1 **Mouse Skeletal Muscle Decrease in the MICOS Complex and Altered Mitochondrial**
2 **Networks with age**
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53   **Graphical Abstract:**
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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.

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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.

Mitochondrial Nanotunnels Change in Size and Amount with Aging in Skeletal Muscle

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To quantify nanotunnels in skeletal muscle, we performed 3D reconstruction in mice at
three ages, measuring 550 mitochondria per mouse (n=3) using four different regions of interests
(ROIs). Although nanotunnels are reported to exist primarily in mitochondrial disease states [12],
we found them in all three ages of mice (Figures 3A–C). The diversity and heterogeneity in the
organization, sizing, and branching of nanotunnels necessitated mito-otyping (Figure 3D). We
found nanotunnels in a greater proportion of mitochondria in 1-year-old mice than in 2-year-old
mice (Figure 3E). However, the number of nanotunnels in each mitochondrion was significantly
less in the 2-year-old mice than the 3-month-old and 1-year-old mice (Figure 3F). The length and
diameter of nanotunnels increased in the older mice compared to the 3-month-old mice (Figures
3G–H), but the increase was significantly greater in the 1-year-old mice than the 2-year-old mice
(Figures 3G–J). These data show that nanotunnel structure, frequency, and length change during
aging.
Since we observed mitochondrial dynamics and cristae change with aging , we measured
transcripts for *Opa-1*, the gene associated with mitochondrial fusion, and *CHCHD3* (*Mic19*),
CHCHD6 (*Mic25*)*,* and *Mitofilin* (*Mic60*), the genes for the principal MICOS complex subunits.
We used reverse-transcriptase qPCR (RT-qPCR) to determine the effect of aging on
transcription. Since aging causes fragmentation of mitochondria, a hallmark for this process is
loss of *Opa-1* and MICOS complex proteins. We hypothesized that aging would also lead to
decreases in the associated MICOS complex proteins, confirming prior studies which have
found Opa-1 loss across aging [19,23–25]. We found progressive loss of both *Opa-1* and MICOS
complex subunits, as measured by loss of transcripts for the proteins (SFigure 3A–D). In 1- and
2-year-old mice, the amount of mRNA made for the MICOS complex subunit genes or the *Opa-*
1 gene was less than half of that made in 3-month-old mice (SFigure 3A–C). Furthermore, for
all MICOS genes, there was a statistically significant decrease in the amount of mRNA over
time with a higher level of significance in 2-year-old mice. These data suggest a correlation
between mitochondrial morphologic changes and decreases in expression of genes for *Opa-1*
and MICOS subunits *CHCHD3*, *CHCHD6*, and *Mitofilin* during aging; however, they are not
sufficient to demonstrate causation, therefore we did additional measurements to show that
reduced protein expression levels may be responsible for the morphologic changes.

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Changes in Cristae and Mitochondria in Myotubes and Oxygen Respiration Rate Upon
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Knockout of MICOS complex and *Opa-1*
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The MICOS complex and OPA-1 are key players in mitochondrial biogenesis [15,19,26], but
how their interactions regulate aging and mitochondrial ultrastructures is poorly understood. To
determine the role of OPA-1 and the MICOS complex in mitochondrial structure and
networking, we ablated the genes for *Opa-1* and the MICOS complex in isolated primary skeletal
muscle cells from 3-month-old mice. We isolated primary satellite cells, then differentiated
myoblasts to myotubes. Using CRISPR/Cas9 method (Table 2), and a control plasmid, we
knocked out the genes for MICOS complex components and *Opa-1* from skeletal muscle cells.
As observed previously, in vitro deletion of OPA-1 altered mitochondrial morphology [16,27,28].
We measured 1250 mitochondria across 10 cells, with loss of *Opa-1* as a positive control for
mitochondrial morphological changes. Although *Opa-1* expression decreases with age [29], the
effect of the MICOS complex being lost in mitochondria morphology is unknown. Using
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.
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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).
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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].

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

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

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

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
CRISPR-Cas9 Knockouts
After three days, myotubes were infected with adenovirus to produce the following knockouts—
control CRISPR/Cas9 (sc-418922), *CHCHD6* (Mic25) CRISPR (sc-425817), *CHCHD3* (Mic19)
CRISPR (sc-425804), and *mitofilin* (Mic60) CRISPR (sc-429376) (Santa Cruz Biotechnology,
California, US), with the use of guide RNA (Table 2). We incubated 2.5% relevant CRISPR,
2.5% RNAiMax (ThermoFisher Scientific; cat # 13778075), and 95% Opti-MEM (Gibco; cat
#31985070) in a tube for 20 minutes. Cells were washed twice with PBS after removal of the
medium and 800 µL of OPT-MEM and 200 µL of the CRISPR mixture were added to each well,
ran in triplicates. Cells were incubated for 4 hours at 37 C, 1.0 mL of DMEM medium was
added, cells were incubated overnight. The myotubes were then washed with PBS and the
medium was replaced. Experiments were performed between 3 and 7 days after infection for a
total of 6 days of differentiation.
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
Serial Block-Face Scanning Electron Microscope (SBF-SEM) Processing of Mouse Muscle
Fibers
SBF-SEM preparation was performed as described previously [17,27,74]. Running on a FEI/Thermo
Scientific Volumescope 2 SEM, a state-of-the-art SBF imaging system, we obtained 300−400
ultrathin (0.09 µm) serial sections from the blocks that were processed for conventional TEM.
All sections were collected onto formvar-coated slot grids (Pella, Redding CA), stained, and
imaged as previously described [17,27,74].
460
Quantification of TEM Micrographs and Parameters Using ImageJ
Quantification of TEM images was performed as described previously using the
NIH *ImageJ* software [16,27]. Cells were divided into four quadrants and two quadrants were
selected randomly for complete analysis. From each individual, a minimum of 10 cells were
measured with three analyses to obtain accurate and reproducible values. If variability occurred,
the number of cells was expanded to 30 cells per individual to reduce the variability.
467
Segmentation and Quantification of 3D SBF-SEM Images Using Amira
Intermyofibrillar (IMF) mitochondria are located between myofibrils, arranged in pairs at the z-
band of each sarcomere, with 2D elongated tubular shapes [75]. However, it is not known how
aging affects mitochondrial orientation, the structure of C-band sarcomeres, or the morphological
changes in incomplete fission known as nanotunnels. For each region of interest across the three
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)
507 LC-MS was performed for myotubes as previously described[76]. TraceFinder 4.1 software was
508 used for analysis and metabolites were identified based on an in-house library. Drift was
509 corrected for as described above [77]. Data were normalized and further visualization and analysis
510 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.

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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

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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.,

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571   **REFERENCES**
572
1.   Bratic A, Larsson N-G. The role of mitochondria in aging. *The Journal of clinical*
investigation. 2013;123:951–957.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

77. Li B, Tang J, Yang Q, Li S, Cui X, Li Y, Chen Y, Xue W, Li X, Zhu F. NOREVA:
normalization and evaluation of MS-based metabolomics data. *Nucleic acids research*.
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≤ 0.05, **** $p ≤ 0.0001$.

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 **** $p ≤ 0.0001$.

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. ** $p ≤ 0.01$, **** $p ≤ 0.0001$.

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 ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
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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 * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, ****
$p \leq 0.0001$.
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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 **** $p \leq 0.0001$.
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905 **Supplementary:**
906
907 **Figure 1:** The (**A**) volume (**B**) 3D area, and (**C**) perimeter of the average mitochondria in each of
908 the three individual mice for the age cohorts, all shown on log scale. The (**D**) volume (**E**) 3D
909 area, and (**F**) perimeter quantified on log scale with each dot representing one of the
910 approximately 550 mitochondria analyzed for each age cohorts.
911
912 **Figure 2:** The (**A**) mitochondria branching index (MBI) and (**B**) sphericity of the average
913 mitochondria in each of the three individual mice for the age cohorts. Combined data of mice for
914 (**C**) MBI and (**D**) sphericity with each dot representing one of the approximately 550
915 mitochondria analyzed for each age cohorts.
916
917 **Figure 3.** Transcription of *Opa-1* and MICOS genes in aging mouse muscle.
918 (**A-D**) Fold changes in the amount of OPA-1 and MICOS gene transcripts in mitochondria of
919 myocytes of 3-month-old, 1-year-old, and 2-year-old mice as measured by RT-qPCR. (**A**) *Opa-1*
920 transcripts. (**B**) *Mitofilin* transcripts. (**C**) *CHCHD3* transcripts. (**D**) *CHCHD6* transcripts.
921 Significance values * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.
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923 **Figure 4.** (**A**) Metabolite PCA and (**B**) T-test comparing myotubes for control to *Mitofilin*
924 knockout mice. (**C**) Heatmap showing the relative abundance of ions and (**D**) Enrichment
925 analysis of metabolites, which links together several similarly functioning metabolites, with the
926 relative abundance for *Mitofilin* knockout.
927
928 **Figure 5.** (**A**) Metabolite PCA and (**B**) ANOVA test comparing control to myotubes of
929 *CHCHD3* and *CHCHD6* knockout mice (**C**) Heatmap showing the relative abundance of ions for
930 control and (**D**) enrichment analysis metabolite for *CHCHD3* and *CHCHD6* knockout mice.
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