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MFN2 overexpression in skeletal muscles of young and old mice causes a mild hypertrophy without altering mitochondrial respiration and H_2O_2 emission

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Abstract

Aim: Sarcopenia, the aging-related loss of muscle mass and function, is a debilitating process negatively impacting the quality of life of affected individuals. Although the mechanisms underlying sarcopenia are incompletely understood, impairments in mitochondrial dynamics, including mitochondrial fusion, have been proposed as a contributing factor. However, the potential of upregulating mitochondrial fusion proteins to alleviate the effects of aging on skeletal muscles remains unexplored. We therefore hypothesized that overexpressing Mitofusin 2 (MFN2) in skeletal muscle in vivo would mitigate the effects of aging on muscle mass and improve mitochondrial function.

Methods: MFN2 was overexpressed in young (7 mo) and old (24 mo) male mice for 4 months through intramuscular injections of an adeno-associated viruses. The impacts of MFN2 overexpression on muscle mass and fiber size (histology), mitochondrial respiration, and H_2O_2 emission (Oroboros fluororespirometry), and various signaling pathways (qPCR and western blotting) were investigated.

Results: MFN2 overexpression increased muscle mass and fiber size in both young and old mice. No sign of fibrosis, necrosis, or inflammation was found upon MFN2 overexpression, indicating that the hypertrophy triggered by MFN2 overexpression was not pathological. MFN2 overexpression even reduced the

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proportion of fibers with central nuclei in old muscles. Importantly, MFN2 overexpression had no impact on muscle mitochondrial respiration and H_2O_2 emission in both young and old mice. MFN2 overexpression attenuated the increase in markers of impaired autophagy in old muscles.

Conclusion: MFN2 overexpression may be a viable approach to mitigate aging-related muscle atrophy and may have applications for other muscle disorders.

KEYWORDS

autophagy, mitochondria, mitochondrial dynamics, mitochondrial fusion, mitofusin 2, sarcopenia, skeletal muscle aging

1 | INTRODUCTION

Accounting for over 40% of total body mass, skeletal muscles represent the most abundant type of tissue in mammals. Skeletal muscles are particularly affected during aging as skeletal muscle mass and strength gradually decline, a process called sarcopenia.[1] These aging-related alterations in skeletal muscle biology can have serious consequences on the quality of life of older adults, as they are associated with mobility alterations, physical frailty, falls, and, ultimately, death.[2,3] There is currently a lack of effective therapies to either prevent or treat sarcopenia, due in large part to the fact that its molecular and cellular bases are still only partly understood. Among the mechanisms that have been put forward, robust evidence has positioned the accumulation of mitochondrial impairments as a likely contributor of sarcopenia.[4–7] Although not a universal finding,[8,9] studies in both animal models and humans have shown that mitochondrial energetics is altered in aged skeletal muscles.[10–15] Several studies have shown that the mitochondrial permeability transition pore, a pore regulating mitochondrial apoptosis as well as multiple proteolytic pathways,[7] becomes dysfunctional with aging.[16,17] In line with these findings, multiple markers of mitochondrial-mediated apoptosis increase with skeletal muscle aging.[16,18–20]

Although impairments in multiple aspects of mitochondrial function are often considered hallmarks of muscle aging, the underlying mechanisms remain only partly understood. Alterations in mitochondrial dynamics have been proposed as potential contributing mechanisms. Mitochondria are dynamic organelles that can undergo fusion and fission. Mitochondrial fission is mainly regulated by Fission 1 (FIS1) and the GTPase dynamin-1-like protein (Drp1).[21] Mitochondrial fusion is driven by two GTPases located on the outer mitochondrial membrane, Mitofusins 1 and 2, (MFN1 and MFN2),[22] and a GTPase located on the inner mitochondrial membrane, the optic atrophy protein 1 (OPA1).[23] The balance between these processes of fusion and fission is critical to maintaining mitochondrial health.[24] In skeletal muscles, the deletion or depletion of proteins involved in mitochondrial fusion/fission results in impaired mitochondrial bioenergetics and morphology and altered muscle mass and function.[25–31] In the specific context of aging, Sebastian and collaborators showed that deletion of MFN2 in aged skeletal muscle induced a sarcopenic phenotype characterized by reduced muscle fiber size, decreased muscle strength, and altered running performance.[27] These deleterious morphological and functional changes were linked to an increase in mitochondrial fragmentation, impaired mitochondrial respiration, increased production of reactive oxygen species, and an inhibition of autophagy/mitophagy.[27] Based on these findings, it was proposed that the downregulation of MFN2 expression in the aged skeletal muscle of wild type mice likely contributed to sarcopenia.[27] These findings raise the possibility that overexpressing MFN2 in aged mice might counter sarcopenia. Further strengthening this view, MFN2 overexpression has been shown in cultured cells to improve mitochondrial bioenergetics[32,33] and to prevent muscle atrophy in a mouse model of cancer cachexia.[34] However, whether enhancing mitochondrial fusion can prevent skeletal muscle atrophy with aging has never been investigated. In this setting, we hypothesized that overexpressing MFN2 in vivo in skeletal muscle using intramuscular injections of an Adeno-Associated Virus (AAV) would mitigate the negative effects of aging on skeletal muscle mass and improve mitochondrial respiration and H_2O_2 production.

2 | RESULTS

2.1 | Successful overexpression of MFN2 in skeletal muscles of young adults and old mice

To investigate the effect of MFN2 overexpression in skeletal muscles, intramuscular injections of AAVs were performed in the TA and EDL (Figure 1A). As shown in Figure 1A, the left leg muscles were injected with

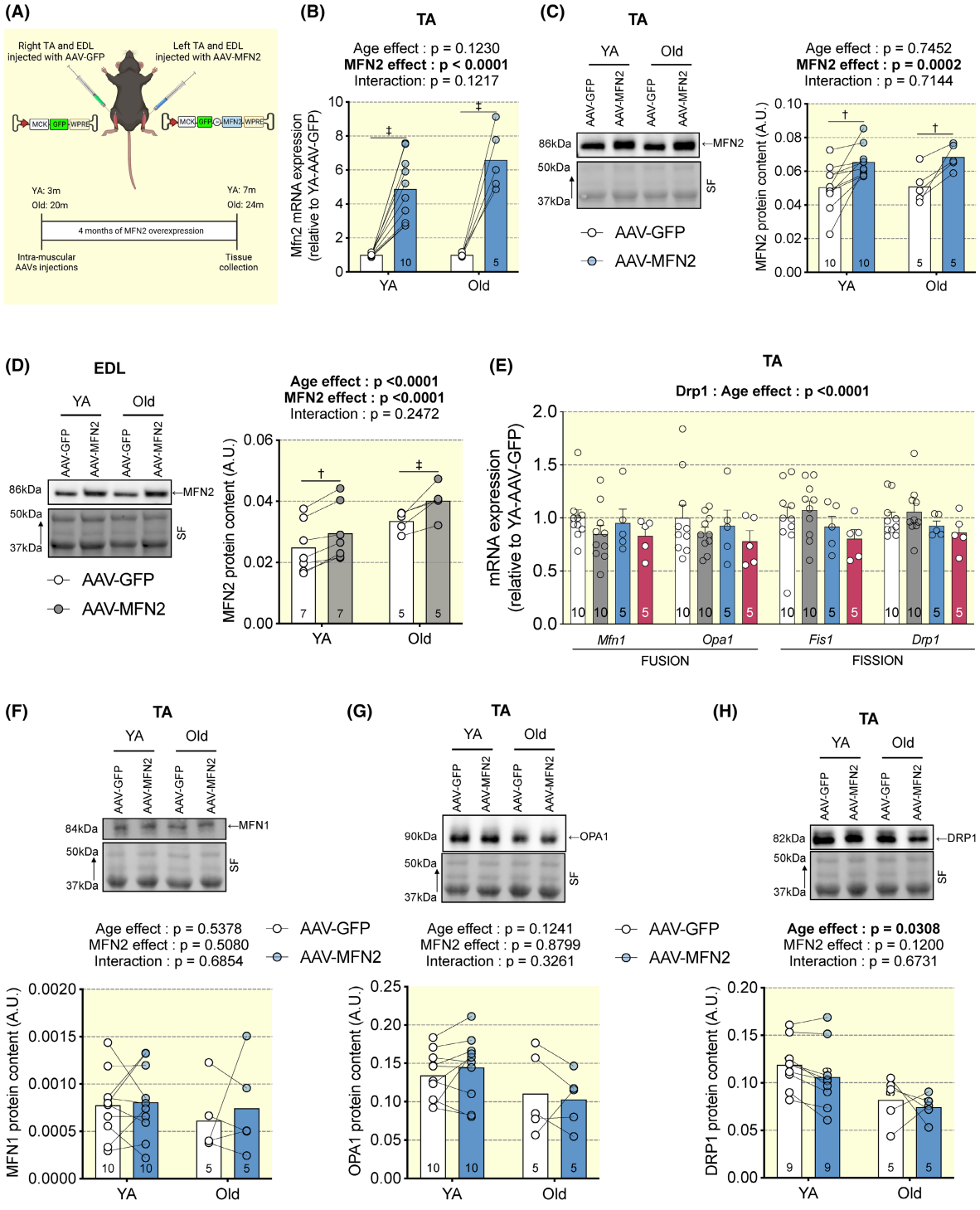


FIGURE 1 Successful overexpression of MFN2 in skeletal muscles of young adult and old mice. (A) Description of AAV construction and experimental design. Animals at 3 months (Young adults: YA) and at 20 months (Old) received two intramuscular injections of AAVs. After 4 months of MFN2 overexpression, mice were sacrificed and muscles collected. (B) Quantification of *Mfn2* mRNAs expression level in TA muscles injected either with AAV-GFP or AAV-MFN2 in YA and Old mice. Values are normalized to AAV-GFP values from YA ($n = 10$ for YA, $n = 5$ for Old). (C) Immunoblot detections of MFN2 protein and quantifications in the TA ($n = 7$ for YA, $n = 5$ for Old) and (D) EDL muscles ($n = 7$ for YA and $n = 5$ for Old). (E) *Mfn1, Opa1* (fusion markers), *Drp1*, and *Fis1* (fission markers) mRNA expressions in TA muscles injected with either AAV-GFP or AAV-MFN2. Values were normalized to AAV-GFP values from YA ($n = 10$ for YA, $n = 5$ for Old). Quantifications and representative blots of MFN1 (F), OPA1 (G), and DRP1 (H) protein content for YA and Old groups in TA muscles injected either with AAV-GFP or AAV-MFN2 ($n = 10$ for YA, $n = 5$ for Old). *p*-values retrieved from a two-way ANOVA are displayed above each bar graph. $^{*}q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

an AAV overexpressing MFN2, while the contralateral leg received a control AAV expressing GFP. One of the main strengths of this experimental design is that each animal is its own control when assessing the impact of MFN2 overexpression. Importantly, we have previously reported that prolonged overexpression of GFP does not affect muscle mass (Leduc Gaudet et al.[35]). As shown in Figure 1A, AAVs were injected in skeletal muscles of young adult (YA; 3 months old (mo) at the time of injections) and old (20 mo at the time of injections) mice. Muscles were collected after 4 months of MFN2 overexpression, that is, at 7 mo for YA and 24 mo for old mice. To validate MFN2 overexpression, *Mfn2* mRNA levels were first assessed in the TA by RT-qPCR (Figure 1B). *Mfn2* mRNA levels were significantly increased in the TA injected with AAV-MFN2 in both YA and old mice. Successful MFN2 overexpression was also confirmed at the protein level in the TA (Figure 1C) and EDL muscles (Figure 1D). No effect of age was found for MFN2 mRNA or protein level in the TA (Figure 1B,C). In contrast, a main effect of age was found for MFN2 protein content in the EDL, indicative of higher MFN2 content in this muscle with aging (Figure 1D).

Because MFN2 is a key regulator of mitochondrial dynamics, we investigated whether its overexpression would impact the mRNA expression and protein content of other key regulators of mitochondrial dynamics by RT-qPCR and western blot, respectively. As shown in Figure 1E, MFN2 overexpression had no effect on the mRNA expression of *Mfn1*, *Opa1*, *Fis1*, or *Drp1*. In line with these findings, MFN2 overexpression also did not affect the protein content of MFN1, OPA1, or DRP1 (Figure 1F–H). Only an age effect for *Drp1* mRNA expression and protein content was found, indicative of lower DRP1 expression levels in the skeletal muscles of old mice (Figure 1E,H).

2.2 | MFN2 overexpression induces muscle hypertrophy and reduces the proportion of fibers with central nuclei in old mice

To investigate the impact of MFN2 overexpression on skeletal muscles, we first assessed the effects on the masses of the TA and EDL collected from young and old mice (Figure 2A,B). As expected, the masses of TA and EDL normalized to body mass were significantly reduced with aging (Figure 2A,B). MFN2 overexpression resulted in a mild increase in the mass of TA in both young and old mice (Figure 2A,B). A main effect of MFN2 overexpression on muscle mass was also observed in the EDL (Figure 2B), although the impact of MFN2 overexpression was only significant in old mice (Figure 2B). In line with these data, MFN2 overexpression increased the myofiber cross-sectional area in the TA of both young and old mice (Figure 2C).

To investigate whether the mild hypertrophy triggered by MFN2 overexpression was pathological, several histological indicators of muscle health and integrity were assessed on TA cross-sections, and the mRNA expression of several markers of inflammation was also assessed in the TA. We first quantified the proportion of myofibers with central myonuclei on TA muscle cross-sections. The proportion of fibers with central myonuclei was significantly increased with aging (Figure 3A), an effect attenuated by MFN2 overexpression (Figure 3A). We next generated a muscle health index by quantifying, on H&E images, the relative area of each cross-section with signs of necrosis, fibrosis, and inflammation. As can be seen in Figure 3B, no significant impact of MFN2 overexpression on this muscle health index was observed in both young and old mice. To strengthen our histological observations, we quantified by RT-qPCR, the mRNA expression of *Il-6*, *Il1-ß*, and *Tnf-α* in TA muscles injected either with AAV-GFP or AAV-MFN2 from young and old mice (Figure 4A–C). As illustrated in Figure 4, no impact of MFN2 overexpression or aging was observed on *Il-6*, *Il1-ß*, and *Tnf-α* mRNA expression levels. Taken altogether these observations suggest that the mild hypertrophy triggered by MFN2 overexpression was not pathological.

2.3 | MFN2 overexpression does not affect mitochondrial respiration of H_2O_2 emission

The impact of MFN2 overexpression on mitochondrial respiration was assessed in permeabilized myofibers prepared from EDL muscles using high resolution respirometry (Figure 5A). Neither the impact of aging nor MFN2 overexpression on mitochondrial respiration was observed (Figure 5B). The acceptor control ratio (ACR), an index of mitochondrial coupling efficiency, was also unaltered by aging or MFN2 overexpression (Figure 5C). Mitochondrial H_2O_2 emission, a surrogate of ROS production, was measured in permeabilized myofibers prepared from EDL muscles using the Amplex Red-HRP enzymatic system (Figure 5D). Similarly, to our data on respiration, no effect of aging or MFN2 overexpression on mitochondrial H_2O_2 emission was observed (Figure 5D). In line with the respirometry and H_2O_2 emission data collected in the EDL, there was no impact of aging or MFN2 overexpression on the activity of SDH (Figure 5E; assessed in situ on cross sections) and 4-HNE content (Figure 5F—a marker of lipid peroxidation) in the TA.

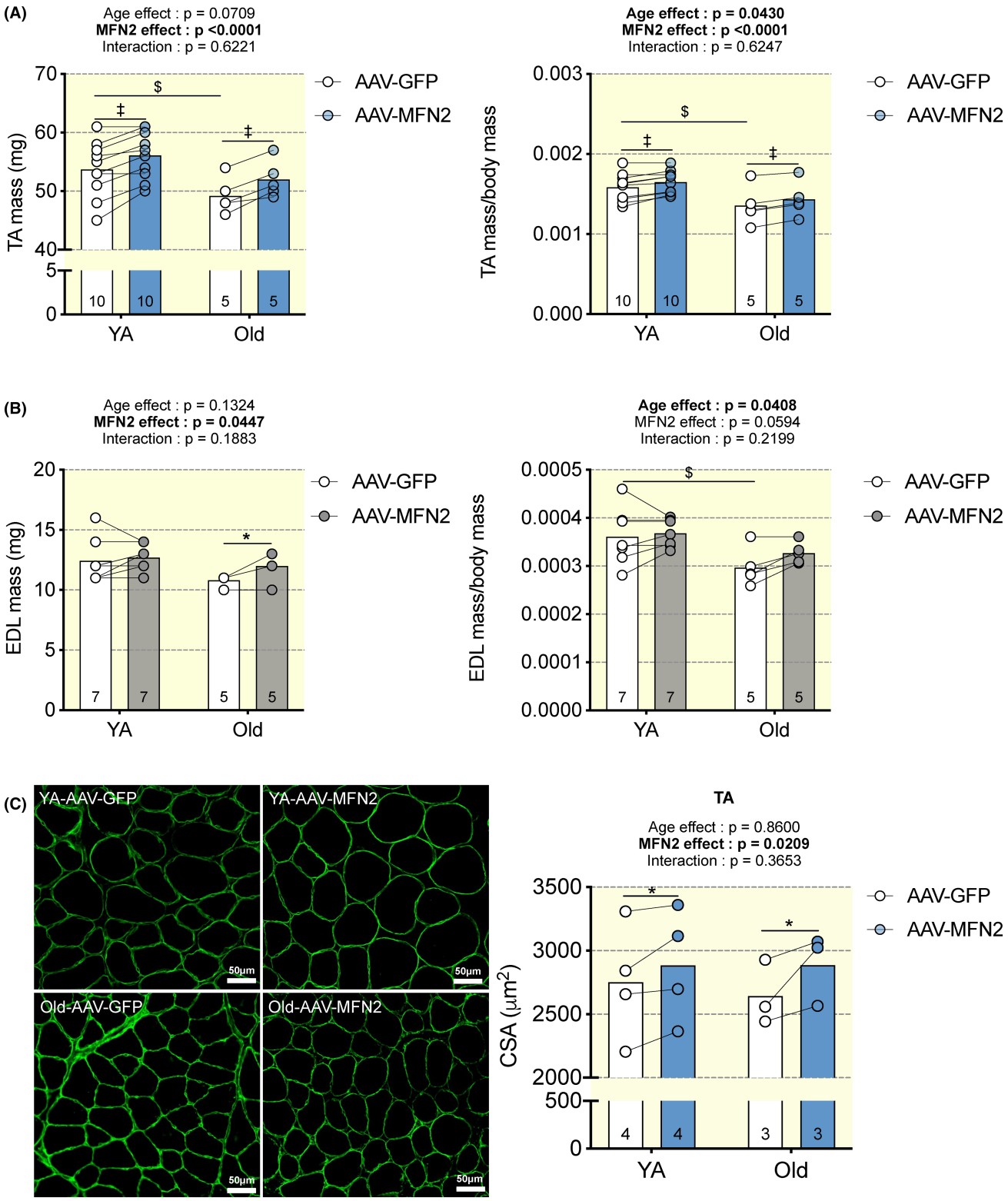


FIGURE 2 The impact of MFN2 overexpression on skeletal muscle mass and fiber size. Impact of MFN2 overexpression on muscle mass and relative to body mass for the TA (A) and EDL (B) from YA and Old mice (TA: $n = 10$ for YA, $n = 5$ for Old; EDL: $n = 7$ for YA, $n = 5$ for Old). (C) Representative dystrophin immunolabeling from cross-sections of TA muscles injected with AAV-GFP or AAV-MFN2 of YA and old groups and quantification of the impact of MFN2 overexpression and aging on the average cross section of TA (CSA, µm^2) ($n = 4$ for YA, $n = 3$ for Old). p-values retrieved from two-way ANOVA are displayed above each bar graph. $^{*}q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

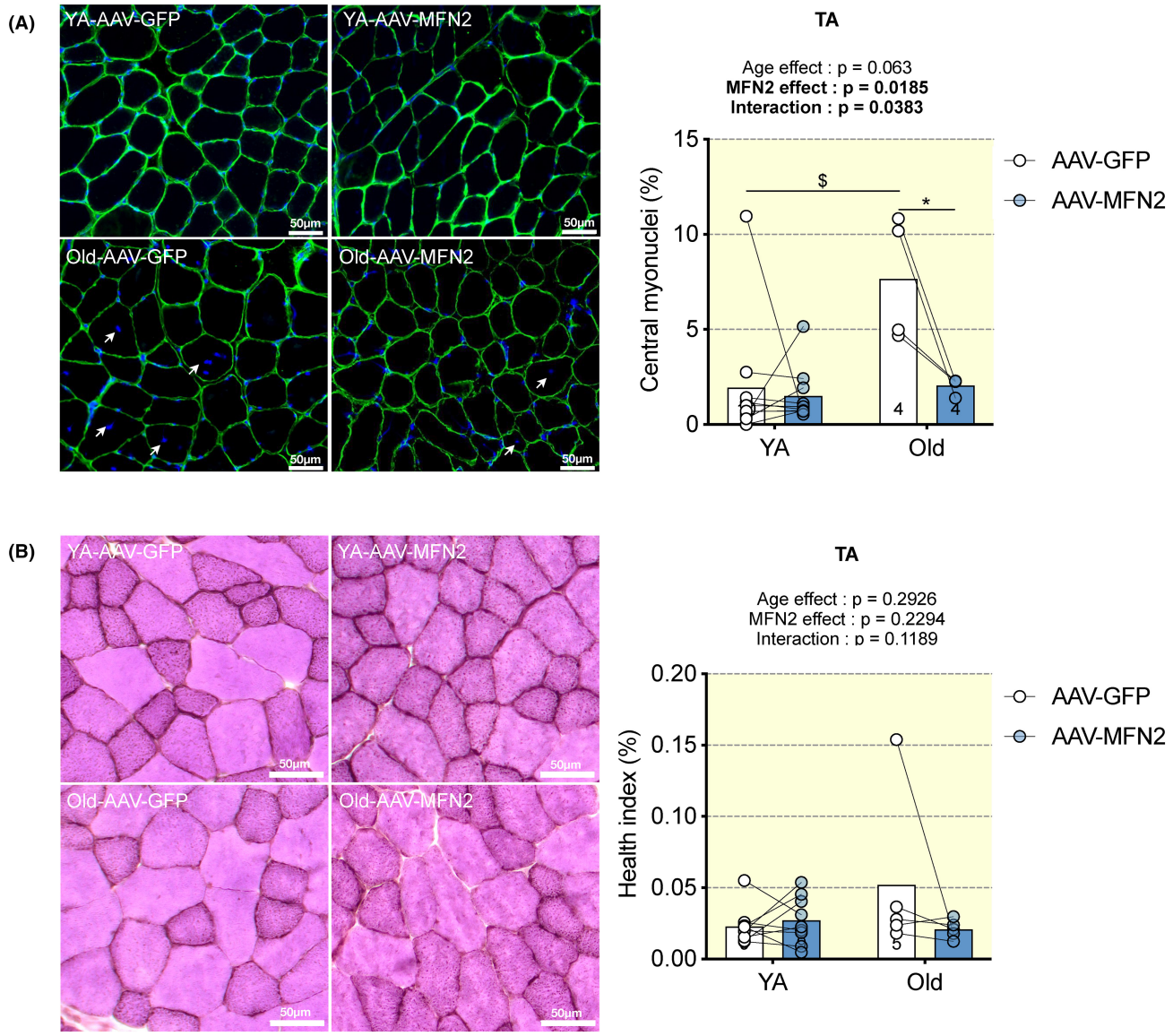


FIGURE 3 MFN2 overexpression alleviates central myonuclei proportion in aged muscles. (A) Representative dystrophin immunolabeling and DAPI staining from cross-sections of TA muscles injected either with AAV-GFP or AAV-MFN2 of YA and Old groups (scale bar: 50 µm; white arrows show fibers with centrally located nuclei) and quantification of the impact of MFN2 overexpression and aging on the proportion of fibers displaying central myonuclei ($n = 10$ for YA, $n = 4$ for Old). (B) Representative Hematoxylin and Eosin staining from cross-sections of TA muscles injected either with AAV-GFP or AAV-MFN2 of the YA and Old groups (scale bar: 50 µm) and % of health index including necrosis, fibrosis, and inflammation features ($n = 9$ for YA, $n = 5$ for Old). *p*-values retrieved from a two-way ANOVA are displayed above each bar graph. *$q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

To assess the impact of aging and MFN2 overexpression on mitochondrial content, several markers were quantified by western blots, including citrate synthase (CS, enzyme located in the mitochondrial matrix), the translocase of the outer mitochondrial membrane 20 (TOMM20, marker of the outer mitochondrial membrane), the voltage-dependent anion channel 1 (VDAC1, marker of the outer mitochondrial membrane), and the representative subunits of the oxidative phosphorylation (OXPHOS; markers of the inner mitochondrial membrane), in EDL (Figure 6A–E) and TA (Figure 6F–J) of YA and Old mice. While VDAC1 protein content was unaffected by aging, CS and TOMM20 contents were significantly lower in EDL of old mice. While MFN2 overexpression in the EDL did not impact CS protein content, it resulted in a significant reduction in VDAC1 and TOMM20 contents in old mice. Old mice displayed higher overall OXPHOS content (main effect) versus young mice (Figure 6E). While MFN2 overexpression had no impact in muscles of young mice, it resulted in an overall decrease (main effect) in the overall OXPHOS content in old mice (Figure 6E). In the TA, no impact of aging or MFN2 overexpression could be

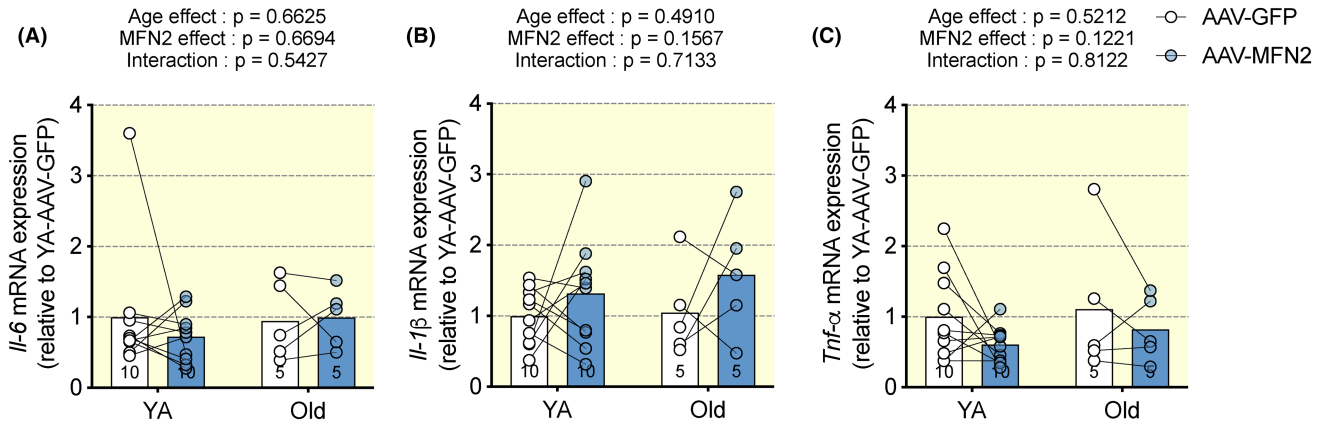


FIGURE 4 MFN2 overexpression does not alter inflammatory markers in the skeletal muscle of young and old mice. Quantification of (A) *Il-6*, (B) *Il-1ß*, and (C) *Tnf-α* mRNA expression levels in TA muscles injected either with AAV-GFP or AAV-MFN2 in YA and Old mice. Values are normalized to AAV-GFP values from YA ($n = 10$ for YA, $n = 5$ for Old). *p*-values retrieved from a two-way ANOVA are displayed above each bar graph. $^{*}q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

evidence for all markers of mitochondrial content, that is, CS, VDAC1, TOMM20, and OXPHOS proteins remained unchanged by aging and specific MFN2 overexpression in all groups (Figure 6F–J). These data suggest that MFN2 overexpression may have muscle- and age-specific impacts on mitochondrial content.

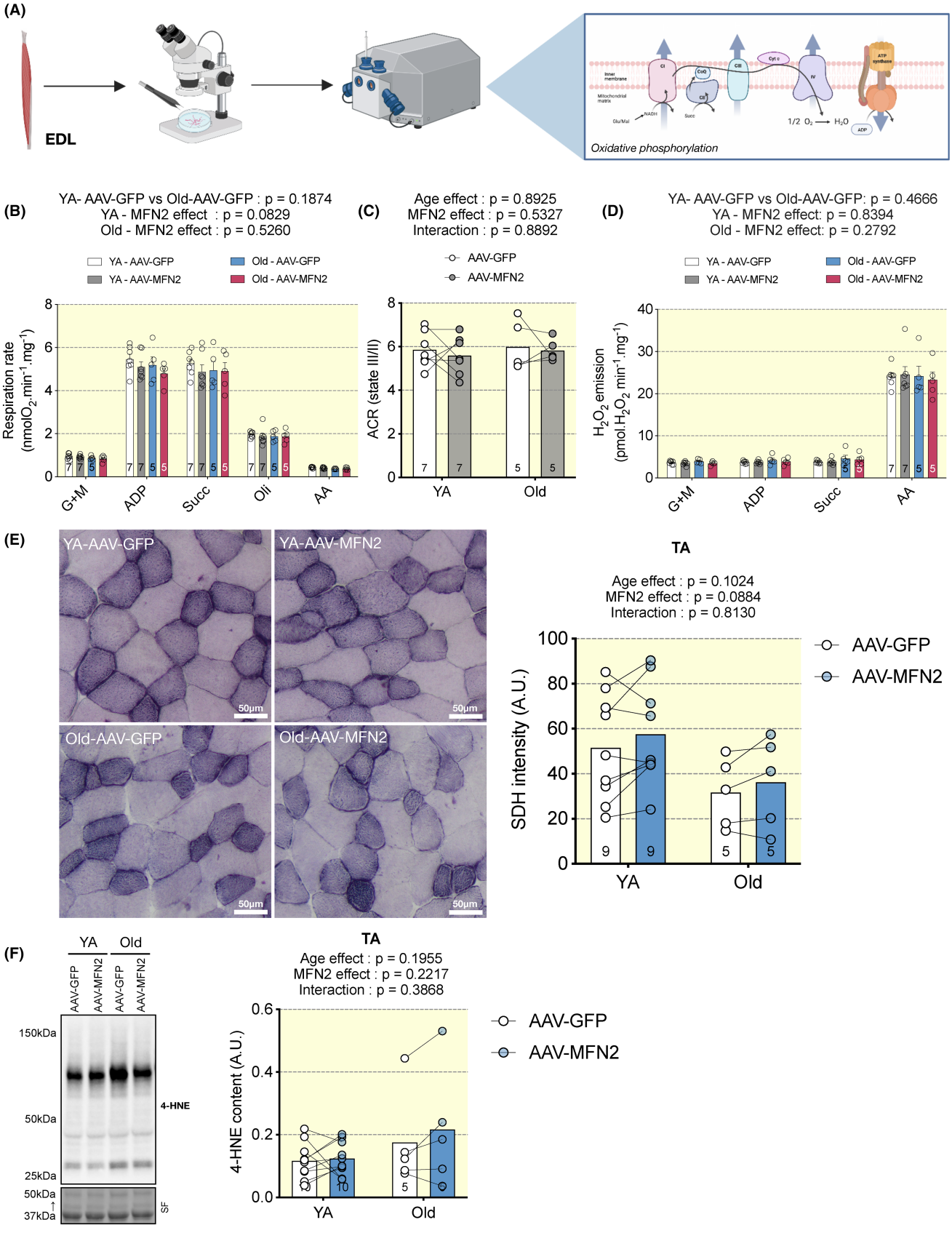
Since multiple markers of mitochondrial content were assessed in the present study, we created a composite index of mitochondrial content to normalized respiration and H_2O_2 emission data to assess whether aging or MFN2 overexpression would alter mitochondrial quality. As shown in Figure 7, no impact of aging on mitochondrial respiration (Figure 7A) and H_2O_2 emission (Figure 7B) normalized to mitochondrial content could be evidence. No significant impact of MFN2 overexpression on mitochondrial respiration and H_2O_2 emission was observed in young mice (Figure 7A,B). However, MFN2 overexpression resulted in a significant increase in mitochondrial respiration (Figure 7A) normalized to mitochondrial content in old mice without altering H_2O_2 emission (Figure 7B), suggesting that MFN2 overexpression might have improved mitochondrial quality in old mice.

2.4 | The impacts of aging and MFN2 overexpression on known regulators of muscle mass

With the aim of gaining insights into the mechanisms underlying the hypertrophic effects of MFN2 overexpression, we assessed the expression and content of various known regulators of muscle mass. It was recently reported that mitochondrial calcium entry through the mitochondrial calcium uniporter (MCU) could regulate skeletal muscle mass, in part by upregulating the peroxisome proliferator-activated receptor gamma coactivator 1-alpha isoform 4 (PGC-1α 4).[36–38] Considering the role played by MFN2 in tethering mitochondria to the endoplasmic reticulum (ER),[39] we reasoned that MFN2 overexpression might have exerted its hypertrophic impact through the modulation of mitochondrial calcium entry and upregulation of PGC-1α4. To indirectly assess whether MFN2 overexpression impacted mitochondrial calcium entry, we quantified the MCU in the TA of young and old mice. As shown in Figure 8A, we found a trend for an increase in MCU content in old skeletal muscles injected with AAV-GFP versus young muscles injected with AAV-GFP (Figure 8A). MFN2 overexpression had no impact of the MCU content in both young and old mice (Figure 8A). Aging and MFN2 overexpression had no impact on *PGC-1α4* mRNA levels in both young and old mice (Figure 8B). These results indicate that the MCU-PGC-1α4 pathway is unlikely to explain the mild hypertrophy caused by MFN2 overexpression. We next investigated whether MFN2 overexpression resulted in an activation of the mammalian target of rapamycin complex 1 (mTORC1) pathway, one of the best characterized pathways in the control of protein synthesis and muscle mass.[38] To this end, we quantified the total and phosphorylated content of S6, a downstream target of mTORC1. The contents of total and phosphorylated S6 (pS6) protein and the pS6 to total S6 ratio were unaffected by aging or MFN2 overexpression in the skeletal muscles of young and old mice (Figure 8C).

2.5 | The impact of aging and MFN2 overexpression on markers of ER-stress

Because MFN2 plays a critical role during ER stress,[40] and because ER stress and altered mitochondria-endoplasmic



reticulum contacts have been proposed as potential contributors to cellular aging,[41] we investigated the impacts of muscle aging and MFN2 overexpression on markers of ER stress, including activating transcription factor 4 (ATF4), ATF6, C/EBP homologous protein (CHOP), and protein disulfide isomerase (PDI). Aging did not impact

FIGURE 5 MFN2 overexpression does not alter mitochondrial respiration and H_2O_2 emission in the skeletal muscle of young and old mice. (A) Schematic representation of the experimental design used to assess mitochondrial respiration and H_2O_2 emission. Fibers from EDL muscles injected with AAV-GFP or AAV-MFN2 were separated and permeabilized before measuring mitochondrial respiration and H_2O_2 emission using an Oroboros O2K high resolution fluorespirometer. (B) Quantification of mitochondrial respiration using different substrates and inhibitors (AA, antimycin A; ADP, adenosine diphosphate; G + M, Glutamate + Malate; Oli, oligomycin; Succ, succinate). (C) ACR: acceptor control ratio (state III/II). (D). Quantification of H_2O_2 emission, a ROS production indicator, using the sequential addition of substrates and inhibitors described above ($n = 7$ for YA, $n = 5$ for Old). (E) Representative images of succinate dehydrogenase activity (SDH) of TA muscle cross sections and SDH intensity quantification (scale bar: 50 μm, $n = 9$ for YA, $n = 5$ for Old). (F) Representative immunoblots and quantifications of 4-Hydroxynonenal (4-HNE) content in TA muscles injected either with AAV-GFP or AAV-MFN2 in YA ($n = 10$) and Old ($n = 5$) mice. *p*-values retrieved from a two-way ANOVA are displayed above each bar graph. $^{*}q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

the mRNA levels or protein contents of Atf6, Atf4, and Chop (Figure 9A–E). Only the protein content of PDI was increased in old skeletal muscles (Figure 9F). A trend for an increase in *Atf6* mRNA expression upon MFN2 overexpression (MFN2 overexpression main effect) was observed (Figure 9A). An overall decrease in *Atf4* and *Chop* mRNA levels upon MFN2 overexpression (main effect) was observed (Figure 9B,C). Post hoc testing revealed that *Chop* mRNA levels significantly decreased in both young and old muscles upon MFN2 overexpression (Figure 9C). At the protein level, a trend for a decrease in CHOP content upon MFN2 overexpression (MFN2 overexpression main effect) was observed (Figure 9D,E). PDI protein content was significantly increased in aged skeletal muscle (Figure 9F). No impact of MFN2 overexpression on PDI content was observed (Figure 9F). While the increase in PDI content in aged skeletal muscles might suggest the presence of ER stress, the overall impact of aging on markers of ER stress was minimal. Although our data suggest that MFN2 overexpression might modulate the ER stress, the absence of a significant impact of MFN2 overexpression on CHOP and PDI protein contents suggests that the hypertrophic effect of MFN2 overexpression in aged skeletal muscles was not consequential to an attenuation of ER stress.

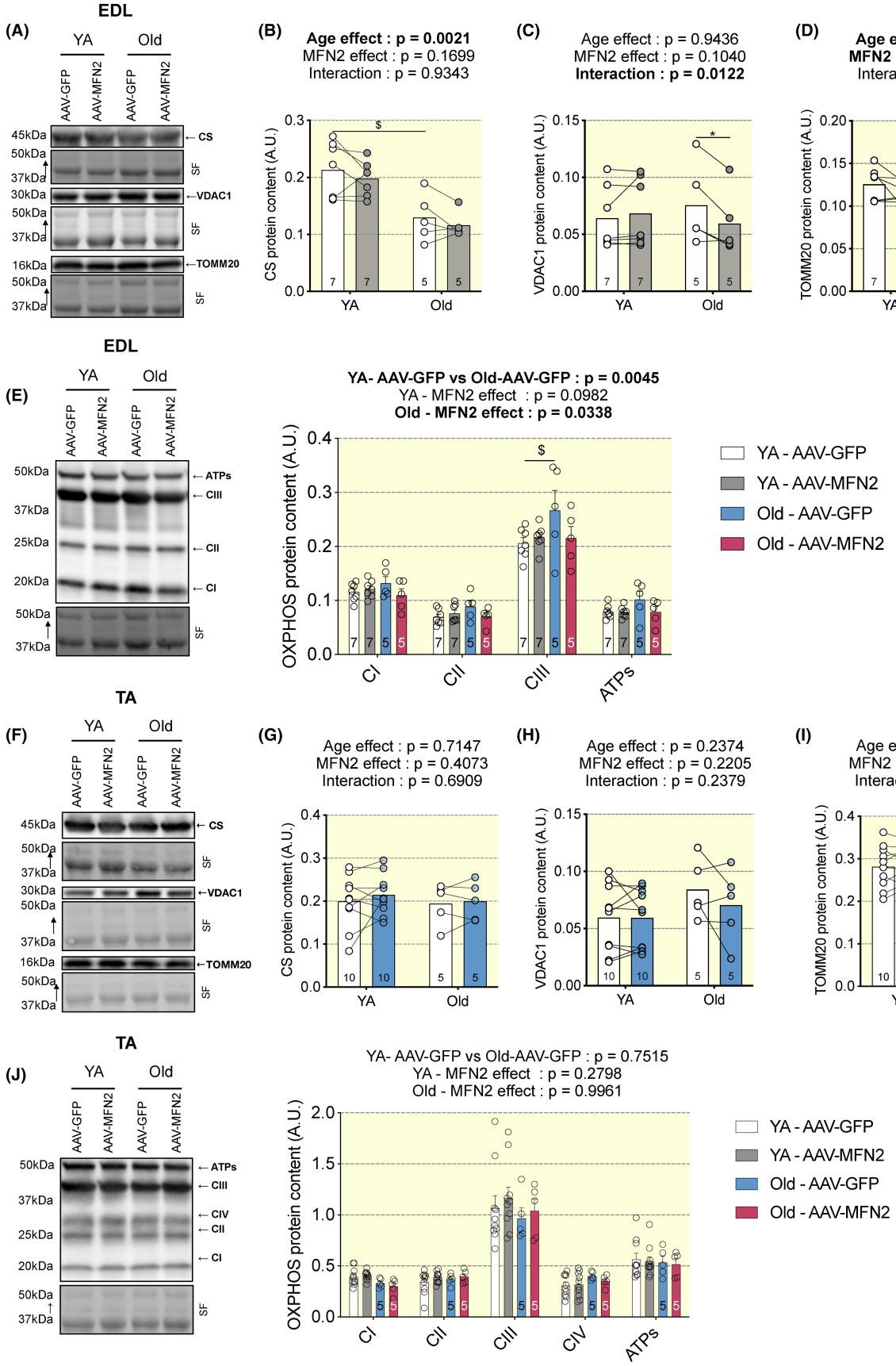
2.6 | The impacts of aging and MFN2 overexpression on markers of autophagy and mitophagy

Because MFN2 has been implicated in the regulation of autophagy, and because altered autophagy is believed to play an important role in the muscle aging process,[27,35,42,43] we assessed whether MFN2 overexpression could impact autophagy and mitophagy markers in the skeletal muscles of young and old mice. To this end, the content of p62 and of the lipidated (LC3-II) and non-lipidated (LC3-I) forms of LC3, key proteins involved in autophagy (Figure 10A–E), were assessed. Old skeletal muscle displayed an increase in the content of p62 and LC3-I (Figure 10A–C). A trend for an increase in the LC3-I content in aged skeletal muscle was observed (main effect of aging; Figure 10C). No impact of aging on the LC3-II protein content was observed (Figure 10D). MFN2 overexpression significantly reduced p62 content in old skeletal muscles (Figure 9B). No impact of MFN2 overexpression on LC3-I, LC3-II, and the LC3-II/I ratio was observed (Figure 10E). To assess the impact of aging and MFN2 overexpression on mitophagy markers, we quantified *Park2* mRNA expression and BNIP3 protein content (Figure 10F,G). Neither aging nor MFN2 overexpression altered *Park2* mRNA levels (Figure 10F). BNIP3 content was significantly increased in aged skeletal muscle (Figure 10G). MFN2 overexpression had no impact on BNIP3 content (Figure 10F,G).

Overall, our data suggest that aging is associated with an accumulation of autophagy and mitophagy markers, potentially due to impaired autophagy/mitophagy. The decrease in p62 content upon MFN2 overexpression in aged skeletal muscles suggests that MFN2 might partly rescue the aging-related impairments in autophagy.

3 | DISCUSSION

Multiple processes involved in the regulation of mitochondrial dynamics have emerged in recent years as critical to the maintenance of skeletal muscle mass, integrity, and function. Silencing or overexpressing the mitochondrial fission protein DRP1 was found to be detrimental to skeletal muscle health.[29–31,44] Conversely, deletion of proteins regulating mitochondrial fusion, such as OPA1 or MFN1/MFN2 induced sarcopenic phenotypes characterized by muscle atrophy and mitochondrial defects.[27,28] However, whether upregulating proteins involved in mitochondrial fusion could positively impact muscle health and counter the sarcopenic process remained unknown. With the aim of filling this gap in knowledge, we tested the impact of MFN2 overexpression in skeletal muscle of young and old mice. We show that AAV-mediated overexpression of MFN2 results in a mild hypertrophy in both young and old skeletal muscles. No signs of fibrosis, necrosis, and inflammation were found upon MFN2 overexpression, indicating that the hypertrophy triggered by MFN2



overexpression was not pathological. MFN2 overexpression in old skeletal muscles even decrease the proportion of fibers with central nuclei. These findings provide the proof of concept that raising the expression of MFN2 late in life, either through pharmaceutical or nonpharmaceutical means, could represent an effective strategy

FIGURE 6 Mitochondrial content in EDL and TA muscles is differentially impacted by MFN2 overexpression. The first part of the panel corresponds to EDL muscle data. (A) Representative immunoblots and quantifications of citrate synthase (CS), VDAC1, and TOMM20 proteins in EDL muscles injected either with AAV-GFP or AAV-MFN2. Quantifications of CS (B), VDAC1 (C), and TOMM20 (D) protein content. (E) Representative immunoblots of OXPHOS protein (complexes I, II, III, IV, and ATP synthase) and protein quantifications for YA and Old groups in EDL muscles injected either with AAV-GFP or AAV-MFN2 ($n = 7$ for YA, $n = 5$ for Old). The second part of the panel corresponds to TA muscle data. (F) Representative immunoblots and quantifications of CS (G), VDAC1 (H), and TOMM20 (I) proteins in TA muscles injected either with AAV-GFP or AAV-MFN2. (J) Representative immunoblots of OXPHOS protein (complex I, II, III, IV, ATP synthase) and protein quantifications for YA and Old groups in TA muscles injected either with AAV-GFP or AAV-MFN2 ($n = 10$ for YA, $n = 5$ for Old). p-values retrieved from a two-way ANOVA are displayed above each bar graph. *$q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

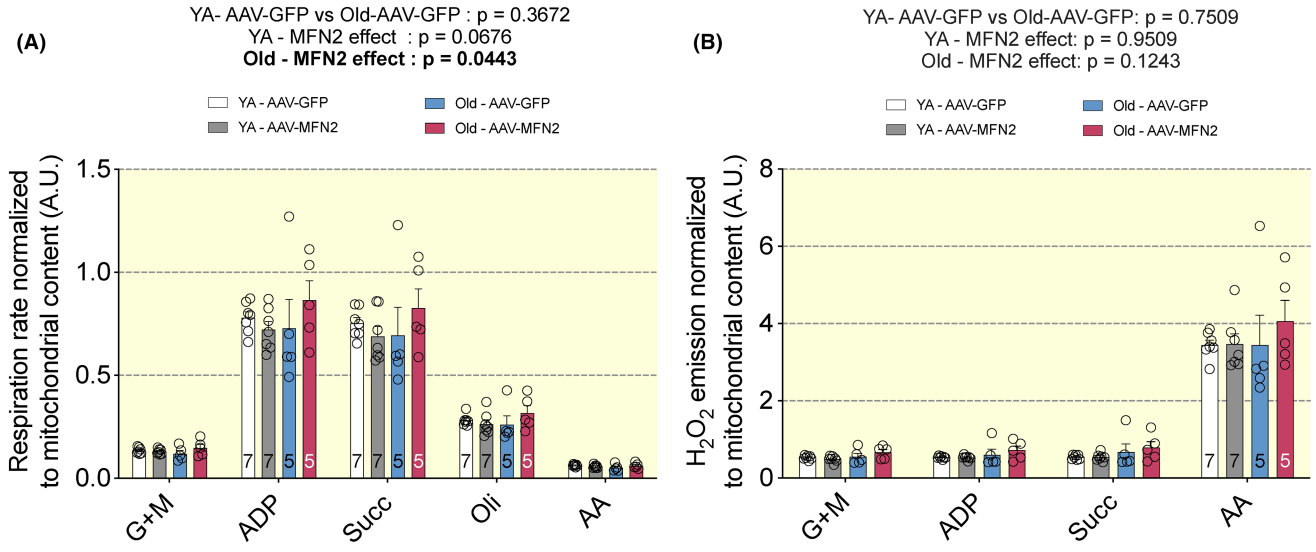


FIGURE 7 The impact of aging and MFN2 overexpression on mitochondrial respiration and H_2O_2 emission normalized to mitochondrial content. (A) Mitochondrial respiration rate and (B) H_2O_2 emission normalized to a composite index of mitochondrial content of YA and old EDL muscles injected either with AAV-GFP or AAV-MFN2. p-values retrieved from a two-way ANOVA are displayed above each bar graph. *$q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

in mitigating the aging-related loss of muscle mass. It is noteworthy to highlight that the increase in MFN2 content seen 4 months after AAV injections falls within the range of what is achievable through high intensity interval training in older adults.[45] The hypertrophic effect of MFN2 overexpression that we report in the present study in young and old mice, associated with the protective impact of MFN2 overexpression on skeletal muscle mass in a mouse model of cancer cachexia[34] position MFN2 as a potential therapeutic target to mitigate muscle atrophy and weakness seen under multiple catabolic conditions. Whether our findings translate to female mice and ultimately to humans will require further study.

Because MFN2 has been proposed to regulate the levels of proteins involved in oxidative phosphorylation[33] and because MFN2 overexpression was shown in cultured cells to improve mitochondrial bioenergetics,[32] we reasoned that MFN2 overexpression in skeletal muscle would increase mitochondrial respiration and markers of mitochondrial content. In striking contrast with our initial hypotheses, we report that 4 months of MFN2 overexpression did not alter mitochondrial respiration in the EDL muscles and SDH activity in the TA muscles in both young and old mice. Similarly, MFN2 overexpression had no impact on mitochondrial H_2O_2 emission in permeabilized myofibers. These findings are in line with a previous study by Lally et al. which showed that 10 weeks of muscle specific MFN2 overexpression in young rats did not alter mitochondrial respiration and H_2O_2 emission rates in permeabilized fibers.[46] Consistent with an absence of impact on the activity of SDH, MFN2 overexpression had no impact on multiple markers of mitochondrial content in the TA. Similarly, no impact of MFN2 overexpression was found in the EDL of young mice. However, several markers of mitochondrial content, namely VDAC1, TOMM20, and representative OXPHOS subunits, were decreased upon MFN2 overexpression in old skeletal EDL muscles. This unexpected impact of MFN2 overexpression on markers of mitochondrial content in the EDL, associated with an absence of effect on mitochondrial

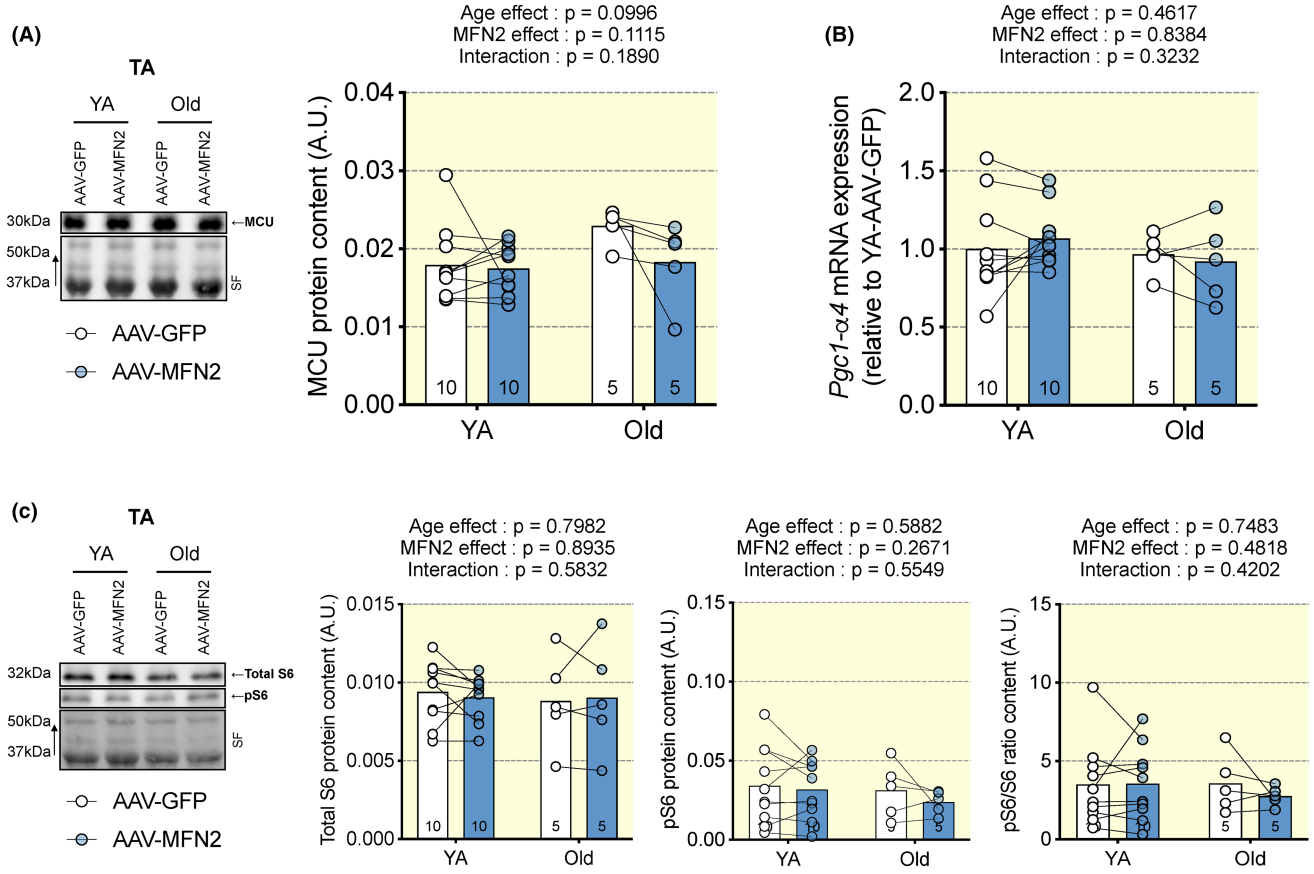


FIGURE 8 The impacts of aging and MFN2 overexpression on known regulators of muscle mass. (A) Representative immunoblots of mitochondrial calcium uniport (MCU) content and corresponding MCU quantifications in TA muscles injected either with AAV-GFP or AAV MFN2 from YA and Old mice. (B) Quantification of peroxisome proliferator-activated receptor gamma coactivator 1-alpha isoform (PGC-1α4) mRNA expression in TA muscles injected either with AAV-GFP or AAV-MFN2 from YA and Old mice. (C) Representative immunoblots of total S6 and phosphorylated S6 (pS6) proteins and corresponding quantifications of total S6, pS6 protein contents and pS6/totalS6 ratio, in TA muscles injected either with AAV-GFP or AAV-MFN2 from YA and Old mice. In all graphs, $n = 10$ for YA and $n = 5$ for Old. *p*-values retrieved from two-way ANOVA are displayed above each bar graph. $^{*}q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

respiration assessed in this muscle, suggest that MFN2 overexpression might have improved the quality of mitochondria in old EDL muscles, perhaps by enhancing the recycling or degradation of damaged/impaired mitochondria. Providing support for this view, the mitochondrial respiration rate normalized to our composite score of mitochondrial content was increased upon MFN2 overexpression in old mice. The differential impact of MFN2 overexpression in the TA and EDL muscles on markers of mitochondrial content remains unexplained and would require further investigation. It is worth noting that we did not observe any impact of aging on mitochondrial respiration and H_2O_2 emission. While several studies have reported a decline in mitochondrial respiration with muscle aging,[10,13,14,19,20] there also exist a non-negligible number of studies that found negligible to no impact of aging on mitochondrial respiration or content, both in humans and in rodents.[8,9,16,47–51] Our manuscript therefore adds to the literature showing that muscle atrophy can develop with aging in the absence of changes in mitochondrial respiration. Similarly, and in line with our findings, an absence of effect of aging on mitochondrial H_2O_2 emission was also reported in several studies.[16,47–49,52] In contrast to previous reports,[27–29] we also did not detect a decrease in the expression or content of mitochondrial fusion proteins with aging. We even noted a trend for an increase in MFN2 content in old EDL muscles and a main effect indicative of lower DRP1 expression with aging in the EDL without changes in the expression of MFN1 and OPA1, findings that are consistent with previously published data in glycolytic skeletal muscles.[53]

With the aim of uncovering the mechanisms underlying the hypertrophic effect of MFN2 overexpression, several pathways known to regulate muscle mass were investigated. Because of the role played by MFN2 in tethering mitochondria to the endoplasmic reticulum,[39] we

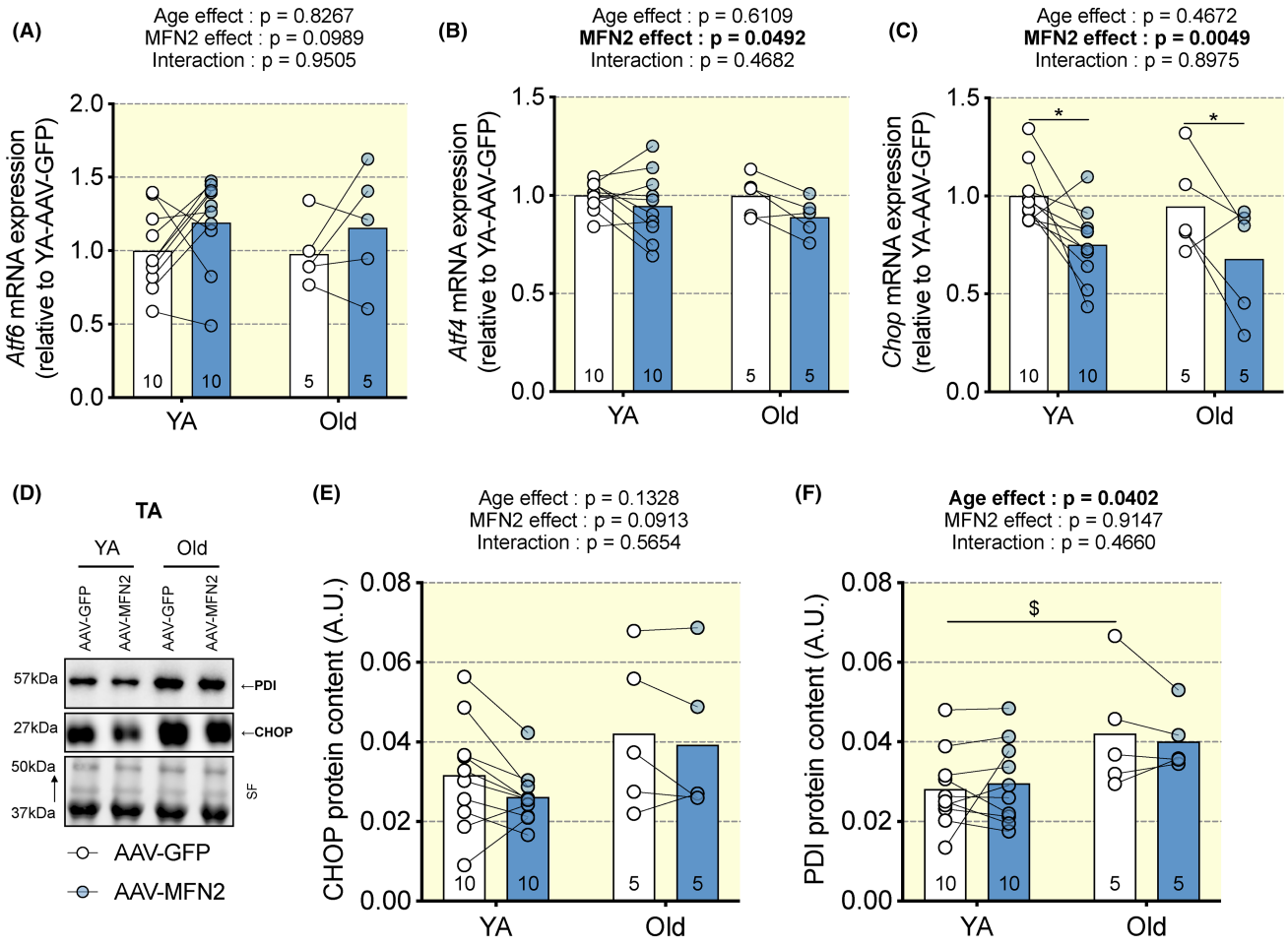


FIGURE 9 The impacts of aging and MFN2 overexpression on markers of ER-stress. (A) Quantification of *Atf4*, (B) *Atf6*, and (C) *Chop* mRNAs fold changes from TA muscles injected either with AAV-GFP or AAV-MFN2 in YA and Old mice. Values are normalized to AAV-GFP values from YA. (D) Representative immunoblots of PDI and CHOP proteins and PDI (E), CHOP (F) quantifications ($n = 10$ for YA, $n = 5$ for Old). *p*-values retrieved from a two-way ANOVA are displayed above each bar graph. $^{*}q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

first explored whether MFN2 overexpression could have exerted its impact through the MCU-PGC-1α4 pathway, a pathway known to positively regulate muscle mass when activated.[36–38] While a trend for an increase in MCU with muscle aging was found, no impact of MFN2 overexpression on the MCU protein content or PGC-1α4 mRNA levels was observed, indicating that the MCU-PGC-1α4 pathway was not upregulated after 4 months of MFN2 overexpression. Similarly, the phosphorylation status of S6 was unaffected by aging or MFN2 overexpression, indicating that the mTORC1 signaling pathway was not activated after 4 months of MFN2 overexpression. While the data suggest that the MCU-PGC-1α4 and mTORC1 pathways were not involved in the hypertrophic impact of MFN2 overexpression remains, their potential contribution cannot be completely ruled out. One important limitation of our study design is the lack of time course data on the impact of MFN2 overexpression. It is therefore possible that the mechanisms underlying the mild hypertrophy triggered by MFN2 overexpression might no longer be detectable after 4 months of overexpression. Investigating the impact of MFN2 overexpression on a larger number of signaling pathways regulating muscle mass at earlier time points would be required to establish the molecular events leading to hypertrophy. It would also be interesting to assess whether MFN2 overexpression still has positive impacts if initiated at a later time point when muscle atrophy and weakness become more severe.[54]

It was recently proposed that ER stress might contribute to the progression of sarcopenia.[55] Because MFN2 is involved in the regulation of ER stress,[40,56,57] we investigated whether MFN2 overexpression would attenuate the impact of aging on markers of ER stress. While the expression or content of ATF4, ATF6, and CHOP were similar in young and old mice, the content of PDI, an ER chaperone upregulated during ER stress, was significantly increased in old skeletal muscles. This result suggests that mild ER stress might develop during muscle

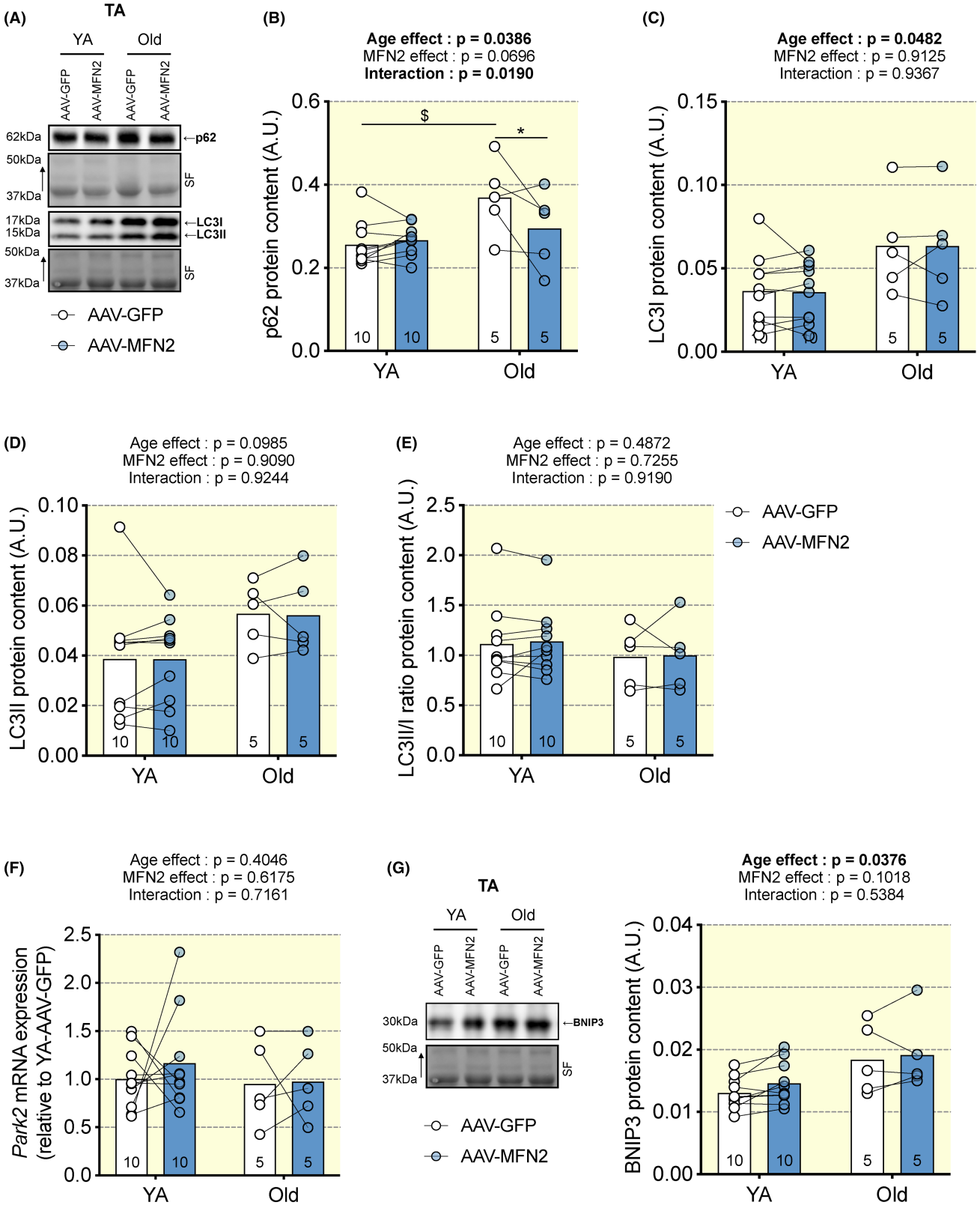


FIGURE 10 The impacts of aging and MFN2 overexpression on markers of autophagy and mitophagy. (A) Representative immunoblots of p62 and LC3 lipidation (LC3I and LC3II) in TA muscles injected either with AAV-GFP or AAV-MFN2 from YA and Old mice. (B) Quantifications of p62, (C) LC3I, (D) LC3II and (E) LC3II/LC3I ratio protein content in TA muscles injected either with AAV-GFP or AAV-MFN2 from YA and Old mice. (F) Quantification of *Park2* mRNAs fold changes from TA muscles injected either with AAV-GFP or AAV-MFN2 in YA and Old mice. (G) Representative immunoblots and quantification of BNIP3 protein content in TA muscles injected either with AAV-GFP or AAV-MFN2 from YA and Old mice ($n = 10$ for YA, $n = 5$ for Old). *p*-values retrieved from a two-way ANOVA are displayed above each bar graph. $^{*}q < 0.05$, $^{\dagger}q < 0.01$, $^{\ddagger}q < 0.001$, and $^{\$}p < 0.05$: statistically significant.

aging. The lack of impact of MFN2 overexpression on CHOP and PDI protein contents suggests that its hypertrophic effect in aged skeletal muscles was unlikely the result of an attenuation of ER stress. A note-worthy finding of the present study is the downregulation of ATF4 upon MFN2 overexpression. ATF4 has been shown to promote muscle atrophy during fasting and has been proposed as a contributor to muscle atrophy and weakness with aging.[58,59] While 6 mo ATF4 KO mice displayed comparable muscle mass and strength versus their wild-type aged-matched littermates, 22 mo ATF4 KO mice were protected from aging-related muscle atrophy and weakness.[59] It is therefore tempting to speculate that the higher muscle mass and fiber size seen in old mice upon MFN2 overexpression might have been caused, at least in part, by a downregulation of ATF4.

Because MFN2 has been implicated in the regulation of autophagy,[60,61] and because altered autophagy is believed to play an important role in the muscle aging process,[27,35,42,43] we investigated whether MFN2 overexpression would attenuate the impact of muscle aging on markers of autophagy. Consistent with previous studies,[27,62] we found that muscle aging was associated with an accumulation of autophagic proteins. Although MFN2 overexpression did not alter the content of LC3I and LC3II, it normalized the content of p62 in old skeletal muscles. This finding suggests that MFN2 overexpression increased autophagy in old skeletal muscles. Such an impact on autophagy might in part explain the similar respiration found in the EDL of old mice despite lower markers of mitochondrial content. However, it should be noted here that we cannot definitively concluded on the impact of MFN2 overexpression on autophagy due to our study design. The proper testing of such a hypothesis would require the assessment of the autophagic flux using an autophagy inhibitor such as bafilomycin-A1, colchicine, leupeptin, or chloroquine.[63] A last noteworthy limitation of our study relates to the fact that we could not assess the impact of aging and MFN2 overexpression on muscle fiber size and autophagic and mitophagic markers in the EDL muscles due to the limited amount of tissue available.

4 | MATERIALS AND METHODS

4.1 | Animal procedures and AAV injections

Experiments were conducted on 3- and 20-month-old ($n=10$/group) male C57BL/6J mice, purchased from Jackson Laboratories. Two to three mice were housed per cage under a 12:12 h light/dark photocycle at 24 ± 1°C and 50%–60% relative humidity, with access to a standard chow diet and water available ad libitum. To assess the impact of MFN2 overexpression in skeletal muscle, AAVs purchased from Vector BioLabs (Malvern, PA, USA) were used. AAVs were of Serotype 1, a serotype with a proven tropism for skeletal muscle cells.[35] To overexpress MFN2, an AAV containing a muscle specific promoter (tMCK; triple tandem of MCK enhancer ligated to the basal promoter of the muscle creatine kinase,[64] a sequence coding for the expression of the *Mfn2* gene, a sequence coding for the auto-cleavable 2A peptide and a sequence coding for the enhanced Green Fluorescent Protein (GFP) the was used (30 µL per site; 1.5×10^{11} gc). This AAV is referred to hereafter as AAV-MFN2. A control AAV containing a sequence coding for the expression of the enhanced GFP under the control of the tMCK promoter was injected in the contralateral leg. This AAV is hereafter referred to as AAV-GFP. Details on AAV construction are available in Figure 1A. After a 7-day acclimatization period, intra-muscular injections were performed under general anesthesia using ~2% isoflurane into the right tibialis anterior (TA) and extensor digitorum longus (EDL) muscles of young adult (YA) and old mice. Using similar experimental procedures, we have previously shown that such intra-muscular injections of serotype 1 AAVs result in the transduction of virtually all TA and EDL fibers.[30,35,65] Four months after the AAV injections, mice were anesthetized with isoflurane and subsequently euthanized with CO_2. The TA and EDL from both legs were carefully removed and weighed. The EDLs were then directly used for the assessment of mitochondrial respiration and H_2O_2 emission. The TAs were divided into three parts; first two were frozen in liquid nitrogen and stored (−80°C) for RT-qPCR and immunoblotting. The last part of the TA was prepared for histology, as described below. The present study was carried out in strict accordance with standards established by the Canadian Council of Animal Care and the guidelines and policies of UQAM. All procedures were approved by the animal ethics committees of UQAM (CIPA#2022-370).

4.2 | Animal number consideration

At the beginning of the protocol, 10 animals per group received AAV injections. However, 4 mice in the old group died or were euthanized due to their critical clinical state before the end of the 4 months of MFN2 overexpression. Among the remaining 6 mice in the old group, 1 was not successfully transduced by the AAVs overexpressing GFP and MFN2, likely due to a technical error. As such, this old mouse was excluded from data analyses. The final maximal sample sizes for young and old animals were therefore $n=10$ and $n=5$, respectively.

4.3 | Skeletal muscle sample sectioning for histological analyses

TA muscle samples were mounted in tragacanth (Sigma, G1128), frozen in cooled liquid isopentane, and stored at −80°C. Ten-micron-thick serial cross-sections were cut in a cryostat at −20°C and mounted on lysine-coated slides (Superfrost).

4.4 | Fiber size determination and central myonuclei

Briefly, muscle cross-sections were first allowed to reach room temperature and fixed in an acetone solution for 15 min. Muscle sections were rehydrated with Phosphate Buffer solution (PBS, Sigma, 4417) and then permeabilized in PBS containing 0.1% of Triton X100 for 15 min. After 3 washing with PBS, muscle slices were blocked with PBS + 10% of goat serum (GS) for 30 min at room temperature and then incubated for 2 h at room temperature with the following primary antibody, dystrophin diluted in PBS containing 10% of GS (see Table 1 for details on the antibody). Muscle cross-sections were then washed 3 times with PBS before being incubated for 1 h with the secondary antibody Alexa Fluor 488 Ig2b goat anti-mouse (see Table 1 for details on antibody). Sections were then washed 3 times in PBS and cover slipped using Prolong Gold containing DAPI to label the nucleus (Thermo Fisher, P36931) as mounting medium. Slides were imaged using an Olympus IX83 Ultra Sonic fluorescence microscope (Olympus, Japan). Analysis of fiber size and percentage of central myonuclei were performed manually using ImageJ (NIH, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/). To assess muscle fiber cross-sectional area, a minimum of 200 myofibers per muscle were manually traced (Detailed number of fibers manually traced per group [Mean ± SD]: YA-AAV-GFP: 264 ± 48; YA-AAV-MFN2: 264 ± 80; Old-AAV-GFP: 247 ± 48; Old-AAV-MFN2: 256 ± 94).

4.5 | Hematoxylin and Eosin staining

The Hematoxylin and Eosin (H&E) staining was realized with the H&E kit according to the manufacturer recommendation (Abcam, ab245880). Briefly, muscle cross-sections were allowed to reach room temperature and completely covered with Hematoxylin Mayer's reagent for 5 min. Muscle sections were rinsed twice in distilled water and incubated for 15 s in Bluing reagent. After two rinses in distilled water, muscle sections were dipped

TABLE 1 List of antibodies used for immunohistochemistry (IHC) and immunoblotting (IB).

Antibody	Source/product no.	Use	Dilution
Rabbit anti-MFN2	Abcam, #Ab124773	IB	1/1000
Rabbit anti-MFN1	ProIntech, #13798-1-AP	IB	1/1000
Rabbit anti-DRP1	Abcam, #Ab184247	IB	1/1000
Mouse anti-OPA1	BDbiosciences, #BD612606	IB	1/1000
Mouse Anti-4HNE	Biotechne, #MAB3249	IB	1/1000
Rabbit anti-TOMM20	Abcam, #Ab186735	IB	1/1000
Rabbit anti-LC3A/B	Cell signaling, #2217	IB	1/1000
Rabbit anti-dystrophin	Sigma-Aldrich, #D8168	IHC	1/500
Rabbit anti-Citrate synthase	Abcam, #Ab96600	IB	1/1000
Mouse anti-p62	Abnova, #H00008878-M01	IB	1/1000
Mouse anti-BNIP3	Sigma-Aldrich #B7931	IB	1/1000
Mouse anti-CHOP	Cell signaling, #2895	IB	1/2000
Rabbit anti-PDI	Cell signaling, #3501	IB	1/1000
Rabbit anti-MCU	Cell signaling, #14997	IB	1/1000
Rabbit anti-Total S6	Cell signaling, #2217	IB	1/1000
Rabbit anti-p-S6 (Ser240/244)	Cell signaling, #2215	IB	1/1000
Mouse anti-OXPHOS	Abcam, #Ab110413	IB	1/1000
Mouse anti-VDAC1	Abcam, #Ab14734	IB	1/1000
Goat anti-mouse IgG	Abcam, #Ab6728	IB	1/10000
Goat anti-rabbit IgG	Abcam, #Ab6721	IB	1/10000
AF 488 IgG2b goat anti-mouse	ThermoFisher, #A-21141	IHC	1/500

in absolute alcohol and directly incubated in Eosin Y solution for 3 min. After three rinses in absolute alcohol baths, muscle sections were cover-slipped using an aqueous mounting medium (Vector Labs, VectaMount AQ Medium, H-5501). Slides were imaged using an Olympus IX83 Ultra Sonic fluorescence microscope (Olympus, Japan). A muscle health index was generated by manually quantifying the cumulative area of regions with signs of necrosis (fibers with abnormal color on H&E images or clearly infiltrated with macrophages), fibrosis (abnormal accumulation of connective tissue between muscle fibers visible on H&E images), and inflammation (infiltration of immune cells visible on H&E images) with image J software (NIH, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/).

4.6 | Succinate dehydrogenase staining

Sections were stained for succinate dehydrogenase (SDH, complex II of the respiratory chain) activity. Muscle cross-sections were first allowed to reach room temperature and then incubated in a solution containing nitroblue tetrazolium (1.5 mM), sodium succinate (130 mM), phenazine methosulphate (0.2 mM), and sodium azide (0.1 mM) for 10 min at room temperature. Cross sections were then washed 3 times (3 × 5min) in distilled water, and cover-slipped using an aqueous mounting medium (Vector Labs, VectaMount AQ Medium, H-5501). Slides were imaged using an Olympus IX83 Ultra Sonic fluorescence microscope (Olympus, Japan). Analysis was performed manually using ImageJ (NIH, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/).

4.7 | Preparation of permeabilized muscle fibers for in situ assessment of mitochondrial respiration and H_2O_2 emission

Mitochondrial function was assessed in freshly dissected EDL muscles. Once dissected, muscles were weighted with a precision scale and then rapidly immersed in ice-cold stabilizing buffer A (2.77 mM CaK_2 ethylene glycol-bis-(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA), 7.23 mM K_2 EGTA, 6.56 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 50 mM 2-(N-morpholino) ethanesulfonic acid potassium salt (K-MES), 20 mM imidazol, 20 mM taurine, 5.3 mM Na_2 ATP, and 15 mM phosphocreatine, pH 7.3). EDL muscles were separated into small fiber bundles using fine forceps under a surgical dissecting microscope (Leica S4 E, Germany). Muscle fiber bundles were incubated in a glass scintillation vial for 30 min at low rocking speed containing buffer A supplemented with 1.5 mg/mL saponin to selectively permeabilize the sarcolemma. Fiber bundles were then washed 3 times in 10 min at low rocking speed in the MiR05 buffer (110 mM sucrose, 20 mM HEPES, 10 mM KH_2PO_4, 20 mM taurine, 60 mM K-lactobionate, 3 mM $MgCl_2$, 0.5 mM EGTA, and 1 g/L of fatty acid free BSA, pH 7.4).

4.8 | Assessment of mitochondrial respiration

The assessment of mitochondrial respiration in permeabilized EDL myofibers was performed using an Oroboros O2K high-resolution fluororespirometer (Oroboros Instruments, Austria) at 37°C in 2 mL of buffer MiR05. Briefly, 3 to 6 mg (wet weight) of EDL permeabilized fiber bundles were weighed and added to the respiration chambers. The following substrates and inhibitors were added sequentially: 10 mM glutamate +5 mM malate (G + M), 2 mM ADP, 10 mM succinate, 1 μM oligomycin, and 10 μM antimycin A. Respiration rates were normalized as nanomoles of dioxygen per minute per mg of wet muscle mass. To assess the intrinsic respiration rates (respiration rates normalized to mitochondrial content), respiration rates were normalized with a composite index of mitochondrial content that was created by integrating markers of the mitochondrial matrix (CS), inner membrane (OXPHOS subunits), and outer membrane (VDAC1 and TOMM20). To calculate this composite index of mitochondrial content, data for each marker (i.e., CS, OXPHOS subunits, TOMM20, and VDAC1) were normalized to the mean of YA-AAV-GFP and added together for each sample. All respiration experiments were analyzed with MitoFun,[66] a homemade code to analyze mitochondrial function data in the Igor Pro 8 software (Wavemetrics, OR, USA).

4.9 | Mitochondrial H_2O_2 emission in permeabilized muscle fibers

The H_2O_2 emission from EDL myofiber bundles was assessed by monitoring the rate of H_2O_2 release using the Amplex Ultra Red-horseradish peroxidase (HRP) system. This was performed along with respiration assessment in the Oroboros O2K high-resolution fluororespirometer (Oroboros Instruments, Austria) at 37°C in 2 mL of buffer MiR05 supplemented with Amplex Ultra Red (10 μM), SOD (5 U/mL), and HRP (1 U/mL) at 37°C. A calibration curve was generated daily using successive additions of known [H_2O_2] in the absence of tissue. H_2O_2 emission was normalized as picomoles per minute per milligram of

wet muscle mass. H_2O_2 emission data were also normalized with the composite index of mitochondrial content detailed at the end of the subsection "Assessment of mitochondrial respiration." All H_2O_2 emission experiments were analyzed with MitoFun,[66] a homemade code to analyze mitochondrial function data in the Igor Pro 8 software (Wavemetrics, OR, USA).

4.10 | Quantitative real-time PCR

Quantitative real-time PCR experiments were performed on snap frozen TA samples. Total RNA was obtained after a Trisol/Chloroform extraction (Invitrogen-Lifetechnologies, 15596–018) followed by purification, binding, and elution on purification columns from Ambion PureLink™ RNA Mini Kit (Invitrogen-Lifetechnologies, 12183018A). The total RNA was reverse transcribed using a Superscript IV reverse transcriptase (Invitrogen-Lifetechnologies, 18090-050) following manufacturer conditions with Random primers (Invitrogen-Lifetechnologies, 48190-011), RNase out (Invitrogen-Lifetechnologies, 48190-019) and dNTP's (Invitrogen-Lifetechnologies, 18727-013). The qPCR was performed with the Power SYBR™ Green PCR Master Mix (Invitrogen-Lifetechnologies, 4368702) using an ABI Prism® 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following parameters: 95°C for 10 min, and 40 cycles of 95°C for 15 s, 57°C for 30 s, 72°C for 34 s. Real-time PCR experiments were performed in triplicate. Relative mRNA quantifications of the genes were determined normalizing data to the housekeeping gene *pgk1*. The primer sequences for all genes are listed in Table 2.

4.11 | Immunoblotting

The content of multiple proteins (listed in Table 2) was quantified in muscle homogenates prepared from TA (snap frozen sample) muscles and EDL (permeabilized myofibers recovered post mitochondrial function assessment). Approximately 15–30 mg for TA and 6–10 mg for EDL, of muscle tissue were homogenized in 10 volumes of an extraction buffer composed of tris base 50 mM, NaCl 150 mM, triton X-100 1%, sodium deoxycholate 0.5%, SDS 0.1%, and 10 μL/mL of a protease and phosphatase inhibitor cocktail (Thermo Fisher, A32959). The homogenates were centrifuged at 12000*g* for 15 min at 4°C. Protein content in the supernatant was determined using the Bradford method. Aliquots of supernatant were mixed with Laemmli buffer 4× (Biorad) containing β-mercaptoethanol, and subsequently boiled at 95°C for 5 min. Approximately 20 μg of proteins were loaded into gradient (4%–15%) and stain-free gels (Mini PROTEAN® TGX Stain-Free TM Gels, Biorad, 4568086), electrophoresed by SDS-PAGE, and then transferred to polyvinylidene fluoride membranes (PVDF, Biorad). A stain-free blot image was taken using the ChemiDocTM Touch Imaging System for total protein measurement in each sample lane. Membranes were blocked in TBS + 1% Tween® 20 (TBS-T) + 5% BSA for 1 hour at room temperature and then incubated with the specific primary antibodies for 1 h 30. The complete list of antibodies used for immunoblotting analyses can be found in Table 2. All antibodies were diluted in blocking buffer. Membranes were washed in TBS-T (3 × 5 min) and incubated with HRP-conjugated secondary anti-rabbit or anti-mouse secondary antibodies for 1 h at room temperature, before further washing in TBS-T (3

TABLE 2 List of primers.

Genes	Forward primer (5′–3′)	Reverse primer (3′–5′)
Il-6	CACGGCCTTCCCTACTTCAC	TGCAAGTGCATCATCGTTGT
Il-1ß	TCGCAGCAGCACATCAACAA	TGGAAGGTCCACGGGAAAGA
Tnf-α	ACTGGCAGAAGAGGCACTCC	CTCCAGCTGCTCCTCCACTT
Park2	TCTTCCAGTGTAACCACCGTC	GGCAGGGAGTAGCCAAGTT
Atf4	GGAATGGCCGGCTATGG	TCCCGGAAAAGGCATCCT
Atf6	TCCTCGGTTCCCCCTTATCT	AACGACTCAGGGATGGTGCT
Chop	GGAGCTGGAAGCCTGGTATG	TGTGCGTGTGACCTCTGTTG
Pgc1α-4	AGTCTCCCCGTGGATGAAGA	GAGCTGAGTGTTGGCTGGTG
Drp1	CCTCAGATCGTCGTAGTGGGA	GTTCCTCTGGGAAGAAGGTCC
Fis1	TGTCCAAGAGCACGCAATTTG	CCTCGCACATACTTTAGAGCCTT
Opa1	CAGAGGATGGTGCTCGTGGA	TCCGTCTTGGATGCACAGGA
Mfn2	GCTCAGGAGCAGCGGGTTTA	TGTGGACACCTGCCTTTCCA
Mfn1	ATGGCAGAAACGGTATCTCCA	CTCGGATGCTATTCGATCAAGTT
Pgk1	CAAGGCTTTGGAGAGTCCAG	TGTGCCAATCTCCATGTTGT

×5 min). Signals were detected using an enhanced chemiluminescence substrate (Biorad, Clarity ECL substrate, 170-5061) and the ChemiDocTM Touch Imaging System. All images were analyzed using the ImageLab software (Biorad). For each sample, the ECL signal for the protein of interest was normalized to the intensity of the stain-free blot image of the corresponding sample (i.e., the intensity of the stain-free blot image was used as a loading control).

4.12 | Data analysis and statistics

All statistical analyses were performed using GraphPad Prism 10. The impacts of aging and MFN2 overexpression on parameters of interest in young and old mice were analyzed using two-way repeated measures analysis of variance (ANOVA). The impacts of aging on parameters of interest were also assessed by comparing YA-AAV-GFP and Old-AAV-GFP using unpaired student *t*-tests. For all ANOVA and student *t*-tests, $p < 0.05$ was considered significant. Corrections for multiple comparisons following ANOVA were performed by controlling for the false discovery rate using the two-stage step-up method of Benjamini and Krieger and Yekutieli ($q < 0.1$ was considered significant). The exact number of animals within each group in all figures is indicated in the Figure legends. Individual data are displayed in each graph.

5 | CONCLUSION

The present study shows that overexpressing MFN2 in the skeletal muscle of young and old mice increases muscle mass and fiber size. This hypertrophic effect of MFN2 overexpression occurs without signs of fibrosis, inflammation, or necrosis, indicating that the positive impact of MFN2 overexpression on muscle mass and myofiber size is not pathological. MFN2 overexpression even decreases the proportion of fibers with central nuclei in old skeletal muscles, which we interpret as a marker of improved muscle health. Importantly, MFN2 overexpression did not alter mitochondrial respiration and H_2O_2 emission in both young and old skeletal muscles. While MFN2 overexpression had no impact on mitochondrial content in the TA of young and old mice, it decreased several markers of mitochondrial content in the EDL of old mice. Although further research is needed, our data also suggest that MFN2 overexpression might attenuate the effects of aging on skeletal muscle autophagy. While the exact mechanisms conferring MFN2 its hypertrophic impact remains to be established, our data position increased MFN2 expression as a potential therapeutic approach and/or potential pharmacological target to mitigate aging-related muscle atrophy, strategies that may have applications for other muscle disorders.

AUTHOR CONTRIBUTIONS

Marina CEFIS: Conceptualization; validation; formal analysis; data curation; methodology; writing – original draft; writing – review and editing; investigation; visualization; supervision. **Manon Dargegen:** Data curation; formal analysis; writing – review and editing; investigation; validation. **Vincent Marcangeli:** Data curation; formal analysis; writing – review and editing; investigation. **Shima Taherkhani:** Writing – review and editing; data curation; formal analysis; investigation. **Maude Dulac:** Data curation; formal analysis; writing – review and editing; investigation. **Jean-Philippe Leduc-Gaudet:** Methodology; investigation; writing – review and editing; validation; formal analysis. **Dominique Mayaki:** Formal analysis; investigation; writing – review and editing. **Sabah Hussain NA:** Writing – review and editing; supervision; resources; validation; methodology. **Gilles Gouspillou:** Conceptualization; methodology; data curation; supervision; resources; writing – review and editing; writing – original draft; project administration; funding acquisition; validation; software; visualization; formal analysis.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

DATA AVAILABILITY STATEMENT

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

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