FEI/Thermo
426 Scientific Volumescope 2 SEM, a state-of-the-art SBF imaging system, we obtained 300−400
427 ultrathin (0.09 µm) serial sections from the blocks that were processed for conventional TEM.
428 All sections were collected onto formvar-coated slot grids (Pella, Redding CA), stained, and
429 imaged as previously described [17,27,74].
430
431 *Quantification of TEM Micrographs and Parameters Using ImageJ*
432 Quantification of TEM images was performed as described previously using the
433 NIH *ImageJ* software [16,27]. Cells were divided into four quadrants and two quadrants were
434 selected randomly for complete analysis. From each individual, a minimum of 10 cells were
435 measured with three analyses to obtain accurate and reproducible values. If variability occurred,
436 the number of cells was expanded to 30 cells per individual to reduce the variability.
437
438 *Segmentation and Quantification of 3D SBF-SEM Images Using Amira*
439 Intermyofibrillar (IMF) mitochondria are located between myofibrils, arranged in pairs at the z-
440 band of each sarcomere, with 2D elongated tubular shapes [75]. However, it is not known how
441 aging affects mitochondrial orientation, the structure of C-band sarcomeres, or the morphological
442 changes in incomplete fission known as nanotunnels. For each region of interest across the three
443 age groups, we analyzed 300 slices at 50 µm intervals at the transverse intervals.

For 3D reconstruction, SBF-SEM images were manually segmented in Amira as described previously [17,27]. All serial sections (300−400 slices) were loaded onto Amira and structural features were traced manually on sequential slices of micrograph blocks. Structures in mice were collected from 30–50 serial sections that were then stacked, aligned, and visualized using Amira to make videos and quantify volumetric structures. An average of 500 total mitochondria across four ROIs from 3 mice were collected for quantification. For 3D reconstruction of myotubes, approximately 20 mitochondria from a minimum of 10 cells were collected. Quantification of SBF-SEM images was performed as described previously [17] using the Amira software (Thermo Scientific).

Data Analysis

All SBF-SEM and TEM data were presented as the mean of at least three independent experiments with similar outcomes. Results were presented as mean ± standard error with individual data points shown. Data with only two groups were analyzed using an unpaired, t-test. For nanotunnel quantification, a Mann-Whitney test (unpaired, nonparametric) t-test was performed between two groups. If more than two groups were compared, one-way ANOVA was performed, and significance was assessed using Fisher's protected least significant difference (LSD) test. GraphPad Prism software package was used for t-tests and ANOVA analyses (La Jolla, CA, USA). For all statistical analyses, $p < 0.05$ indicated a significant difference. Higher degrees of statistical significance (**, ***, ****) were defined as $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively.

Gas Chromatography-Mass Spectrometry (GC-MS)

Samples were extracted for metabolites and prepared as previously designed[76]. The profiling of the metabolites was performed using TraceFinder 4.1 with standard verified peaks and retention times. TraceFinder was used to compare metabolite peaks in each sample against an in-house library of standards. TraceFinder was also used for GC-MS peak integration to obtain peak areas for each metabolite. After this analysis, we used previously described protocols [77] to correct for drift over time by using QC samples run at both the beginning and end of the sequence. The data was then normalized to an internal standard to control for extraction, derivatization, and/or loading effects.

Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS was performed for myotubes as previously described[76]. TraceFinder 4.1 software was used for analysis and metabolites were identified based on an in-house library. Drift was corrected for as described above [77]. Data were normalized and further visualization and analysis were performed on MetaboAnalyst 5.0 [78].

Analyzing Metabolomic Data

Metabolomic analysis was performed as described previously [76] using the web service MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/MetaboAnalyst/ModuleView.xhtml, last accessed on 8 February 2022) that combines machine learning methods and statistics to group data using PCA, heat mapping, metabolite set enrichment analysis, and statistical analysis. One-way ANOVA and Fisher's LSD multiple comparison test were also used. PCA uses score plots to provide an overview of variance for the principal components. Heatmaps separate hierarchical clusters leading to progressively larger clusters. Clusters are based on similarity using Euclidean

distance and Ward's linkage to minimize the clustering needed. Metabolite Set Enrichment Analysis (MSEA), which determines whether a set of functionally related metabolites is altered, can be used to identify consistent changes across many metabolites with similar roles. Overrepresentation analysis determines whether a group of compounds is overrepresented in comparison to pure chance and whether a group of metabolites have similar changes. In this analysis, the fold enrichment was calculated by dividing the observed hits by the expected metabolites. Expected number of hits are calculated by MetaboAnalyst 5.0. GraphPad Prism software (La Jolla, CA, USA) was used for statistical analysis with data expressed as mean ± standard deviation, and one-tailed p-values ≤ 0.01 were considered significant.

Measurement of OCR Using Seahorse

Using an XF24 extracellular flux (XF) bioanalyzer (Agilent Technologies/Seahorse Bioscience, North Billerica, MA, USA), OCR was measured for *Opa-1*, *CHCHD3*, *CHCHD6*, or *Mitofilin* knocked down cells as previously described[28]. Three independent experiments were performed with four to six replicates for each time and for each condition and representative data from the replicates are shown.

ACKNOWLEDGEMENTS

We would like to thank Melanie McReynolds for her advice on processing metabolomic samples and Mariya Sweetwyne for her advice as an aging expert. This work was supported by UNCF/BMS EE United Negro College Fund/Bristol-Myers Squibb E.E. T-32, number DK007563 entitled Multidisciplinary Training in Molecular Endocrinology to Z.V.; Just Postgraduate Fellowship in the Life Sciences Fellowship to H.K.B.; NSF MCB #2011577I to S.A.M.; The UNCF/BMS E.E. Just Faculty Fund, Burroughs Wellcome Fund Career Awards at the Scientific Interface Award, Burroughs Wellcome Fund Ad-hoc Award, NIH Small Research Pilot Subaward to 5R25HL106365-12 from the NIH PRIDE Program, DK020593, Vanderbilt Diabetes and Research Training Center for DRTC Alzheimer's Disease Pilot & Feasibility Program to A.H.J. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of the NIH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

CONSENT FOR PUBLICATION

All authors have agreed to the final version of this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

Z.V., E.G., L.V., J.S., H.K.B., S.A.M., M.A.P., A.H.J., J.A.G., and D.D. conceived and designed research; A.G.M., A.C., L.V., Z.V., T.A.C, B.C.M., J.L., H.K.B., B.R, C.E., D.D., A.H.J., and

534 J.A.G. performed experiments; J.D., K.N, J.S., E.G., Z.V., J.L., B.R., T.A.C., A.K.R., A.M.Q.,
535 V.E., E.G., D.D., , J.A.G., and A.H.J. analyzed data; B.T, K.N, J.S., E.G., Z.V., S.A.M., A.M.Q.,
536 V.E., H.K.B., A.C., A.G.M., J.D., M.A.P., D.D., J.A.G., and A.H.J.. interpreted results of
537 experiments and prepared figures; K.N, E.G., Z.V., J.S., S.A.M., L.V., A.G.M., M.A.P., A.K.R.,
538 B.C.M., B.T., C.E., A.C., H.K.B., D.D., J.A.G., and A.H.J. drafted manuscript, edited, and
539 revised manuscript; A.H.J., D.D., and J.A.G. approved final version of manuscript.
540
541 **REFERENCES**
542

543 1. Bratic A, Larsson N-G. The role of mitochondria in aging. *The Journal of clinical*
544 *investigation*. 2013;123:951–957.

545 2. Barja G. The mitochondrial free radical theory of aging. *Progress in molecular biology and*
546 *translational science*. 2014;127:1–27.

547 3. Rowland AA, Voeltz GK. Endoplasmic reticulum–mitochondria contacts: function of the
548 junction. *Nature reviews Molecular cell biology*. 2012;13:607–615.

549 4. Dai D-F, Santana LF, Vermulst M, Tomazela DM, Emond MJ, MacCoss MJ, Gollahon K,
550 Martin GM, Loeb LA, Ladiges WC, Rabinovitch PS. Overexpression of catalase targeted to
551 mitochondria attenuates murine cardiac aging. *Circulation*. 2009;119:2789–2797.

552 5. Xu H, Ranjit R, Richardson A, Van Remmen H. Muscle mitochondrial catalase expression
553 prevents neuromuscular junction disruption, atrophy, and weakness in a mouse model of
554 accelerated sarcopenia. *J Cachexia Sarcopenia Muscle*. 2021;12:1582–1596.

555 6. Friederich M, Hansell P, Palm F. Diabetes, oxidative stress, nitric oxide and mitochondria
556 function. *Current diabetes reviews*. 2009;5:120–144.

557 7. Renken CW. The structure of mitochondria. 2004.

558 8. Cogliati S, Enriquez JA, Scorrano L. Mitochondrial cristae: where beauty meets
559 functionality. *Trends in biochemical sciences*. 2016;41:261–273.

560 9. Kühlbrandt W. Structure and function of mitochondrial membrane protein complexes. *BMC*
561 *biology*. 2015;13:1–11.

562 10. Otera H, Ishihara N, Mihara K. New insights into the function and regulation of
563 mitochondrial fission. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*.
564 2013;1833:1256–1268.

565 11. Favaro G, Romanello V, Varanita T, Desbats MA, Morbidoni V, Tezze C, Albiero M,
566 Canato M, Gherardi G, De Stefani D. DRP1-mediated mitochondrial shape controls calcium
567 homeostasis and muscle mass. *Nature communications*. 2019;10:1–17.

568 12. Vincent AE, Turnbull DM, Eisner V, Hajnóczky G, Picard M. Mitochondrial Nanotunnels.
569 *Trends Cell Biol*. 2017;27:787–799.

570 13. Zhang L, Trushin S, Christensen TA, Bachmeier BV, Gateno B, Schroeder A, Yao J, Itoh K,
571 Sesaki H, Poon WW. Altered brain energetics induces mitochondrial fission arrest in
572 Alzheimer's Disease. *Scientific reports*. 2016;6:1–12.

573 14. Zhang J, He Z, Fedorova J, Logan C, Bates L, Davitt K, Le V, Murphy J, Li M, Wang M,
574 Lakatta EG, Ren D, Li J. Alterations in mitochondrial dynamics with age-related
575 Sirtuin1/Sirtuin3 deficiency impair cardiomyocyte contractility. *Aging Cell*. 2021;20:e13419.

576 15. Kozjak-Pavlovic V. The MICOS complex of human mitochondria. *Cell and tissue research*.
577 2017;367:83–93.

578 16. Lam J, Katti P, Biete M, Mungai M, AshShareef S, Neikirk K, Lopez EG, Vue Z,
579 Christensen TA, Beasley HK. A Universal Approach to Analyzing Transmission Electron
580 Microscopy with ImageJ. *bioRxiv*. 2021.

581 17. Garza-Lopez E, Vue Z, Katti P, Neikirk K, Biete M, Lam J, Beasley HK, Marshall AG,
582 Rodman TA, Christensen TA, Salisbury JL, Vang L, Mungai M, AshShareef S, Murray SA,
583 Shao J, Streeter J, Glancy B, Pereira RO, Abel ED, Hinton A. Protocols for Generating
584 Surfaces and Measuring 3D Organelle Morphology Using Amira. *Cells*. 2022;11:65.

585 18. Rampelt H, Zerbes RM, van der Laan M, Pfanner N. Role of the mitochondrial contact site
586 and cristae organizing system in membrane architecture and dynamics. *Biochimica et*
587 *Biophysica Acta (BBA)-Molecular Cell Research*. 2017;1864:737–746.

588 19. Hu C, Shu L, Huang X, Yu J, Gong L, Yang M, Wu Z, Gao Z, Zhao Y, Chen L. OPA1 and
589 MICOS Regulate mitochondrial crista dynamics and formation. *Cell death & disease*.
590 2020;11:1–17.

591 20. Friedman JR, Mourier A, Yamada J, McCaffery JM, Nunnari J. MICOS coordinates with
592 respiratory complexes and lipids to establish mitochondrial inner membrane architecture.
593 *Elife*. 2015;4:e07739.

594 21. Vincent AE, White K, Davey T, Philips J, Ogden RT, Lawless C, Warren C, Hall MG, Ng
595 YS, Falkous G. Quantitative 3D mapping of the human skeletal muscle mitochondrial
596 network. *Cell reports*. 2019;26:996–1009.

597 22. Koopman WJH, Visch H-J, Verkaart S, van den Heuvel LWPJ, Smeitink JAM, Willems
598 PHGM. Mitochondrial network complexity and pathological decrease in complex I activity
599 are tightly correlated in isolated human complex I deficiency. *Am J Physiol Cell Physiol*.
600 2005;289:C881-890.

601 23. Zheng J, Croteau DL, Bohr VA, Akbari M. Diminished OPA1 expression and impaired
602 mitochondrial morphology and homeostasis in Aprataxin-deficient cells. *Nucleic acids*
603 *research*. 2019;47:4086–4110.

604 24. Varanita T, Soriano ME, Romanello V, Zaglia T, Quintana-Cabrera R, Semenzato M,
605 Menabò R, Costa V, Civiletto G, Pesce P. The OPA1-dependent mitochondrial cristae
606 remodeling pathway controls atrophic, apoptotic, and ischemic tissue damage. *Cell*
607 *metabolism*. 2015;21:834–844.

608 25. Khin PP, Hong Y, Yeon M, Lee DH, Lee JH, Jun H-S. Dulaglutide improves muscle
609 function by attenuating inflammation through OPA-1-TLR-9 signaling in aged mice. *Aging*
610 *(Albany NY)*. 2021;13:21962–21974.

611 26. Li H, Ruan Y, Zhang K, Jian F, Hu C, Miao L, Gong L, Sun L, Zhang X, Chen S.
612 Mic60/Mitofilin determines MICOS assembly essential for mitochondrial dynamics and
613 mtDNA nucleoid organization. *Cell Death & Differentiation*. 2016;23:380–392.

614 27. Hinton A, Katti P, Christensen TA, Mungai M, Shao J, Zhang L, Trushin S, Alghanem A,
615 Jaspersen A, Geroux RE. A comprehensive approach for artifact-free sample preparation and
616 assessment of mitochondrial morphology in tissue and cultured cells. *bioRxiv*. 2021.

617 28. Pereira RO, Tadinada SM, Zasadny FM, Oliveira KJ, Pires KMP, Olvera A, Jeffers J,
618 Souvenir R, Mcglauflin R, Seei A. OPA 1 deficiency promotes secretion of FGF 21 from
619 muscle that prevents obesity and insulin resistance. *The EMBO journal*. 2017;36:2126–2145.

620 29. Tezze C, Romanello V, Desbats MA, Fadini GP, Albiero M, Favaro G, Ciciliot S, Soriano
621 ME, Morbidoni V, Cerqua C, Loefler S, Kern H, Franceschi C, Salvioli S, Conte M, Blaauw
622 B, Zampieri S, Salviati L, Scorrano L, Sandri M. Age-Associated Loss of OPA1 in Muscle
623 Impacts Muscle Mass, Metabolic Homeostasis, Systemic Inflammation, and Epithelial
624 Senescence. *Cell Metab*. 2017;25:1374-1389.e6.

625 30. John GB, Shang Y, Li L, Renken C, Mannella CA, Selker JM, Rangell L, Bennett MJ, Zha J.
626 The mitochondrial inner membrane protein mitofilin controls cristae morphology. *Molecular*
627 *biology of the cell*. 2005;16:1543–1554.

628 31. Ding C, Wu Z, Huang L, Wang Y, Xue J, Chen S, Deng Z, Wang L, Song Z, Chen S.
629 Mitofilin and CHCHD6 physically interact with Sam50 to sustain cristae structure. *Scientific*
630 *reports*. 2015;5:1–11.

631 32. Darshi M, Taylor SS. Mitochondrial ChChD3 acts as a Scaffold for Mitofilin, Sam50 and
632 PKA. 2008.

633 33. An J, Shi J, He Q, Lui K, Liu Y, Huang Y, Sheikh MS. CHCM1/CHCHD6, novel
634 mitochondrial protein linked to regulation of mitofilin and mitochondrial cristae
635 morphology. *Journal of Biological Chemistry*. 2012;287:7411–7426.

636 34. Ott C, Dorsch E, Fraunholz M, Straub S, Kozjak-Pavlovic V. Detailed analysis of the human
637 mitochondrial contact site complex indicate a hierarchy of subunits. *PloS one*.
638 2015;10:e0120213.

639 35. Kondadi AK, Anand R, Reichert AS. Functional interplay between cristae biogenesis,
640 mitochondrial dynamics and mitochondrial DNA integrity. *International journal of*
641 *molecular sciences*. 2019;20:4311.

642 36. Stephan T, Brüser C, Deckers M, Steyer AM, Balzarotti F, Barbot M, Behr TS, Heim G,
643 Hübner W, Ilgen P. MICOS assembly controls mitochondrial inner membrane remodeling
644 and crista junction redistribution to mediate cristae formation. *The EMBO journal*.
645 2020;39:e104105.

646 37. Wang L, Hsu T, Lin H, Fu C. Drosophila MICOS knockdown impairs mitochondrial
647 structure and function and promotes mitophagy in muscle tissue. *Biology Open*.
648 2020;9:bio054262.

649 38. Torres MJ, Kew KA, Ryan TE, Pennington ER, Lin C-T, Buddo KA, Fix AM, Smith CA,
650 Gilliam LA, Karvinen S, Lowe DA, Spangenburg EE, Zeczycki TN, Shaikh SR, Neufer PD.
651 17β-Estradiol Directly Lowers Mitochondrial Membrane Microviscosity and Improves
652 Bioenergetic Function in Skeletal Muscle. *Cell Metabolism*. 2018;27:167-179.e7.

653 39. Kitajima Y, Ono Y. Estrogens maintain skeletal muscle and satellite cell functions. *Journal*
654 *of Endocrinology*. 2016;229:267–275.

655 40. Wasilewski M, Semenzato M, Rafelski SM, Robbins J, Bakardjiev AI, Scorrano L. Optic
656 Atrophy 1-Dependent Mitochondrial Remodeling Controls Steroidogenesis in Trophoblasts.
657 *Current Biology*. 2012;22:1228–1234.

658 41. Sarzi E, Seveno M, Angebault C, Milea D, Rönnbäck C, Quilès M, Adrian M, Grenier J,
659 Caignard A, Lacroux A, Lavergne C, Reynier P, Larsen M, Hamel CP, Delettre C, Lenaers
660 G, Müller A. Increased steroidogenesis promotes early-onset and severe vision loss in
661 females with OPA1 dominant optic atrophy. *Human Molecular Genetics*. 2016;25:2539–
662 2551.

663 42. Chao de la Barca JM, Fogazza M, Rugolo M, Chupin S, Del Dotto V, Ghelli AM, Carelli V,
664 Simard G, Procaccio V, Bonneau D, Lenaers G, Reynier P, Zanna C. Metabolomics
665 hallmarks OPA1 variants correlating with their in vitro phenotype and predicting clinical
666 severity. *Hum Mol Genet*. 2020;29:1319–1329.

667 43. Dahl R, Larsen S, Dohlmann TL, Qvortrup K, Helge JW, Dela F, Prats C. Three-dimensional
668 reconstruction of the human skeletal muscle mitochondrial network as a tool to assess
669 mitochondrial content and structural organization. *Acta Physiol (Oxf)*. 2015;213:145–155.

670 44. Glancy B, Hartnell LM, Malide D, Yu Z-X, Combs CA, Connelly PS, Subramaniam S,
671 Balaban RS. Mitochondrial reticulum for cellular energy distribution in muscle. *Nature*.
672 2015;523:617–620.

673 45. Garnier A, Fortin D, Delomenie C, Momken I, Veksler V, Ventura-Clapier R. Depressed
674 mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal
675 muscles. *The Journal of physiology*. 2003;551:491–501.

676 46. Mukund K, Subramaniam S. Skeletal muscle: A review of molecular structure and function,
677 in health and disease. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*.
678 2020;12:e1462.

679 47. Lin I-H, Chang J-L, Hua K, Huang W-C, Hsu M-T, Chen Y-F. Skeletal muscle in aged mice
680 reveals extensive transformation of muscle gene expression. *BMC genetics*. 2018;19:1–13.

681 48. Galpin AJ, Raue U, Jemiolo B, Trappe TA, Harber MP, Minchev K, Trappe S. Human
682 skeletal muscle fiber type specific protein content. *Anal Biochem*. 2012;425:175–182.

683 49. Sanz A, Stefanatos RK. The mitochondrial free radical theory of aging: a critical view.
684 *Current aging science*. 2008;1:10–21.

685 50. Rolo AP, Palmeira CM. Diabetes and mitochondrial function: role of hyperglycemia and
686 oxidative stress. *Toxicology and applied pharmacology*. 2006;212:167–178.

687 51. Lavorato M, Iyer VR, Dewight W, Cupo RR, Debattisti V, Gomez L, Fuente SD la, Zhao Y-
688 T, Valdivia HH, Hajnóczky G, Franzini-Armstrong C. Increased mitochondrial
689 nanotunneling activity, induced by calcium imbalance, affects intermitochondrial matrix
690 exchanges. *PNAS*. 2017;114:E849–E858.

691 52. Liu T, Woo JA, Bukhari MZ, LePochat P, Chacko A, Selenica MB, Yan Y, Kotsiviras P,
692 Buosi SC, Zhao X. CHCHD10-regulated OPA1-mitofilin complex mediates TDP-43-
693 induced mitochondrial phenotypes associated with frontotemporal dementia. *The FASEB*
694 *Journal*. 2020;34:8493–8509.

695 53. de La Barca JMC, Arrázola MS, Bocca C, Arnauné-Pelloquin L, Iuliano O, Tcherkez G,
696 Lenaers G, Simard G, Belenguer P, Reynier P. The metabolomic signature of Opa1
697 deficiency in rat primary cortical neurons shows aspartate/glutamate depletion and
698 phospholipids remodeling. *Scientific reports*. 2019;9:1–8.

699 54. Bocca C, Nzoughet JK, Leruez S, Amati-Bonneau P, Ferré M, Kane M-S, Veyrat-Durebex
700 C, de La Barca JMC, Chevrollier A, Homedan C. A plasma metabolomic signature involving
701 purine metabolism in human optic atrophy 1 (OPA1)-related disorders. *Investigative*
702 *ophthalmology & visual science*. 2018;59:185–195.

703 55. de La Barca JMC, Simard G, Sarzi E, Chaumette T, Rousseau G, Chupin S, Gadras C,
704 Tessier L, Ferré M, Chevrollier A. Targeted metabolomics reveals early dominant optic
705 atrophy signature in optic nerves of Opa1delTTAG/+ mice. *Investigative ophthalmology &*
706 *visual science*. 2017;58:812–820.

707 56. Garcia-Cazarin ML, Snider NN, Andrade FH. Mitochondrial isolation from skeletal muscle.
708 *JoVE (Journal of Visualized Experiments)*. 2011;:e2452.

709 57. Wortel IMN, van der Meer LT, Kilberg MS, van Leeuwen FN. Surviving Stress: Modulation
710 of ATF4-Mediated Stress Responses in Normal and Malignant Cells. *Trends Endocrinol*
711 *Metab*. 2017;28:794–806.

712 58. Cristae undergo continuous cycles of membrane remodelling in a MICOS-dependent
713 manner. *EMBO reports*. 2020;21:e49776.

714 59. Hessenberger M, Zerbes RM, Rampelt H, Kunz S, Xavier AH, Purfürst B, Lilie H, Pfanner
715 N, van der Laan M, Daumke O. Regulated membrane remodeling by Mic60 controls
716 formation of mitochondrial crista junctions. *Nat Commun*. 2017;8:15258.

717 60. Tarasenko D, Barbot M, Jans DC, Kroppen B, Sadowski B, Heim G, Möbius W, Jakobs S,
718 Meinecke M. The MICOS component Mic60 displays a conserved membrane-bending
719 activity that is necessary for normal cristae morphology. *J Cell Biol*. 2017;216:889–899.

720 61. Rieusset J. The role of endoplasmic reticulum-mitochondria contact sites in the control of
721 glucose homeostasis: an update. *Cell death & disease*. 2018;9:1–12.

722 62. Han J, Back SH, Hur J, Lin Y-H, Gildersleeve R, Shan J, Yuan CL, Krokowski D, Wang S,
723 Hatzoglou M. ER-stress-induced transcriptional regulation increases protein synthesis
724 leading to cell death. *Nature cell biology*. 2013;15:481–490.

725 63. Zhang X, Wang X, Yuan Z, Radford SJ, Liu C, Libutti SK, Zheng XFS. Amino acids-
726 Rab1A-mTORC1 signaling controls whole-body glucose homeostasis. *Cell Reports*.
727 2021;34:108830.

728 64. Darshi M, Mendiola VL, Mackey MR, Murphy AN, Koller A, Perkins GA, Ellisman MH,
729 Taylor SS. ChChd3, an inner mitochondrial membrane protein, is essential for maintaining
730 crista integrity and mitochondrial function. *Journal of biological chemistry*. 2011;286:2918–
731 2932.

732 65. Piñero-Martos E, Ortega-Vila B, Pol-Fuster J, Cisneros-Barroso E, Ruiz-Guerra L, Medina-
733 Dols A, Heine-Suñer D, Lladó J, Olmos G, Vives-Bauzà C. Disrupted in schizophrenia 1
734 (DISC1) is a constituent of the mammalian mitochondrial contact site and cristae organizing
735 system (MICOS) complex, and is essential for oxidative phosphorylation. *Human Molecular*
736 *Genetics*. 2016;25:4157–4169.

737 66. Genin EC, Plutino M, Bannwarth S, Villa E, Cisneros-Barroso E, Roy M, Ortega-Vila B,
738 Fragaki K, Lespinasse F, Pinero-Martos E. CHCHD 10 mutations promote loss of
739 mitochondrial cristae junctions with impaired mitochondrial genome maintenance and
740 inhibition of apoptosis. *EMBO molecular medicine*. 2016;8:58–72.

741 67. Park Y-U, Jeong J, Lee H, Mun JY, Kim J-H, Lee JS, Nguyen MD, Han SS, Suh P-G, Park
742 SK. Disrupted-in-schizophrenia 1 (DISC1) plays essential roles in mitochondria in
743 collaboration with Mitofilin. *Proceedings of the National Academy of Sciences*.
744 2010;107:17785–17790.

745 68. Genin EC, Bannwarth S, Lespinasse F, Ortega-Vila B, Fragaki K, Itoh K, Villa E, Lacas-
746 Gervais S, Jokela M, Auranen M, Ylikallio E, Mauri-Crouzet A, Tyynismaa H, Vihola A,
747 Augé G, Cochaud C, Sesaki H, Ricci J-E, Udd B, Vives-Bauza C, Paquis-Flucklinger V.
748 Loss of MICOS complex integrity and mitochondrial damage, but not TDP-43 mitochondrial
749 localisation, are likely associated with severity of CHCHD10-related diseases. *Neurobiology*
750 *of Disease*. 2018;119:159–171.

751 69. Bannwarth S, Ait-El-Mkadem S, Chaussenot A, Genin EC, Lacas-Gervais S, Fragaki K,
752 Berg-Alonso L, Kageyama Y, Serre V, Moore DG, Verschueren A, Rouzier C, Le Ber I,
753 Augé G, Cochaud C, Lespinasse F, N'Guyen K, de Septenville A, Brice A, Yu-Wai-Man P,
754 Sesaki H, Pouget J, Paquis-Flucklinger V. A mitochondrial origin for frontotemporal
755 dementia and amyotrophic lateral sclerosis through CHCHD10 involvement. *Brain*.
756 2014;137:2329–2345.

757 70. Eramo MJ, Lisnyak V, Formosa LE, Ryan MT. The 'mitochondrial contact site and cristae
758 organising system' (MICOS) in health and human disease. *The Journal of Biochemistry*.
759 2020;167:243–255.

760 71. Martin CR, Preedy VR, Rajendram R. Assessments, Treatments and Modeling in Aging and
761 Neurological Disease: The Neuroscience of Aging. Academic Press; 2021: 1-608.

762 72. Kandlur A, Satyamoorthy K, Gangadharan G. Oxidative Stress in Cognitive and Epigenetic
763 Aging: A Retrospective Glance. *Frontiers in Molecular Neuroscience*. 2020;13. Available at
764 https://www.frontiersin.org/article/10.3389/fnmol.2020.00041. Accessed March 17, 2022.

765 73. Boudina S, Sena S, Theobald H, Sheng X, Wright JJ, Hu XX, Aziz S, Johnson JI, Bugger H,
766 Zaha VG, Abel ED. Mitochondrial energetics in the heart in obesity-related diabetes: direct
767 evidence for increased uncoupled respiration and activation of uncoupling proteins.
768 *Diabetes*. 2007;56:2457–2466.

769 74. Neikirk K, Vue Z, Katti P, Shao J, Christensen TA, Lopez EG, Marshall A, Palavicino-
770 Maggio C, Ponce J, Alghanem A. Systematic Transmission Electron Microscopy-Based
771 Identification of Cellular Degradation Machinery. *bioRxiv*. 2021.

772 75. Vendelin M, Béraud N, Guerrero K, Andrienko T, Kuznetsov AV, Olivares J, Kay L, Saks
773 VA. Mitochondrial regular arrangement in muscle cells: a "crystal-like" pattern. *American*
774 *Journal of Physiology-Cell Physiology*. 2005;288:C757–C767.

775 76. Phillips MA, Arnold KR, Vue Z, Beasley HK, Garza-Lopez E, Marshall AG, Morton DJ,
776 McReynolds MR, Barter TT, Hinton A. Combining Metabolomics and Experimental
777 Evolution Reveals Key Mechanisms Underlying Longevity Differences in Laboratory
778 Evolved Drosophila melanogaster Populations. *International Journal of Molecular Sciences*.
779 2022;23:1067.

780 77. Li B, Tang J, Yang Q, Li S, Cui X, Li Y, Chen Y, Xue W, Li X, Zhu F. NOREVA:
781 normalization and evaluation of MS-based metabolomics data. *Nucleic acids research*.
782 2017;45:W162–W170.

78. Chong J, Soufan O, Li C, Caraus I, Li S, Bourque G, Wishart DS, Xia J. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. *Nucleic acids research*. 2018;46:W486–W494.

(13) **SUPPORTING INFORMATION**

(14) TABLES

(15) FIGURE LEGEND

Figure 1: Decreased mitochondrial size and volume in skeletal muscle of aged mice in SBF-SEM 3D reconstructions.

(**A-C**) Representative SBF-SEM orthoslice for skeletal muscle. (**A'-C'**) 3D reconstructions of mitochondria (various colors) in skeletal muscle mouse tissues of different ages overlaid on ortho slices. (**A''-C''**) 3D reconstructed and isolated mitochondria for clear visualization. (**A'-C''**) Pseudo-colored individual mitochondria in skeletal muscle to differentiate micro-level changes. (**A, A', A''**) The 2D ortho slice, the overlay of the 2D ortho slice, and the 3D reconstruction with individually colored mitochondria in tissue from 3-month-old mice. (**B, B', B''**) The 2D ortho slice, the overlay of the 2D ortho slice, and the 3D reconstruction with individually colored mitochondria in tissue from 1-year-old mice. (**C, C', C''**) The 2D ortho slice, the overlay of the 2D ortho slice, and the 3D reconstruction with individually colored mitochondria in tissue from 2-year-old mice. (**D-F**) Quantification of 3D reconstructions, with each dot representing the overall average of all mitochondria quantified for each mouse. (**D**) Mitochondrial volume in skeletal muscle of different ages. (**E-F**) Mitochondrial area and perimeter in skeletal tissues of different ages. Significance values * $p \leq 0.05$, **** $p \leq 0.0001$.

Figure 2: SBF-SEM 3D reconstruction in skeletal muscle of aged mice.

(**A-C**) 3D reconstruction of individually colored mitochondria from a transverse view for mouse skeletal tissues of different ages. (**A'-C'**) 3D reconstruction of individually colored mitochondria from a longitudinal view in skeletal muscle tissues of different ages. (**D**) MBI for mitochondria in tissues of different ages, with each dot representing the overall average mitochondria quantified for each of the three mouses. (**F**) Sphericity of mitochondria in skeletal muscle of different ages, with dots representing the average of all mitochondria quantified for each of the three mouses. (**E**) Representative examples of 3D reconstruction of mitochondria in skeletal muscle of different ages organized by volume. Significance values **** $p \leq 0.0001$.

Figure 3. Mitochondrial nanotunnels in skeletal mouse tissue.

(**A-C**) TEM tracing showing identified nanotunnels across aged cohorts in skeletal mouse tissue. (**D**) Representative examples of 3D reconstruction of nanotunnels in tissues of different ages organized by volume. (**E-H**) Quantification comparing frequency, average nanotunnel amount, nanotunnel length, and diameter of nanotunnels, respectively, across aged cohorts. (**I**) Histogram showing frequency of various nanotunnel lengths for each age group. (**J**) Histogram showing frequency of various nanotunnel diameters for each age group. ** $p \leq 0.01$, **** $p \leq 0.0001$.

Figure 4. Knockout of *Mitofilin* or *Opa-1* in myotubes results in changes in mitochondria, cristae, and oxygen consumption rates.

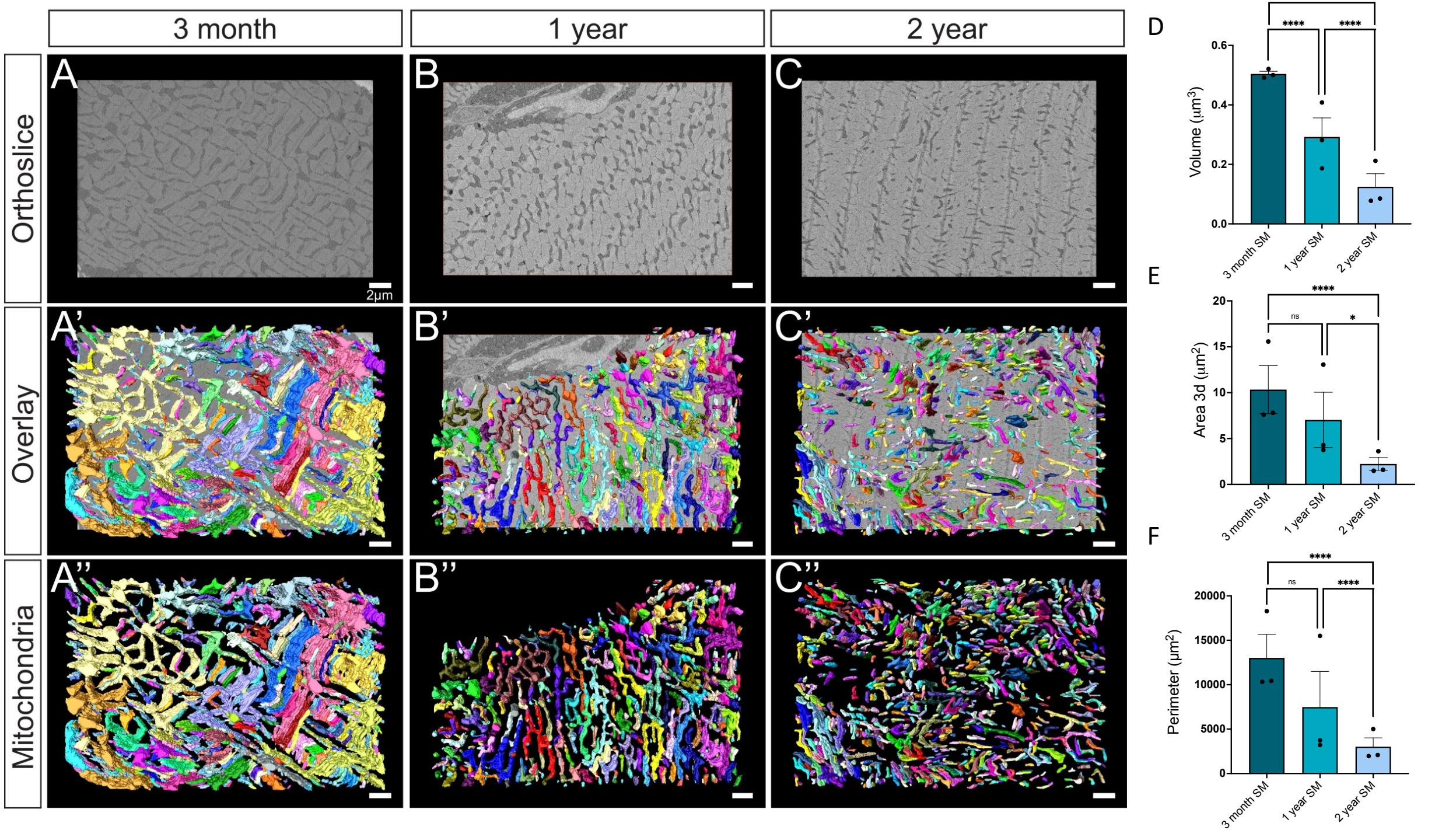
(**A-C**) Representative images of mitochondria and cristae from myotubes of OPA-1 and *Mitofilin* knockout mice compared to WT. (**D**) Mitochondrial area in myotubes of *Opa-1*, and *Mitofilin* knockout mice compared to WT. (**E**) Circularity index, measuring the roundness and symmetry of mitochondria, in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**F**) The

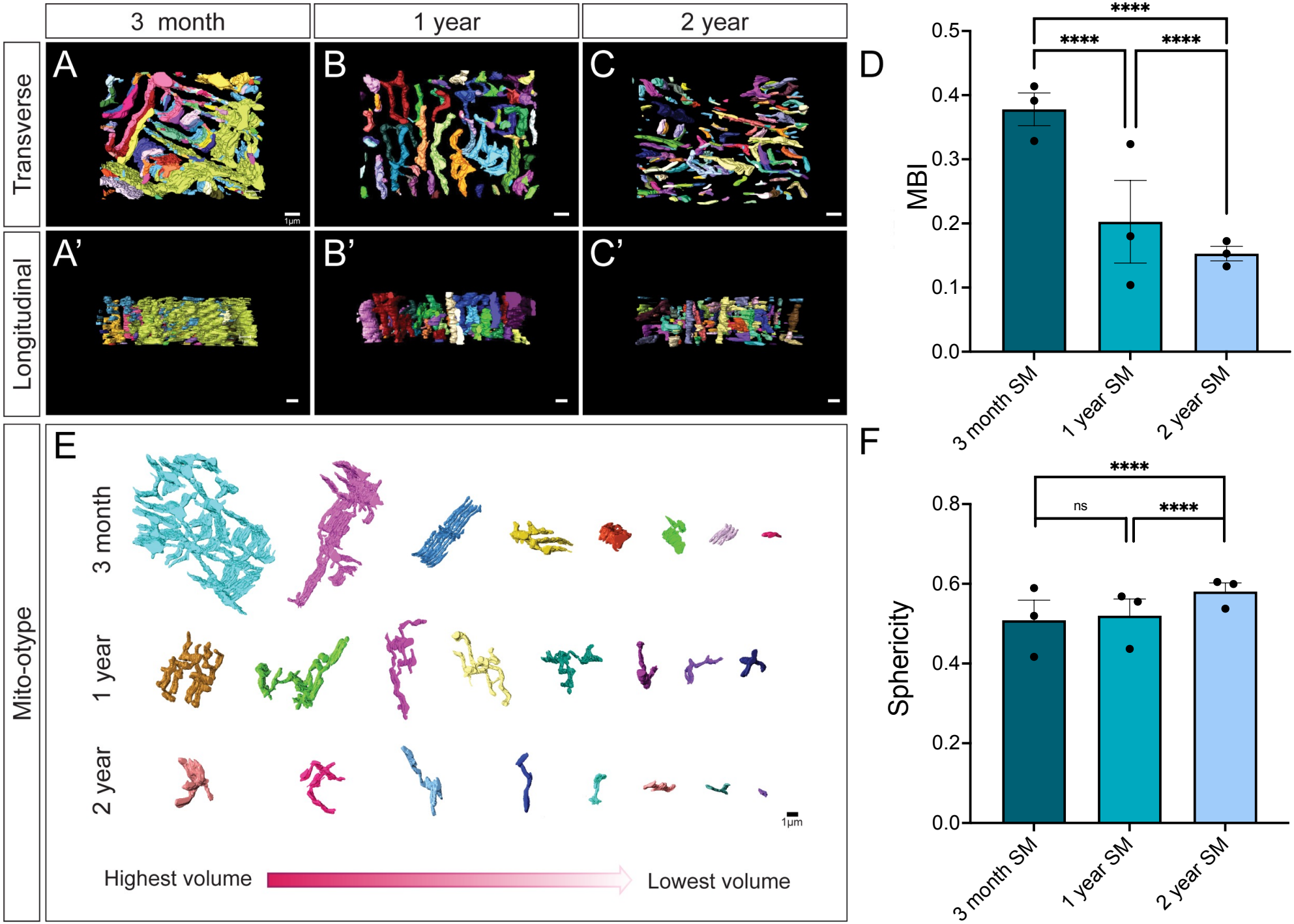
number of mitochondria in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**G**) Quantification of individual cristae in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**H**) Cristae scores measuring the uniformity and idealness of cristae in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**I**) Surface area of the average cristae in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**J**) OCR in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**K**) Basal OCR, the net respiration rate once non-mitochondrial respiration has been removed, in myotubes of *Opa-1*, and *Mitofilin* knockout mice compared to WT. (**L**) ATP-linked respiration, shown from intervals 4−7 in the OCR graphs, was determined by the addition of oligomycin (an inhibitor of respiration), thus representing the respiration dependent on ATP, in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**M**) Maximum OCR, represented by the peak from intervals 7−11 once non-mitochondrial respiration was deducted, in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**N**) The reserve capacity, which is represented by the difference between basal OCR and maximum OCR, in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. (**O**) Proton leak, representing non-phosphorylating electron transfer, in myotubes of *Opa-1* and *Mitofilin* knockout mice compared to WT. Significance values ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

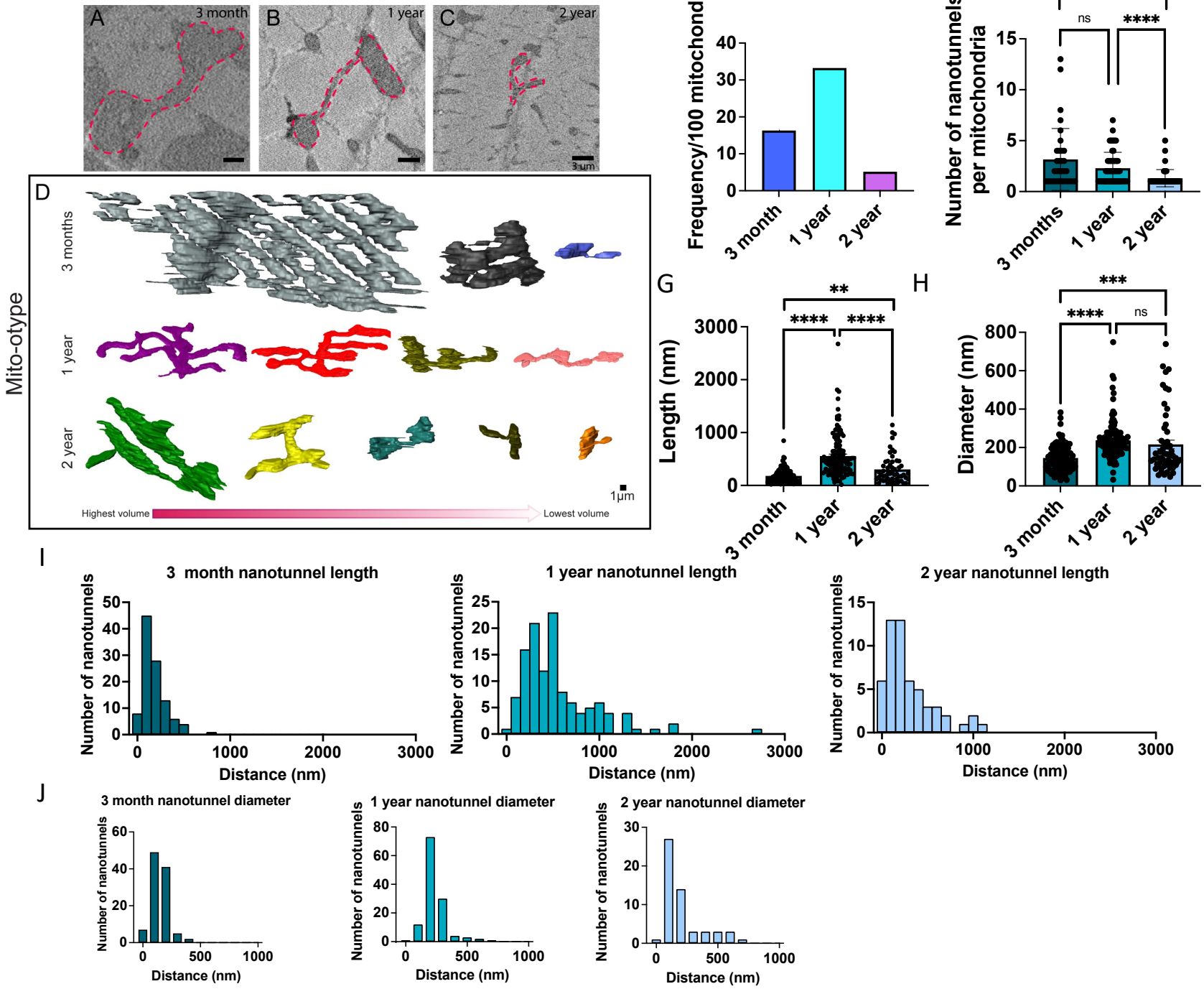
Figure 5. Knockout of MICOS complex proteins in myotubes results in changes in mitochondria, cristae, and oxygen consumption rates.
(**A-D**) Representative images of mitochondria and cristae from myotubes of *Opa-1*, *CHCHD3*, and *CHCHD6* knockout mice compared to WT. (**E**) Mitochondrial area in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**F**) Circularity index in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**G**) Mitochondria in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**H**) Quantification of individual cristae in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**I**) Cristae score in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**J**) Surface area of the average cristae in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**K**) OCR in myotubes of *CHCHD3, CHCHD6*, and *Opa-1* knockout mice compared to WT. **(L)** Basal OCR in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**M**) ATP-linked respiration in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**N**) Maximum OCR in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**O**) The reserve capacity in myotubes of *CHCHD3*, *CHCHD6*, and *Opa-1* knockout mice compared to WT. (**P**) Proton leak in myotubes of *Opa-1*, *CHCHD3*, and *CHCHD6*, knockout mice compared to WT. Significance values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

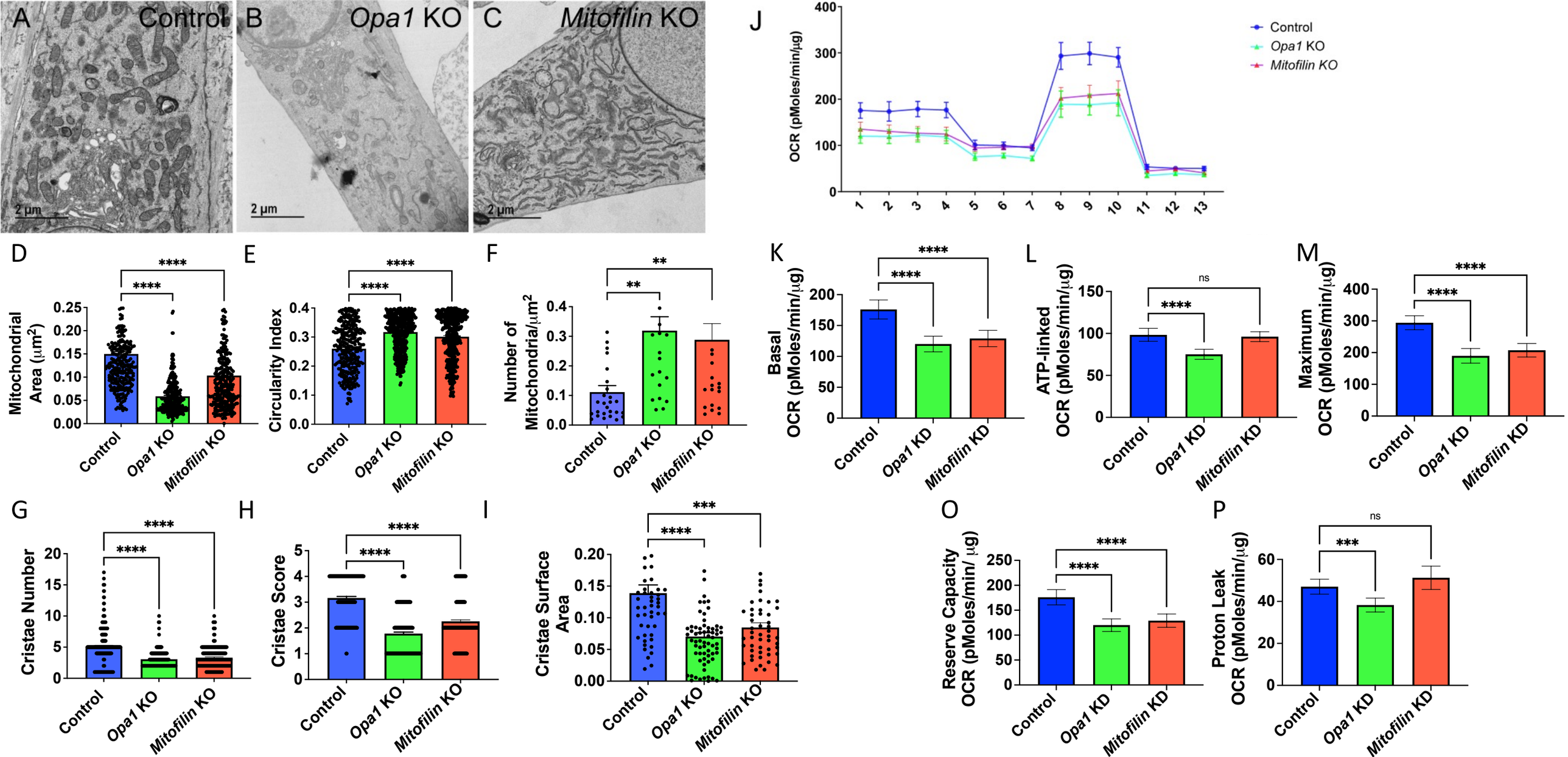
Figure 6. Loss of OPA-1 or MICOS complex proteins in myotubes decreases mitochondrial size and length.
(**A-E**) Representative images showing 3D reconstructions of mitochondria in myotubes of *Opa-1*, *Mitofilin CHCHD3*, and *CHCHD6* knockout mice compared to WT. (**F**) Mitochondrial 3D length in myotubes of *Opa-1***,** *Mitofilin*, *CHCHD3*, and *CHCHD6* knockout mice compared to WT. (**G**) Mitochondrial volume on log scale in myotubes of *Opa-1***,** *Mitofilin, CHCHD3*, and *CHCHD6* knockout mice compared to WT. Significance value **** $p \leq 0.0001$.

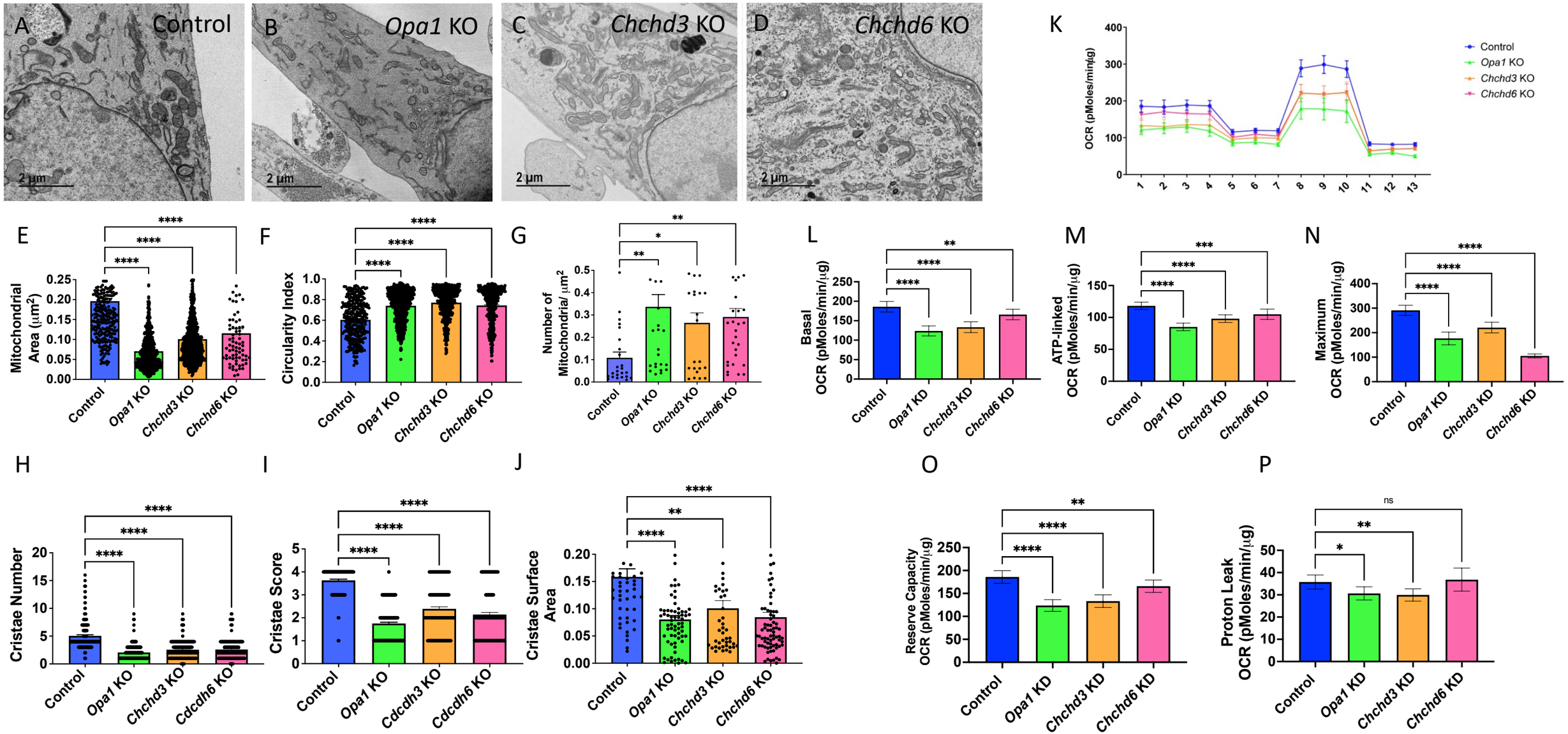
875 **Supplementary:**
876
877 **Figure 1:** The (**A**) volume (**B**) 3D area, and (**C**) perimeter of the average mitochondria in each of
878 the three individual mice for the age cohorts, all shown on log scale. The (**D**) volume (**E**) 3D
879 area, and (**F**) perimeter quantified on log scale with each dot representing one of the
880 approximately 550 mitochondria analyzed for each age cohorts.
881
882 **Figure 2:** The (**A**) mitochondria branching index (MBI) and (**B**) sphericity of the average
883 mitochondria in each of the three individual mice for the age cohorts. Combined data of mice for
884 (**C**) MBI and (**D**) sphericity with each dot representing one of the approximately 550
885 mitochondria analyzed for each age cohorts.
886
887 **Figure 3.** Transcription of *Opa-1* and MICOS genes in aging mouse muscle.
888 (**A-D**) Fold changes in the amount of OPA-1 and MICOS gene transcripts in mitochondria of
889 myocytes of 3-month-old, 1-year-old, and 2-year-old mice as measured by RT-qPCR. (**A**) *Opa-1*
890 transcripts. (**B**) *Mitofilin* transcripts. (**C**) *CHCHD3* transcripts. (**D**) *CHCHD6* transcripts.
891 Significance values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
892
893 **Figure 4.** (**A**) Metabolite PCA and (**B**) T-test comparing myotubes for control to *Mitofilin*
894 knockout mice. (**C**) Heatmap showing the relative abundance of ions and (**D**) Enrichment
895 analysis of metabolites, which links together several similarly functioning metabolites, with the
896 relative abundance for *Mitofilin* knockout.
897
898 **Figure 5.** (**A**) Metabolite PCA and (**B**) ANOVA test comparing control to myotubes of
899 *CHCHD3* and *CHCHD6* knockout mice (**C**) Heatmap showing the relative abundance of ions for
900 control and (**D**) enrichment analysis metabolite for *CHCHD3* and *CHCHD6* knockout mice.
901

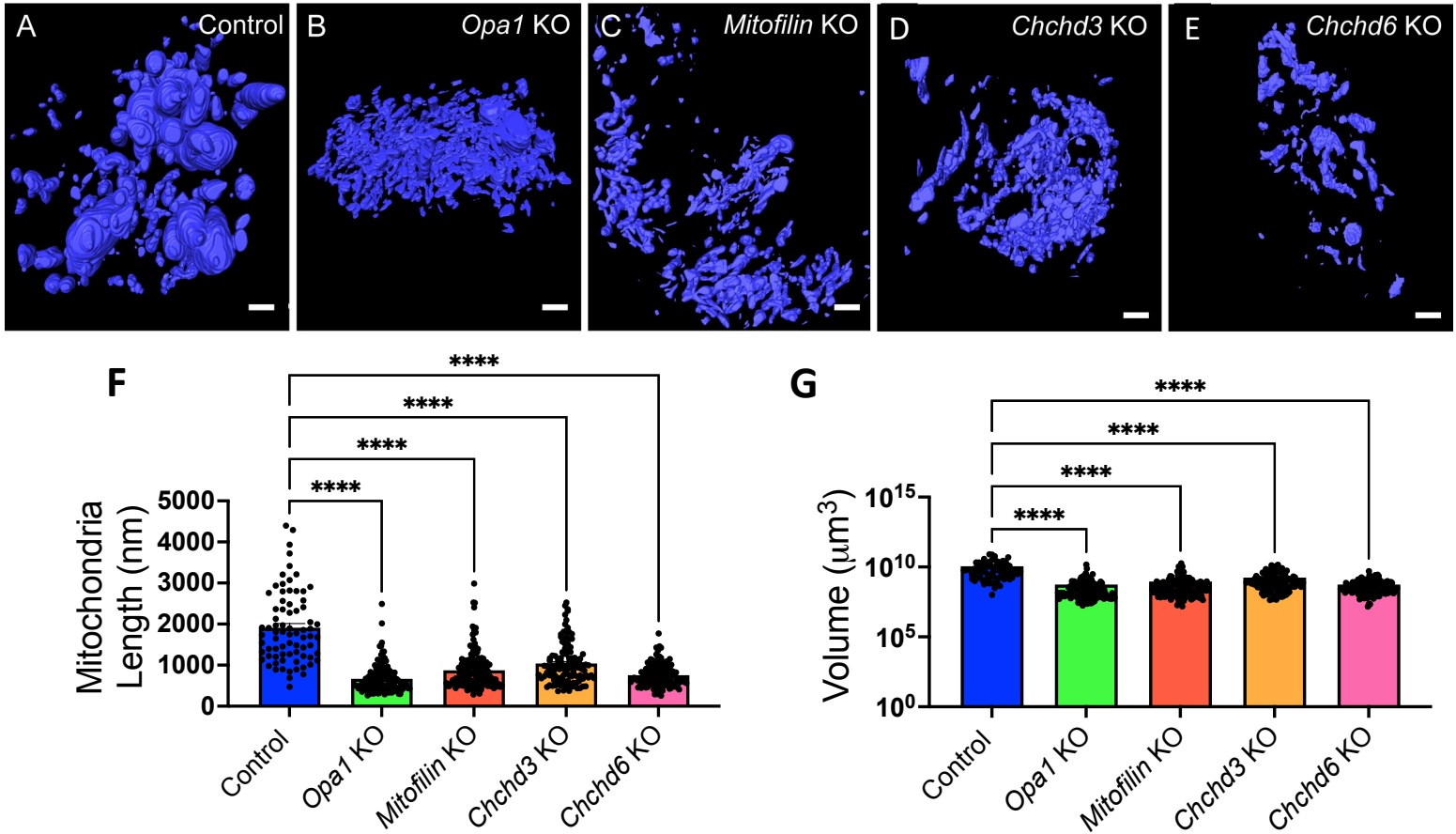

3 month
1 year
2 year
Orthoslice
Overlay
Mitochondria
A
B
C
A'
B'
C'
A''
B''
C''
2µm
D
E
F
Volume (µm³)
Area 3d (µm²)
Perimeter (µm²)
3 month SM
1 year SM
2 year SM
****
ns
*


3 month
1 year
2 year
Transverse
Longitudinal
Mito-otype
A
B
C
A'
B'
C'
1µm
D
MBI
0.0
0.1
0.2
0.3
0.4
0.5
****
****
****
3 month SM
1 year SM
2 year SM
E
3 month
1 year
2 year
1µm
Highest volume
Lowest volume
F
Sphericity
0.0
0.2
0.4
0.6
0.8
****
ns
****
3 month SM
1 year SM
2 year SM


A
3 month
B
1 year
C
2 year
3 µm
D
Mito-otype
3 months
1 year
2 year
1µm
Highest volume
Lowest volume
E
Frequency/100 mitochondria
3 month
1 year
2 year
F
Number of nanotunnels per mitochondria
ns
****
3 months
G
Length (nm)
**
H
Diameter (nm)
***
I
3 month nanotunnel length
1 year nanotunnel length
2 year nanotunnel length
Number of nanotunnels
Distance (nm)
J
3 month nanotunnel diameter
1 year nanotunnel diameter
2 year nanotunnel diameter


A
Control
2 µm
B
Opa1 KO
2 µm
C
Mitofilin KO
2 µm
J
OCR (pMoles/min/µg)
Control
Opa1 KO
Mitofilin KO
D
Mitochondrial Area (µm²)
E
Circularity Index
F
Number of Mitochondria/µm²
K
Basal OCR (pMoles/min/µg)
L
ATP-linked OCR (pMoles/min/µg)
ns
M
Maximum OCR (pMoles/min/µg)
G
Cristae Number
H
Cristae Score
I
Cristae Surface Area
O
Reserve Capacity OCR (pMoles/min/ µg)
P
Proton Leak OCR (pMoles/min/µg)
Control
Opa1 KO
Mitofilin KO
Opa1 KD
Mitofilin KD


A
Control
B
Opa1 KO
C
Chchd3 KO
D
Chchd6 KO
2 µm
K
OCR (pMoles/min/µg)
Control
Opa1 KO
Chchd3 KO
Chchd6 KO
E
Mitochondrial Area (µm²)
F
Circularity Index
G
Number of Mitochondria/ µm²
L
Basal OCR (pMoles/min/µg)
M
ATP-linked OCR (pMoles/min/µg)
N
Maximum OCR (pMoles/min/µg)
Control
Opa1 KO
Chchd3 KO
Chchd6 KO
Opa1 KD
Chchd3 KD
Chchd6 KD
H
Cristae Number
I
Cristae Score
J
Cristae Surface Area
O
Reserve Capacity OCR (pMoles/min/µg)
P
Proton Leak OCR (pMoles/min/µg)
Cdcdh3 KO
Cdcdh6 KO
ns


A
Control
B
Opa1 KO
C
Mitofilin KO
D
Chchd3 KO
E
Chchd6 KO
F
Mitochondria Length (nm)
5000
4000
3000
2000
1000
0
****
****
****
****
Control
Opa1 KO
Mitofilin KO
Chchd3 KO
Chchd6 KO
G
Volume (μm3)
10^15
10^10
10^5
10^0
****
****
****
****
Control
Opa1 KO
Mitofilin KO
Chchd3 KO
Chchd6 KO