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

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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.

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.

169
170 **Mitochondrial Nanotunnels Change in Size and Amount with Aging in Skeletal Muscle**
171 To quantify nanotunnels in skeletal muscle, we performed 3D reconstruction in mice at
172 three ages, measuring 550 mitochondria per mouse (n=3) using four different regions of interests
173 (ROIs). Although nanotunnels are reported to exist primarily in mitochondrial disease states [12],
174 we found them in all three ages of mice (Figures 3A–C). The diversity and heterogeneity in the
175 organization, sizing, and branching of nanotunnels necessitated mito-otyping (Figure 3D). We
176 found nanotunnels in a greater proportion of mitochondria in 1-year-old mice than in 2-year-old
177 mice (Figure 3E). However, the number of nanotunnels in each mitochondrion was significantly
178 less in the 2-year-old mice than the 3-month-old and 1-year-old mice (Figure 3F). The length and
179 diameter of nanotunnels increased in the older mice compared to the 3-month-old mice (Figures
180 3G–H), but the increase was significantly greater in the 1-year-old mice than the 2-year-old mice
181 (Figures 3G–J). These data show that nanotunnel structure, frequency, and length change during
182 aging.
183 Since we observed mitochondrial dynamics and cristae change with aging , we measured
184 transcripts for *Opa-1*, the gene associated with mitochondrial fusion, and *CHCHD3* (*Mic19*),
185 *CHCHD6* (*Mic25*), and *Mitofilin* (*Mic60*), the genes for the principal MICOS complex subunits.
186 We used reverse-transcriptase qPCR (RT-qPCR) to determine the effect of aging on
187 transcription. Since aging causes fragmentation of mitochondria, a hallmark for this process is
188 loss of *Opa-1* and MICOS complex proteins. We hypothesized that aging would also lead to
189 decreases in the associated MICOS complex proteins, confirming prior studies which have
190 found Opa-1 loss across aging [19,23–25]. We found progressive loss of both *Opa-1* and MICOS
191 complex subunits, as measured by loss of transcripts for the proteins (SFigure 3A–D). In 1- and
192 2-year-old mice, the amount of mRNA made for the MICOS complex subunit genes or the *Opa-*
193 *1* gene was less than half of that made in 3-month-old mice (SFigure 3A–C). Furthermore, for
194 all MICOS genes, there was a statistically significant decrease in the amount of mRNA over
195 time with a higher level of significance in 2-year-old mice. These data suggest a correlation
196 between mitochondrial morphologic changes and decreases in expression of genes for *Opa-1*
197 and MICOS subunits *CHCHD3*, *CHCHD6*, and *Mitofilin* during aging; however, they are not
198 sufficient to demonstrate causation, therefore we did additional measurements to show that
199 reduced protein expression levels may be responsible for the morphologic changes.
200
201 **Changes in Cristae and Mitochondria in Myotubes and Oxygen Respiration Rate Upon**
202 **Knockout of MICOS complex and *Opa-1***
203 The MICOS complex and OPA-1 are key players in mitochondrial biogenesis [15,19,26], but
204 how their interactions regulate aging and mitochondrial ultrastructures is poorly understood. To
205 determine the role of OPA-1 and the MICOS complex in mitochondrial structure and
206 networking, we ablated the genes for *Opa-1* and the MICOS complex in isolated primary skeletal
207 muscle cells from 3-month-old mice. We isolated primary satellite cells, then differentiated
208 myoblasts to myotubes. Using CRISPR/Cas9 method (Table 2), and a control plasmid, we
209 knocked out the genes for MICOS complex components and *Opa-1* from skeletal muscle cells.
210 As observed previously, in vitro deletion of OPA-1 altered mitochondrial morphology [16,27,28].
211 We measured 1250 mitochondria across 10 cells, with loss of *Opa-1* as a positive control for
212 mitochondrial morphological changes. Although *Opa-1* expression decreases with age [29], the
213 effect of the MICOS complex being lost in mitochondria morphology is unknown. Using
214 transmission electron microscopy (TEM) images, we compared mitochondria and cristae in

myotubes from wild type (WT) and knockouts of *Opa-1* and *Mitofilin* genes, which are essential for the organization of mitochondrial cristae [30,31] (Figures 4A–C). Mitochondrial average area decreased for both *Opa-1* and *Mitofilin* knockout myotubes (Figure 4D), while mitochondrial circularity (the roundness and symmetry of mitochondria) and number increased for both *Opa-1* and *Mitofilin* knockout myotubes (Figures 4E–F). For both *Opa-1* and *Mitofilin* knockouts, the number of cristae per mitochondrion decreased, as did the cristae score and cristae surface areas compared to the WT (Figures 4G–I). Cristae score measures the uniformity and regularity of cristae compared to typical cristae, with a lower number representing abnormality and dysfunction.

To further elucidate these changes in event of loss of other MICOS proteins, we also compared WT with *Opa-1*, *CHCHD3*, and *CHCHD6* knockout myotubes (Figures 5A–D). Knockout of MICOS subunit *CHCHD3* results in fragmented mitochondria with less *Opa-1* as the cristae lose their normal structure [32]. Similarly, *CHCHD6* is important in maintaining crista structure and its downregulation results in hollow cristae lacking an electron-dense matrix, thereby inhibiting ATP production and cell growth [31,33,34]. Mitochondrial average area decreased for *Opa-1*, *CHCHD3*, and *CHCHD6* knockout myotubes (Figure 5E), while the circularity index and the number of mitochondria, once normalized, increased (Figures 5F–G). For *Opa-1*, *CHCHD3*, and *CHCHD6* knockouts, the number of cristae per mitochondrion decreased, as did the cristae score and cristae surface area compared to the WT (Figures 5H–J). The least significant change was seen in the *CHCHD3* knockout. Together, these data show quantitative and structural changes in both mitochondria and cristae upon loss of MICOS proteins.

Loss of OPA-1 induces bioenergetic stress and decreased electron transport chain function [28] and ablation of the MICOS complex alters mitochondrial capacity [35,36]. We found that loss of OPA-1 or *Mitofilin* in myotubes decreased basal oxygen consumption rate (OCR) (Figures 4J–K) and decreased ATP-linked, maximum, and reserve capacity OCR (Figures 4L–N). Although OPA-1 knockout myotubes exhibited a decrease in proton leak, which represents protons that go from the mitochondria to matrix without producing ATP, *Mitofilin* knockouts showed no significant difference (Figure 4O). In *Opa-1*, *CHCHD3*, and *CHCHD6* knockouts, there was a decrease in basal, ATP-linked, maximum, and reserve capacity OCR compared to the control (Figures 5K-O). Although proton leak OCR decreased in *Opa-1* and *CHCHD3* knockout myotubes (Figure 5P), there was no significant difference between the control and *CHCHD6*. The decrease in OCR may be attributed to smaller and fragmented mitochondria; mitochondrial density decreases as fragmentation targets them for autophagy [29,37]. Together, these results show that MICOS and *Opa-1* are essential for normal respiration of muscle tissue.

TEM provides mitochondrial detail, but not 3D morphology; therefore, we used SBF-SEM to look at the 3D structure of the mitochondria. Using myotubes with ablated genes for *Opa-1* and MICOS complex subunits, as described above, we measured a total of 200 mitochondria across 10 cells. We compared mitochondria in WT, *Opa-1*, *Mitofilin, CHCHD3*, and *CHCHD6* knockout myotubes (Figures 6A-E).

We found that compared to the elongated mitochondria in the WT, the 3D length was much shorter in *Opa-1* and MICOS protein knockouts (Figure 6F). Similarly, the volume of mitochondria was also less in *CHCHD3*, *CHCHD6*, *Mitofilin*, and *Opa-1* knockouts compared to the control (Figure 6G). The 3D reconstruction data, in combination with the prior TEM results, show how mitochondrial dynamics change with the loss of MICOS subunits.

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].

305 Using 3D reconstructions, we found that all parameters of the mitochondria changed
306 during aging including smaller volume, area, and perimeter (Figure 1D-F), and the mitochondria
307 became less interconnected. This increased fragmentation suggests a decreased mitochondrial
308 fusion, in association with age-dependent decreases of OPA-1, which is a regulator of
309 mitochondrial fusion. We also saw a decrease in the MBI during aging, suggesting that
310 mitochondria decrease in networking, but their shape may not radically change as they increase
311 in sphericity as they age (Figure 2).
312 The change in morphology in aging mitochondria could be explained by the MFRTA,
313 which proposes that ROS alter other functions of mitochondria, including ATP production, as
314 they induce aging [49]. Since oxidative stress has been linked to many diseases of aging (diabetes,
315 heart disease, sarcopenia, arthritis, kidney disease)[50], understanding the association between
316 aging and mitochondria is important.
317 We found nanotunnels, which are thin, double-membrane mitochondrial connections that
318 can transport proteins and nutrients, in all skeletal muscle samples. While healthy humans have
319 almost no nanotunnels [21], we unexpectedly found a moderate number of nanotunnels in young 3-
320 month-old mice (Figures 3E–H), and a consistent increase of nanotunnels in the skeletal muscle
321 of 1-year-old mice. These structures may have an unclear beneficial role in skeletal muscle of
322 mice. Given the association of nanotunnels with human diseases, we predicted that the 2-year-
323 old "aged" mouse skeletal muscle would have the most nanotunnels, however the nanotunnel
324 number was quite heterogeneous in 2-year-old mice and less were observed [12,51]. The 2-year-old
325 samples may have shown fewer nanotunnels due to the high rate of fragmentation (Figure 1) and
326 lack of connectedness (Figure 2) preventing nanotunnel formation. In contrast, perhaps the
327 remaining networks permitted the increased number of nanotunnels in 1-year moderately aged
328 samples. The presence of nanotunnels in the healthy 3-month-old mouse skeletal muscle
329 suggests significant differences in mouse versus human muscle [21].
330 MICOS proteins play key regulatory roles in mitochondrial structure and function [19,26,37].
331 We determined by TEM 3D reconstructions that the loss of *Mitofilin*, *CHCHD3*, and *CHCHD6*
332 (Figures 4-5) resulted in fragmentation, disrupted cristae, and smaller mitochondria (Figures 4A–
333 I, Figures 4P–Y; Figure 5), similar to the loss of OPA-1, which is known to cause changes in
334 oxidative phosphorylation (Figures 4Z–AE) [19,23,28]. Overall, mitochondria lacking the MICOS
335 genes had characteristics similar to those of aged mouse skeletal muscle (Figures 1−2). This
336 similarity in phenotype suggests there may be an association. Thus, changes in mitochondrial
337 morphology due to aging may be caused by a lack of MICOS gene expression. This is supported
338 by the RT-qPCR data that showed decreased *CHCHD3*, *CHCHD6*, *Mitofilin*, and *Opa-1*
339 transcription with aging (SFigure 3). Although there is a link between aging and loss of *Opa-1*
340 [24,29], little is known about the role of the MICOS complex in aging. Changes in mitochondrial
341 architecture and loss of integrity may be caused by decreased MICOS proteins; thus, it will be
342 important to develop methods to restore MICOS proteins and *Opa-1* lost during aging to
343 decrease the deleterious effects of mitochondrial dysfunction. Although studies have examined
344 the role of knockouts on mitochondrial dynamics, few studies have examined how loss of
345 MICOS proteins may be restored to mitochondria [23,52].
346 Many studies have analyzed the mitochondrial metabolome using mouse skeletal muscles
347 [45,53–57]. We found that loss of *Mitofilin* affected cristae morphology (Figures 4G–H), decreased
348 oxidative phosphorylation (Figure 4J), and may have increased lipid and steroid synthesis, which
349 may be important for mitochondrial endoplasmic reticulum contact (MERC) regulation and
350 cristae formation. We found an increase in tryptophan and methyl histidine metabolism (SFigure

351 4D) and an increase in taurine metabolism and hypotaurine, a key sulfur-containing amino acid
352 for the metabolism of fats. Loss of *Opa-1* also changes amino acid and lipid metabolism, similar
353 to the loss of *Mitofilin* [40–42]. Steroidogenesis, which makes the membrane less rigid, was
354 increased. Since the loss of *Mitofilin*, *CHCHD6*, or *CHCHD3* caused a loss of oxidative capacity
355 (Figure 4J-O; 5K-P), increased steroid synthesis may allow the cell to recover bioenergetic
356 functions, as steroid compounds decrease membrane viscosity, with the incorporation of estrogen
357 [38]. In the absence of *Mitofilin*, the cristae junctions and contact sites fall apart [58]; thus, *Mitofilin* is
358 critical for maintaining cristae [59,60]. Cells lacking *Mitofilin* may make steroids to help the
359 membrane to reconfigure broken cristae. Since the loss of *Opa-1* causes more MERCs [3], loss of
360 *Mitofilin* may increase phospholipids (SFigure 4D) because of increased smooth MERCs, which
361 are associated with lipid changes [61]. This is supported by the fact that biosynthesis of
362 phosphatidylethanolamine and phosphatidylcholine and metabolism of arachidonic acid and
363 sphingolipid increased with loss of *Mitofilin* (SFigure 4D). Since these phospholipids aggregate
364 around MERCs and may shuffle into the ER, *Mitofilin* may be a critical gene for regulating
365 cristae morphology and with a key role in regulating mitochondrial metabolism, which is novel.
366 *Mitofilin* may be an important target in the future to restore energy production. Loss of
367 *Mitofilin* may lead to ER stress, which, via ATF4, activates amino acid transporters [62] that further
368 activate mTORC1. ER stress activates mTORC as a result of a decrease in glucose [57]. Critically,
369 mTORC1 affects glucose homeostasis [63], and this may lead to inefficiency in energy use. This
370 can result in changes in autophagy. Therefore, as a downstream effect of *Mitofilin* loss increasing
371 mTORC1, this may explain why deletion of MICOS in *Drosophila* increases autophagy [37].
372 Similarly, previously loss of *Opa-1* increases ER stress [28], and loss of *Mitofilin* may cause a
373 similar increase in amino acid catabolism. If ER stress activates amino acid transporters,
374 branched-chain amino acids could increase ER stress, resulting in a positive feedback loop that
375 affects the health of the cell, cellular energy, metabolism, and antioxidants. ER stress may also
376 be responsible for the poor performance and fragmentation of mitochondria (Figure 4-5). Loss of
377 *Mitofilin* may result in the breakdown of protein pathways that regulate ER stress. Other amino
378 acid pathways, such as homocysteine (SFigure 4D), are involved in triglyceride uptake and
379 increased intracellular cholesterol, suggesting that proteins like ATF4 [57] and the MICOS
380 complex [15,26] are important for aging. In particular, the MICOS components may prevent
381 mitochondrial fragmentation by blocking ER stress pathways in aging. Further studies are
382 needed to better understand the role of MICOS in MERC formation and the relation between
383 smooth MERC and lipid synthesis.
384 Although *Mitofilin* is a key component of the MICOS complex, other components are
385 likely also important. The loss of *CHCHD3* or *CHCHD6* leads to a decrease in and disassembly
386 of all *Mitofilin* subcomplex components in mammals, with abnormal cristae morphology and
387 growth defects [33,64–69]. Downregulation of *CHCHD3* is linked to type 2 diabetes [70]. In our
388 metabolomics enrichment dataset (SFigure 5D), loss of *CHCHD3* or *CHCHD6* in mouse
389 myotubes resulted in a preference for alternative fuel sources, such as lactate, lactose, and
390 starches. Supplementation of healthy myotubes with galactose leads to a 30% increase in
391 oxidative capacity (i.e., OCR) due to an increase in AMPK phosphorylation and cytochrome c
392 oxidase (COX) activity, thereby forcing cells to become more oxidative to maintain ATP levels
393 [71]. In our samples, as oxidative metabolism decreased, anaerobic metabolism and lactate levels
394 increased, forcing cells to produce ATP by anaerobic glycolysis. However, long and high-level
395 exposure to D-galactose generates free radicals, which alter MERCs, cause mitochondrial

396 dysfunction, and induce aging [2,72]. This is the likely explanation for mitochondrial fragmentation
397 in aged samples and loss of the MICOS complex, which should be investigated further.
398 In conclusion, we present a quantitative evaluation of mitochondrial and cristae
399 morphology in mouse skeletal muscle using 3D reconstructions and TEM analysis. We found
400 oxidative differences upon loss of MICOS proteins. 3D reconstructions of nanotunnels in
401 mitochondria showed novel patterns with a moderate number of nanotunnels in young skeletal
402 mouse tissue and relatively low numbers in the most aged mouse skeletal muscles. Similar
403 changes in mitochondrial morphology were observed in aging muscles and for loss of MICOS
404 proteins in mouse skeletal muscle, and MICOS proteins decreased with age. This suggests a
405 relationship between the MICOS complex and aging, and further studies through 3D
406 reconstruction could elucidate the linkage between age-related muscle dysfunction, the MICOS
407 complex, and disease states in mitochondria.
408
409 **EXPERIMENTAL PROCEDURES**
410 *Animal Care and Maintenance*
411 All procedures for the care of mice were in accordance with humane and ethical protocols
412 approved by the University of Iowa Animal Care and Use Committee (IACUC) following the
413 National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals as
414 described previously [28]. All experiments used WT male C57Bl/6J mice housed at 22°C on a 12-
415 hour light, 12-hour dark cycle with free access to water and standard chow. Mice were
416 anesthetized with 5% isoflurane/95% oxygen.
417
418 *RNA Extraction and RT-qPCR*
419 Total RNA was extracted from tissue using TRIzol reagent (Invitrogen, cat #), purified with the
420 RNeasy kit (Qiagen Inc, cat #), and quantitated by the absorbance at 260 nm and 280 nm using a
421 NanoDrop 1000 (NanoDrop products, Wilmington, DE, USA) spectrophotometer. Total RNA
422 (~1 µg) was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit
423 (Applied Biosciences, Carlsbad CA, cat #) followed by real-time quantitative PCR (qPCR)
424 reactions using SYBR Green (Life Technologies, Carlsbad, CA, cat #) [73]. Triplicate samples for
425 qPCR (~50 ng) in a 384-well plate were placed into ABI Prism 7900HT instrument (Applied
426 Biosystems) programmed as follows: 1 cycle at 95°C for 10 min; 40 cycles of 95°C for 15 s;
427 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
428 15 s; and one cycle of 95°C for 15 s. Data were normalized to glyceraldehyde-3-phosphate
429 dehydrogenase (*Gapdh*), and results are shown as fold changes. qPCR primers were designed
430 using Primer-Blast or were previously published sequences [28] as shown in Table 1.
431 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'

432

433 *Isolation of Satellite Cells*
434 Satellite cell differentiation was performed as previously described [16,28]. Cells were cultured at
435 37°C, 5% CO_2 Dulbecco's modified Eagle's medium (DMEM; GIBCO, cat #) supplemented
436 with 10% FBS (Atlanta Bio selected, cat #), and 1% penicillin-streptomycin (Gibco, Waltham,
437 MA, USA, cat #).
438
439 *CRISPR-Cas9 Knockouts*
440 After three days, myotubes were infected with adenovirus to produce the following knockouts—
441 control CRISPR/Cas9 (sc-418922), *CHCHD6* (Mic25) CRISPR (sc-425817), *CHCHD3* (Mic19)
442 CRISPR (sc-425804), and *mitofilin* (Mic60) CRISPR (sc-429376) (Santa Cruz Biotechnology,
443 California, US), with the use of guide RNA (Table 2). We incubated 2.5% relevant CRISPR,
444 2.5% RNAiMax (ThermoFisher Scientific; cat # 13778075), and 95% Opti-MEM (Gibco; cat
445 #31985070) in a tube for 20 minutes. Cells were washed twice with PBS after removal of the
446 medium and 800 µL of OPT-MEM and 200 µL of the CRISPR mixture were added to each well,
447 ran in triplicates. Cells were incubated for 4 hours at 37 C, 1.0 mL of DMEM medium was
448 added, cells were incubated overnight. The myotubes were then washed with PBS and the
449 medium was replaced. Experiments were performed between 3 and 7 days after infection for a
450 total of 6 days of differentiation.
451 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

452
453 *Serial Block-Face Scanning Electron Microscope (SBF-SEM) Processing of Mouse Muscle*
454 *Fibers*
455 SBF-SEM preparation was performed as described previously [17,27,74]. Running on a FEI/Thermo
456 Scientific Volumescope 2 SEM, a state-of-the-art SBF imaging system, we obtained 300−400
457 ultrathin (0.09 µm) serial sections from the blocks that were processed for conventional TEM.
458 All sections were collected onto formvar-coated slot grids (Pella, Redding CA), stained, and
459 imaged as previously described [17,27,74].
460
461 *Quantification of TEM Micrographs and Parameters Using ImageJ*
462 Quantification of TEM images was performed as described previously using the
463 NIH *ImageJ* software [16,27]. Cells were divided into four quadrants and two quadrants were
464 selected randomly for complete analysis. From each individual, a minimum of 10 cells were
465 measured with three analyses to obtain accurate and reproducible values. If variability occurred,
466 the number of cells was expanded to 30 cells per individual to reduce the variability.
467
468 *Segmentation and Quantification of 3D SBF-SEM Images Using Amira*
469 Intermyofibrillar (IMF) mitochondria are located between myofibrils, arranged in pairs at the z-
470 band of each sarcomere, with 2D elongated tubular shapes [75]. However, it is not known how
471 aging affects mitochondrial orientation, the structure of C-band sarcomeres, or the morphological
472 changes in incomplete fission known as nanotunnels. For each region of interest across the three
473 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

520 distance and Ward's linkage to minimize the clustering needed. Metabolite Set Enrichment
521 Analysis (MSEA), which determines whether a set of functionally related metabolites is altered,
522 can be used to identify consistent changes across many metabolites with similar roles.
523 Overrepresentation analysis determines whether a group of compounds is overrepresented in
524 comparison to pure chance and whether a group of metabolites have similar changes. In this
525 analysis, the fold enrichment was calculated by dividing the observed hits by the expected
526 metabolites. Expected number of hits are calculated by MetaboAnalyst 5.0. GraphPad Prism
527 software (La Jolla, CA, USA) was used for statistical analysis with data expressed as mean ±
528 standard deviation, and one-tailed p-values ≤ 0.01 were considered significant.
529
530 *Measurement of OCR Using Seahorse*
531 Using an XF24 extracellular flux (XF) bioanalyzer (Agilent Technologies/Seahorse Bioscience,
532 North Billerica, MA, USA), OCR was measured for *Opa-1*, *CHCHD3*, *CHCHD6*, or *Mitofilin*
533 knocked down cells as previously described[28]. Three independent experiments were performed
534 with four to six replicates for each time and for each condition and representative data from the
535 replicates are shown.
536
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552 The authors declare that they have no conflict of interest.
553
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556
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558 The data that support the findings of this study are available from the corresponding author upon
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560
561 **AUTHOR CONTRIBUTIONS**
562 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

563 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

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570
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(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 **** $p \leq 0.0001$.

Supplementary:

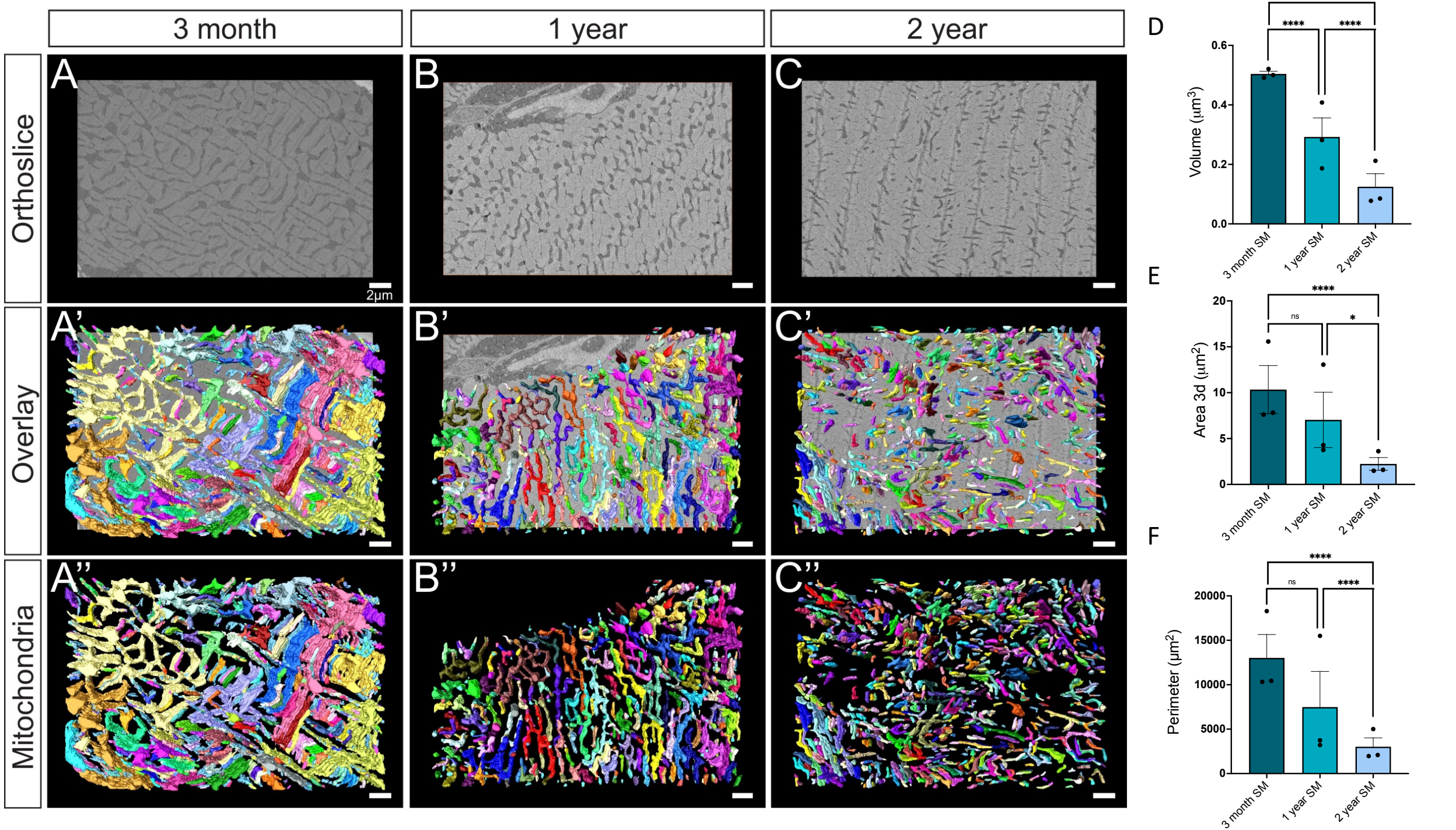
Figure 1: The (**A**) volume (**B**) 3D area, and (**C**) perimeter of the average mitochondria in each of the three individual mice for the age cohorts, all shown on log scale. The (**D**) volume (**E**) 3D area, and (**F**) perimeter quantified on log scale with each dot representing one of the approximately 550 mitochondria analyzed for each age cohorts.

Figure 2: The (**A**) mitochondria branching index (MBI) and (**B**) sphericity of the average mitochondria in each of the three individual mice for the age cohorts. Combined data of mice for (**C**) MBI and (**D**) sphericity with each dot representing one of the approximately 550 mitochondria analyzed for each age cohorts.

Figure 3. Transcription of *Opa-1* and MICOS genes in aging mouse muscle.
(**A-D**) Fold changes in the amount of OPA-1 and MICOS gene transcripts in mitochondria of myocytes of 3-month-old, 1-year-old, and 2-year-old mice as measured by RT-qPCR. (**A**) *Opa-1* transcripts. (**B**) *Mitofilin* transcripts. (**C**) *CHCHD3* transcripts. (**D**) *CHCHD6* transcripts. Significance values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Figure 4. (**A**) Metabolite PCA and (**B**) T-test comparing myotubes for control to *Mitofilin* knockout mice. (**C**) Heatmap showing the relative abundance of ions and (**D**) Enrichment analysis of metabolites, which links together several similarly functioning metabolites, with the relative abundance for *Mitofilin* knockout.

Figure 5. (**A**) Metabolite PCA and (**B**) ANOVA test comparing control to myotubes of *CHCHD3* and *CHCHD6* knockout mice (**C**) Heatmap showing the relative abundance of ions for control and (**D**) enrichment analysis metabolite for *CHCHD3* and *CHCHD6* knockout mice.


3 month
1 year
2 year
Orthoslice
Overlay
Mitochondria
A
B
C
A'
B'
C'
A''
B''
C''
2µm
D
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F
Volume (µm³)
Area 3d (µm²)
Perimeter (µm²)
3 month SM
1 year SM
2 year SM
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ns
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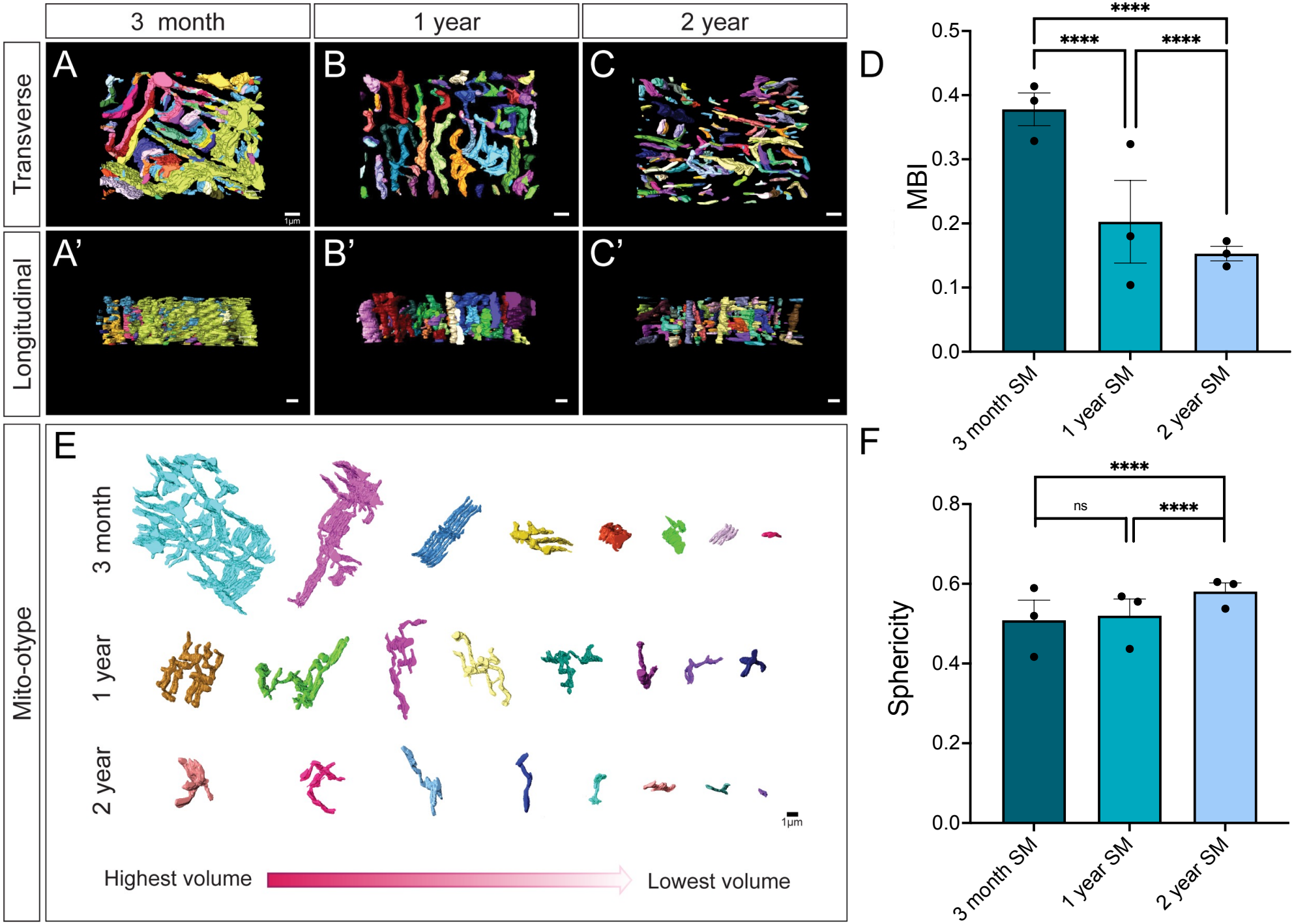
3 month
1 year
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Transverse
Longitudinal
Mito-otype
A
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C
A'
B'
C'
1µm
D
MBI
0.0
0.1
0.2
0.3
0.4
0.5
****
****
****
3 month SM
1 year SM
2 year SM
E
3 month
1 year
2 year
1µm
Highest volume
Lowest volume
F
Sphericity
0.0
0.2
0.4
0.6
0.8
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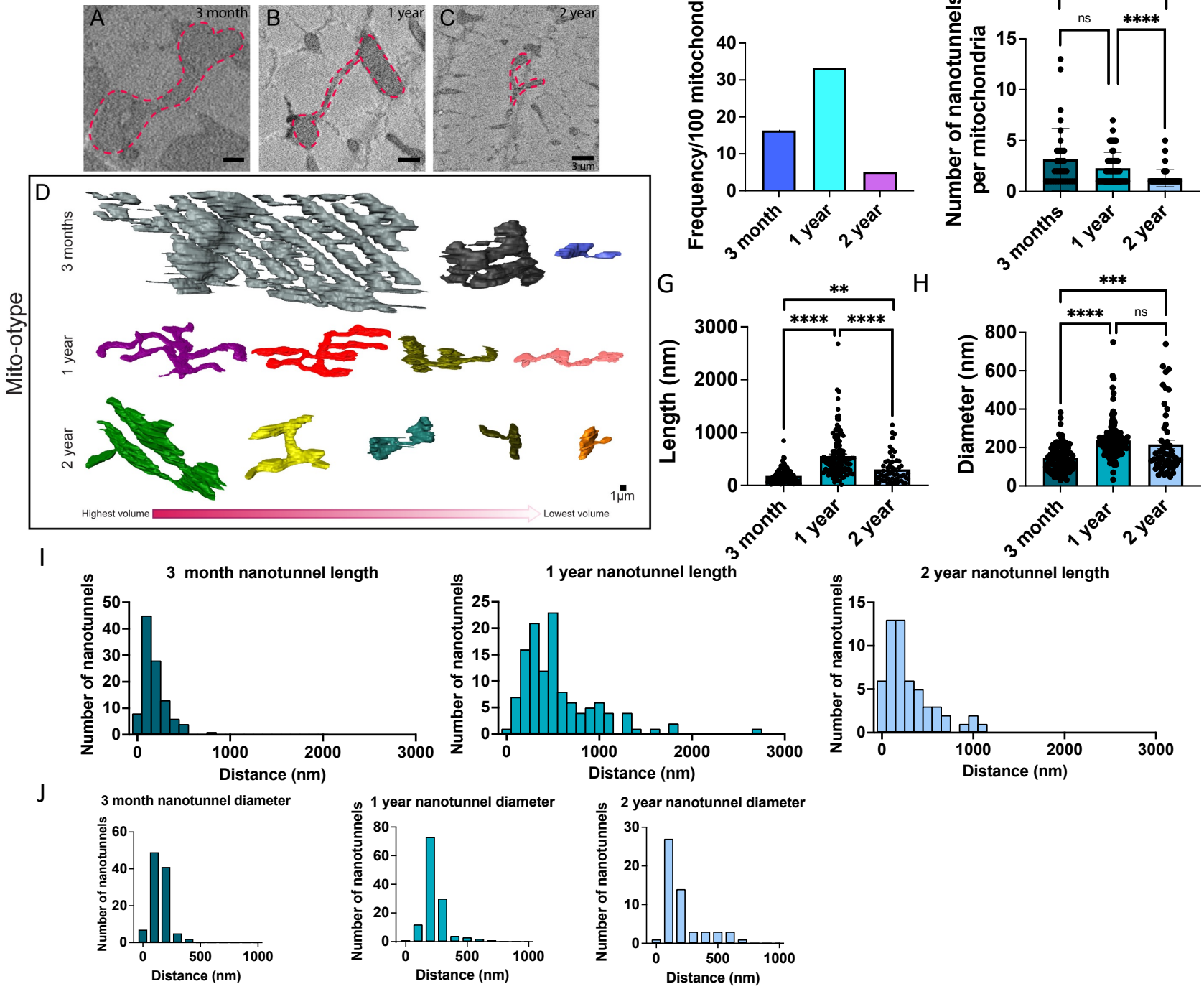
3 month SM
1 year SM
2 year SM


A
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B
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C
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D
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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)
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I
3 month nanotunnel length
1 year nanotunnel length
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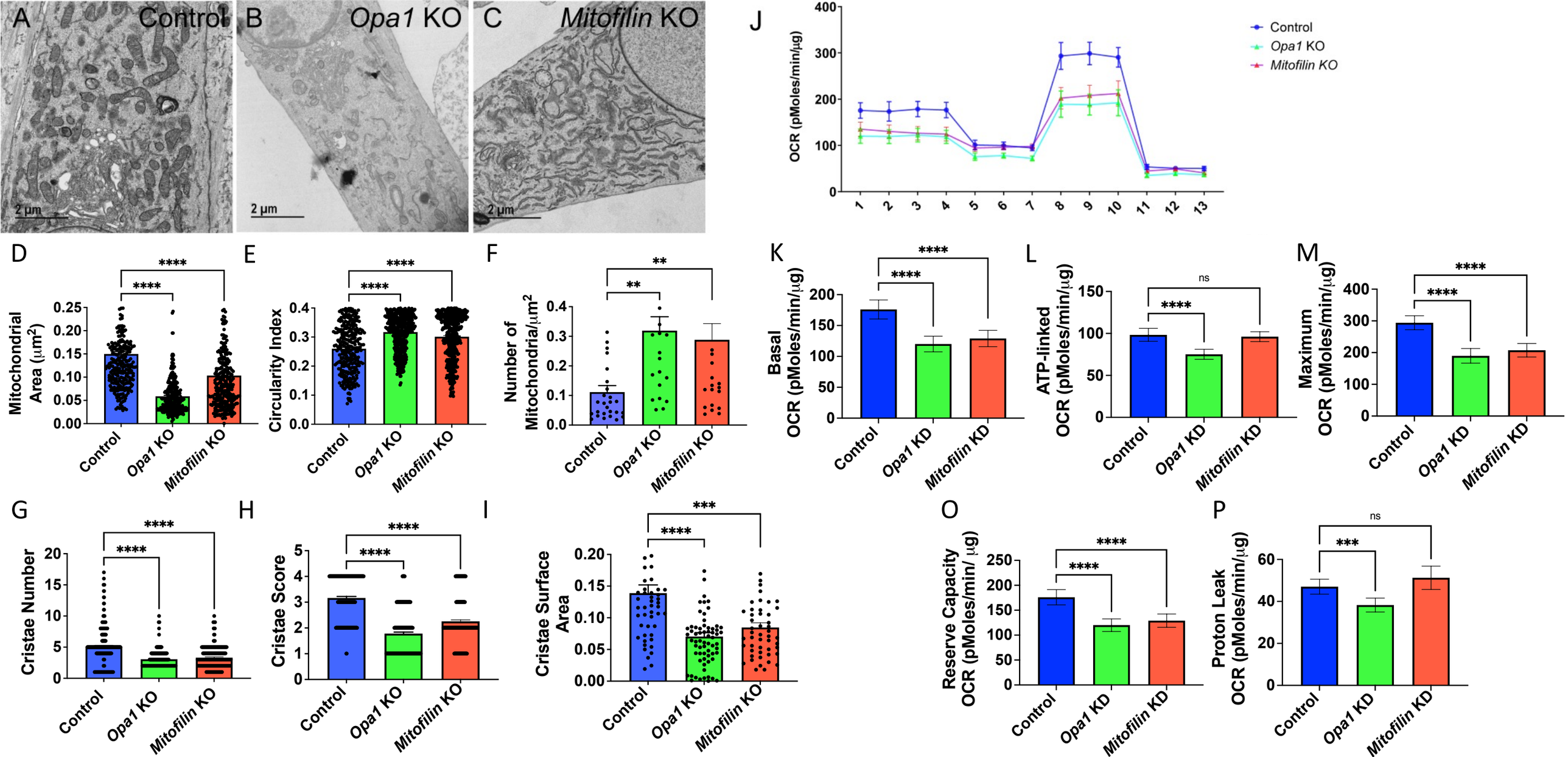
Number of nanotunnels
Distance (nm)
J
3 month nanotunnel diameter
1 year nanotunnel diameter
2 year nanotunnel diameter


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)
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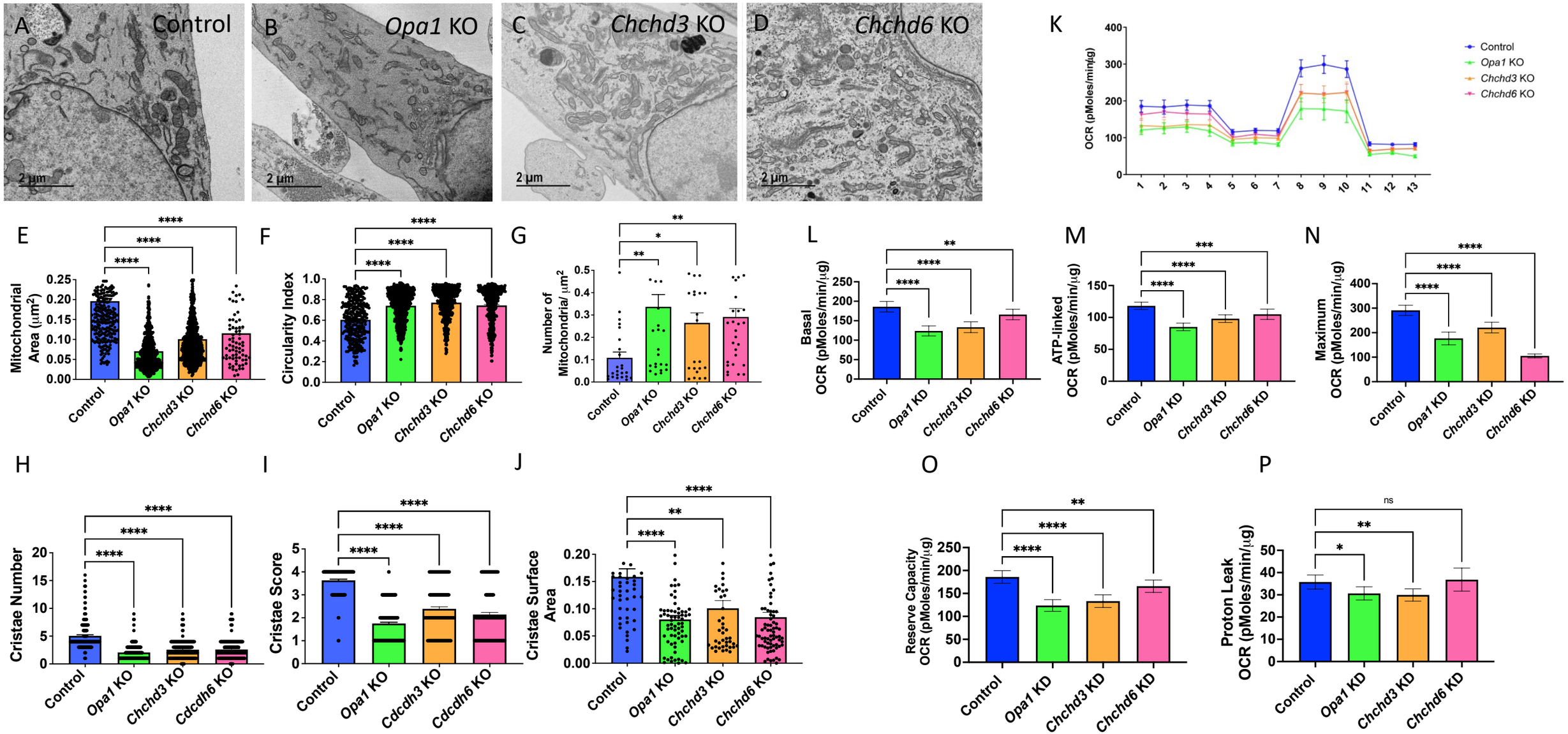
Opa1 KO
Mitofilin KO
Opa1 KD
Mitofilin KD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B
Opa1 KO
C
Chchd3 KO
D
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OCR (pMoles/min/µg)
Control
Opa1 KO
Chchd3 KO
Chchd6 KO
E
Mitochondrial Area (µm²)
F
Circularity Index
G
Number of Mitochondria/ µm²
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
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Cristae Number
I
Cristae Score
J
Cristae Surface Area
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Reserve Capacity OCR (pMoles/min/µg)
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Proton Leak OCR (pMoles/min/µg)
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Cdcdh6 KO
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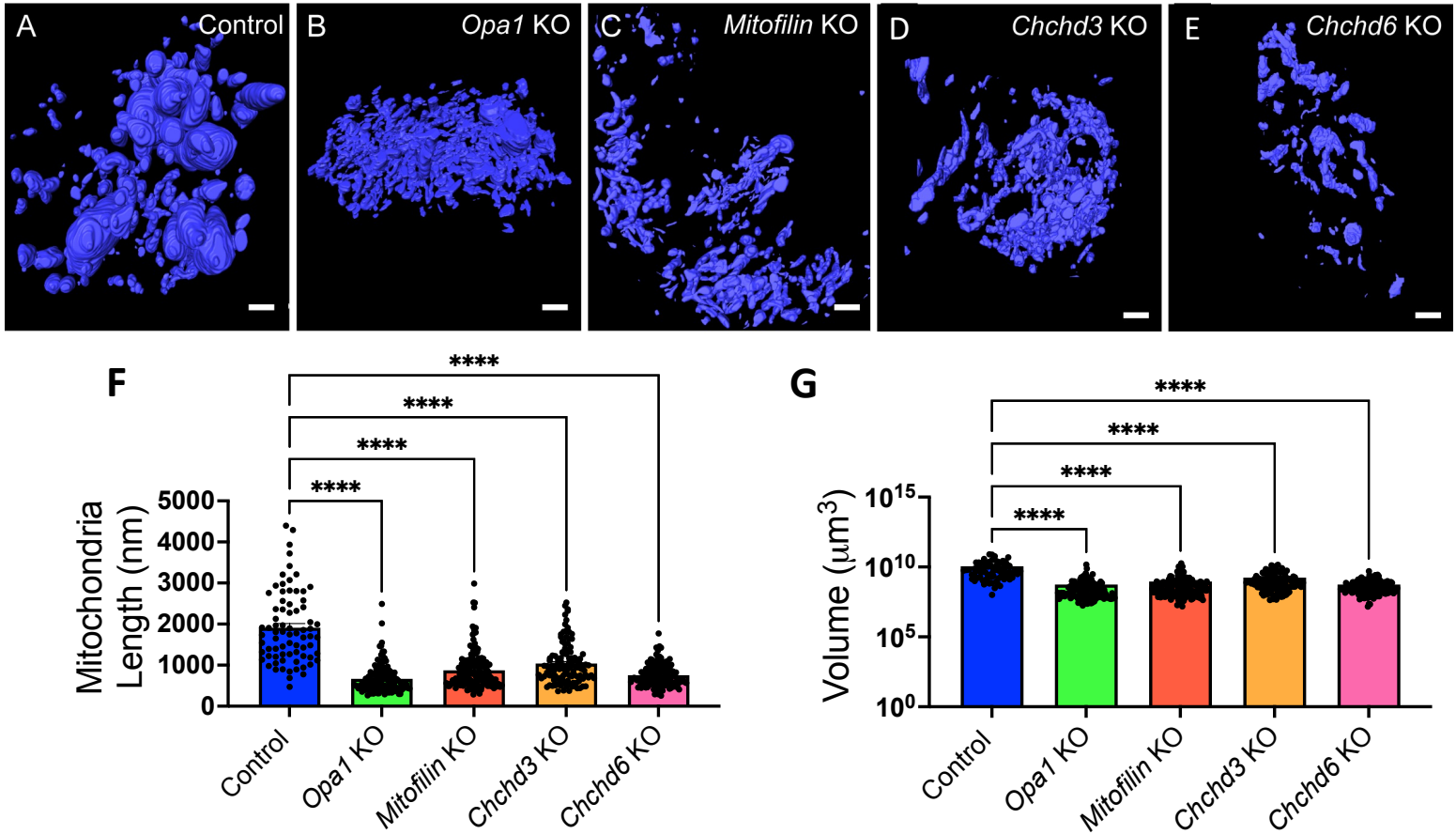
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Control
B
Opa1 KO
C
Mitofilin KO
D
Chchd3 KO
E
Chchd6 KO
F
Mitochondria Length (nm)
5000
4000
3000
2000
1000
0
****
****
****
****
Control
Opa1 KO
Mitofilin KO
Chchd3 KO
Chchd6 KO
G
Volume (μm3)
10^15
10^10
10^5
10^0
****
****
****
****
Control
Opa1 KO
Mitofilin KO
Chchd3 KO
Chchd6 KO