

Drp1-FGF21 axis drives FAPs-mediated fibro-adipogenic degeneration in Duchenne muscular dystrophy

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Article

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Drp1-FGF21 axis drives FAPs-mediated fibro-adipogenic degeneration in Duchenne muscular dystrophy

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ABSTRACT

Mitochondrial dysfunction is an early pathogenic event in dystrophic muscle contributing to muscle damage. Here, we provide an in-depth characterization of the marked induction of mitochondrial fission in *mdx* muscle fibers, driven by excessive activation of the fission regulator Drp1. This aberrant Drp1 activity triggers the integrated stress response (ISR), resulting in muscle-derived secretion of FGF21, which in turn promotes the fibro-adipogenic differentiation of fibro-adipogenic progenitors (FAPs). Importantly, we demonstrate that inhibition of Drp1 is both necessary and sufficient to restore mitochondrial function and suppresses FGF21 upregulation, limiting its detrimental impact on FAPs and contributing to the overall improvement of DMD phenotype. Our study identifies FGF21 as a key mediator of DMD pathology and supports targeting mitochondrial dynamics as a disease-modifying therapeutic strategy.

Running title

Mitochondrial fission drives fibroadipogenic damage

Keywords: mitochondrial fission, Drp1, FGF21, FAPs, Duchenne muscular dystrophy, fibro-adipogenic differentiation.

INTRODUCTION

Duchenne muscular dystrophy (DMD) is a rare, severe X-linked neuromuscular disorder caused by the loss of dystrophin that affects 1:3500/1:5000 boys worldwide and results in markedly reduced life expectancy (1).

Dystrophin ensures the mechanical link between the extracellular matrix and the fiber's cytoskeleton through the dystrophin-associated protein complex; its absence triggers destabilization of the sarcolemma integrity and induces cycles of muscle degeneration and regeneration due to contraction-induced injury (2). These cycles result in a cascade of pathological events that include chronic inflammation and fibrosis (3).

Recent years have marked a transition from symptomatic treatment, glucocorticoid derivatives and givinostat (4), to dystrophin-restoring strategies (5). Nevertheless, questions remain about their clinical efficacy and safety profile (6, 7), and improving these strategies can depend on a more comprehensive understanding of the molecular mechanisms driving DMD pathophysiology.

In this context, a growing body of preclinical and clinical evidence highlights the early contribution of mitochondrial dysfunctions to DMD pathogenesis (8). Indeed, the loss of dystrophin promotes mitochondrial bioenergetic failure (9), excessive mitochondrial calcium uptake, opening of the mitochondrial permeability transition pore (10, 11), and increased production of reactive oxygen species (ROS) (12, 13). In turn, mitochondrial damage accelerates myofiber degeneration and also contributes to the progression of cardiomyopathy in DMD (14).

Mitochondria are highly dynamic organelles whose morphology and function are controlled by the balance between fission and fusion events (15).

During fission, a mitochondrion divides into two or more daughter organelles, often characterized by different membrane potentials. Conversely, fusion involves the close contact of two or more mitochondria and the sequential fusion of the outer and inner mitochondrial membranes (16). Mitochondrial fission is driven by dynamin-related protein 1 (Drp1) and requires specific mitochondrial adaptors, such as mitochondrial fission protein 1 (Fis1), mitochondrial fission factor (Mff), and mitochondrial dynamics proteins of 49 and 51 kDa (Mid49 and Mid51) (16).

Fission can result in either mitochondria degradation or biogenesis in a mechanistically and spatially controlled manner (17). In skeletal muscle, excessive Drp1-mediated mitochondrial fission causes impaired muscle growth and leads to mitochondrial repositioning within myofibers (18, 19). Similarly, Drp1 deficiency results in myopathy, marked muscle atrophy, and weakness (20), highlighting the relevance of physiological levels of Drp1 for muscle homeostasis.

Although mitochondrial dynamics have been only partially explored in DMD, previous studies show mitochondrial fragmentation in myofibers (21, 22). Furthermore, a fragmented mitochondrial network has been described in dystrophic nematodes and zebrafish, and suppression of the *drp-1* gene mitigates muscle degeneration (21). Likewise, the severe DMD mouse model, *mdx/Utr*^{-/-}, displays an imbalance between Drp1 and Mfn2 expression, with aberrantly elevated Drp1 levels indicating activation of the fission machinery (23).

Disrupted mitochondrial dynamics also function as a central signaling hub, capable of initiating molecular cascades that drive adaptive stress responses, including the sustained activation of the eukaryotic initiation factor 2 α (eIF2 α) pathway (19, 24). Activation of eIF2 α enhances the selective translation of stress-responsive genes, such as activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) (25). ATF4 acts as a master regulator of integrated stress response (ISR), coordinating transcriptional programs that promote metabolic adaptation and cellular recovery (19, 24). Both participate in the mammalian mitochondrial unfolded protein response (UPR^{mt}), a conserved pathway that supports protein quality control and recovery (26). The UPR activation, especially in muscle, induces the expression and release of fibroblast growth factor-21 (FGF21) (19, 24), an endocrine/paracrine stress-responsive myokine that can also be released by multiple organs (e.g., liver, adipose tissue). FGF21 is a starvation-like hormone with several metabolic functions globally aimed at overcoming nutrient deprivation and involved in mitochondrial quality control (27-29). FGF21 acts through FGF receptors and typically requires co-receptors, such as β -Klotho (KLB) (30). KLB is markedly upregulated in the diaphragm of dystrophin-null mice (30) and is specifically expressed in fibroadipogenic precursor (FAP) cultures alongside adipogenic markers (31).

FGF21 acts in a dual context-dependent manner: i) as a compensatory signal during acute energetic stress (32), ii) as a deleterious cellular and systemic signal when chronically elevated (19, 24). Indeed, exacerbated FGF21 expression contributes to neuromuscular junction disruption, thereby accelerating muscle atrophy (33). It negatively affects the secretion of growth hormone, impairing muscle growth (19), and accelerates senescence in many tissues (24). Consistently, FGF21 ablation protects from loss of muscle mass and function in fasting-induced muscle atrophy (27). In DMD, FGF21 levels are increased and contribute to the bone defects (34). However, the mechanisms driving FGF21 upregulation and its downstream effects in DMD remain unclear. Here, we show that Drp1 upregulation in DMD muscle drives excessive mitochondrial fission, activating the ISR and increasing muscle-derived FGF21, which promotes adipogenic degeneration of FAPs. We also show that Drp1 inhibition is both necessary and sufficient to suppress aberrant FGF21 upregulation and to ameliorate disease phenotype. These findings identify FGF21 as a key mediator of the DMD condition and support the therapeutic relevance of targeting mitochondrial dynamics as a disease-modifying strategy.

MATERIALS AND METHODS

Mice and treatment

All the procedures complied with the Italian (D.L. 26/2014) and European (2010/63/UE) animal care and were approved by the Italian Ministry of Health (approval no. 761/2022-PR). Dystrophic mice were generated by crossing *mdx* mice (C57BL/10ScSnDmd*mdx*/J) with PhAM mice (C57BL6/129SV) expressing a mitochondrial Dendra2 (35). PhAM mice with a comparable genetic background served as controls. This dystrophic line recapitulated the typical *mdx* muscle phenotype (9, 35) and allowed a more comprehensive assessment of mitochondrial morphology. Mice were housed under controlled conditions ($23 \pm 1^{\circ}\text{C}$, $50 \pm 5\%$ humidity) with a 12-hour light/dark cycle and provided with food and water ad libitum. Only male mice were used and randomly assigned to the experimental groups. Sample size was determined using G*Power, assuming a power of 0.8, an alpha level of 0.05, and an expected within-group variability of 20%. Mice were treated with mdivi-1 or injected with AAV-shDrp1, and the full experimental details are provided in the Supplementary Methods.

Treadmill test

Mice underwent exhaustion test on a standard treadmill according to TREAT-NMD SOP DMD_M.2.1.003. Full experimental details are provided in the Supplementary Methods.

Cell cultures and treatments

Muscle satellite cells and FAPs were isolated from 3-month-old *mdx*-PhAM mice using automated tissue dissociation and magnetic depletion as previously reported (36). Full experimental details are provided in the Supplementary Methods.

Nucleic acid extractions, RT-qPCR.

Cell and muscle RNA were extracted using PureZOL reagent, retrotranscribed, and analyzed by SYBR Green RT-qPCR (37). Full experimental details are provided in the Supplementary Methods

Protein and mitochondria isolation and Western Blotting

Muscle samples were lysed using appropriate buffers with protease and phosphatase inhibitors, and with mechanical homogenization. Lysates were mixed with 4× Laemmli sample buffer and heated or not for downstream analysis. Mitochondria were also isolated by differential centrifugation of muscle homogenates. Full experimental details are provided in the Supplementary Methods.

Proximity Ligation Assays (PLA)

Fixed and permeabilized single gastrocnemius (GS) fibers were processed for Duolink proximity ligation assay and quantified using ImageJ (18). Complete methodological details are reported in the Supplementary Methods.

High-resolution respirometry (HRR)

Mitochondrial oxygen consumption was assessed by high-resolution respirometry on isolated mitochondria using a validated protocol (35, 38). Complete buffer composition, titrations, and analytical details are provided in the Supplementary Methods.

Histology and immunofluorescence

Tibialis anterior (TA) and diaphragm (DP) were immediately frozen to allow the preparation of 10 μm thick sections for both histological and immunofluorescence analysis. Single fibers, obtained from soleus muscles previously fixed, were used for mitochondrial network analysis. Full experimental details are provided in the Supplementary Methods.

Statistical evaluation

Statistical analyses were performed with GraphPad Prism. Normality was assessed by the Shapiro-Wilk test. Two-group comparisons used the unpaired t-test or Mann–Whitney test for non-normal data. Multiple groups were analyzed by one-way ANOVA with Holm–Šidák or Tukey post hoc analyses. For the fragility test (Figure. 2C), mixed-effects analysis was applied. For grouped analyses, multiple t-tests were used. Outliers were checked before any analysis.

RESULTS

Drp1 is upregulated in both patients and *mdx* mice, and it is a relevant target in DMD.

Since Drp1 has been reported to be dysregulated in DMD models (21-23, 39), we assessed its expression in muscle biopsies from DMD patients. Both mRNA and protein levels (Figure 1A) were increased in patients' biopsies compared to healthy muscles, consistent with the upregulation observed in TA of the 12-week-old *mdx* mice compared to control mice (Figure 1B). To sustain the relevance of targeting Drp1 in DMD (40), we downregulated its expression by injecting an adeno-associated viral vector serotype 1 (AAV1) having proven tropism for skeletal muscle cells (41) and carrying a validated shRNA targeting Drp1 (42) (AAV-shDrp1). *Mdx* mice, 12-week-old, were injected in the TA with the AAV1, while PBS was injected as a control. AAV-shDrp1 significantly decreased Drp1 mRNA levels ($P < 0.0001$) (Figure S1A) and protein contents ($P < 0.05$) (Figure 2A), restoring them to WT values (Figure 2A) and excluding adverse effects associated with excessive Drp1 depletion (20, 42). We next examined the expression of other mitochondrial dynamics proteins, revealing a tendency toward Mfns upregulation, whereas Fis1 and Mff showed opposite patterns, with Mff being increased (Figure S1B). To establish whether Drp1 knockdown was associated with changes in muscle function, we measured maximal tetanic force generation during isometric contractions, as well as the susceptibility to contraction-induced injury in muscles 11 weeks post-injection. While the maximal force generation was not modified after Drp1 knockdown (Figure 2B), the force drop resulting from lengthening contraction-induced injury was improved (Figure 2C), suggesting that Drp1 downregulation ameliorates the robustness of dystrophic muscles.

Consistently, Drp1 knockdown abolished the pseudo-hypertrophy observed in PBS-injected *mdx* TA (Figure 2D), improved muscle architecture (Figure S1C), and increased the proportion of smaller fibers (Figure 2E), without affecting fiber number (Figure S1D). This was fully consistent with the increased regeneration (Figure 3A), as indicated by increased MYH3-positive fibers.

Besides, Drp1 knockdown counteracted fibrotic degeneration (Figure 3B), showing decreased collagen content in TA (Figure 3C), as well as reduced expression of fibrosis-related genes (Figure 3D).

Overall, these findings suggest that Drp1 dysregulation contributes to multiple pathological features of dystrophic muscle and that its modulation promotes regeneration, reduces fibrosis, and improves muscle resistance to damage.

Drp1 is more active in *mdx* myofibers, leading to a less interconnected mitochondrial network.

Despite some pieces of evidence (21, 22, 40), the defects associated with dysregulated mitochondrial dynamics are still underexplored in DMD. Consistently, we investigated the expression of mitochondrial shaping proteins throughout disease progression. Starting at 12 weeks of age, both fission and fusion proteins, except for Opa1 (Figure 4A and Figure S2A), were upregulated in *mdx* muscles, indicating a remodeling of the mitochondrial dynamics machinery. In addition, in dystrophic GS fibers at 20 weeks, Drp1 was predominantly localized to mitochondria (Figure S2B) and showed increased binding to its receptor Fis1, as revealed by Drp1:Fis1 proximity ligation assay (Figure 4B). The signal was higher in *mdx* fibers than in WT, indicating enhanced Drp1 activation and the occurrence of excessive mitochondrial fission.

To further characterize this aberrant mitochondrial network, we used our mito-Dendra2 reporter mice. This model allows the visualization of the mitochondrial network in all tissues. In addition, the mito-Dendra2 can switch from green to red fluorescence upon photoconversion (43). When a sub-population of mitochondria is irradiated with the 405 nm laser light, the green signal photoconverts to red fluorescence, enabling measurements of mitochondrial connectivity (43). In fiber bundles, we photoconverted almost at single-mitochondrion resolution and tracked the spread of the red signal to adjacent mitochondria. In *mdx* fibers, fluorescence spread was significantly reduced compared to controls, demonstrating a profound loss of mitochondrial connectivity (Figure 4C). Accordingly, in *mdx* fibers, the network appeared dispersed and disorganized (Figure S2C).

In WT samples, the mitochondrial network displayed a regular spatial organization (Figure 4D, left). Spatial analysis of fluorescence intensity showed periodic oscillations that gradually decayed toward a stable baseline (horizontal asymptote). This parameter reflects how well the oscillatory pattern is maintained over distance and therefore provides a quantitative assessment of mitochondrial spatial organization, with higher asymptote values indicating a more ordered network. In contrast, mitochondria in *mdx* fibers showed a marked disruption of both pattern regularity and periodicity (Figure 4D, right), the oscillations decayed more rapidly, and the horizontal asymptote was significantly reduced in *mdx* compared with WT fibers (Figure 4D, box plot), consistent with a loss of mitochondrial spatial organization. This thorough analysis demonstrates that increased Drp1 activity in dystrophic myofibers is associated with profound remodeling of mitochondrial architecture and reduced network interconnectivity.

Fission enhances the integrated stress response (ISR) in dystrophic muscle, inducing FGF21 production and the activation of an adipogenic program.

Given links between Drp1 and FGF21 induction in various tissues, including liver (44), brain (45), and skeletal muscle (19), we examined this pathway in dystrophic muscle. In 20-week-old *mdx* mice, FGF21 expression selectively increased in skeletal muscle (Figure 5A and Figure S3A), but not in the liver (Figure S3B and S3C), which is normally the primary source of circulating FGF21 (32). Notably, hepatic FGF21 levels were lower in *mdx* mice than in WT, likely reflecting a negative feedback mechanism.

Next, to identify the molecular pathway responsible for the overexpression of FGF21 in dystrophic muscle, we evaluated the level of p-eIF2 α , a central component of the integrated stress response (46). p-eIF2 α levels were increased in dystrophic muscles at 20 weeks of age (Figure 5B) together with the expression of ATF4-dependent genes and UPR^{mt} markers, including CHOP (Figure 5B), FGF21 (Figure. 5A), HSP60 and ClpP (Figure. 5B). These findings suggest that Drp1 overexpression in dystrophic conditions triggers a stress-adaptive response involving p-

eIF2 α and CHOP, culminating in UPR^{mt} and FGF21 induction, as already evidenced in the specific transgenic model (19).

Of note, Drp1 has been described as a modulator of both mitochondrial and ER morphology (47). Consistently, we also detected increased ER stress markers in *mdx* muscles at 20 weeks of age (Figure S3D), suggesting the coexistence of both mitochondrial and ER stress.

The function of FGF21 is complex and remains debated due to its multiple sites of generation and diverse biological actions (32, 34). β -klotho, an obligate coreceptor mediating FGF21 signaling, has been reported as markedly upregulated in FAPs (31). In addition, FGF21 can also affect satellite cell proliferation and differentiation (48). To assess whether elevated FGF21 levels influence the function of muscle-resident cell populations, we isolated both FAPs and satellite cells from dystrophic muscles and performed *in vitro* differentiation in the presence of recombinant FGF21 (34) (rFGF21) or vehicle. rFGF21 (1 ng/mL) treatment significantly enhanced FAP differentiation into adipocytes as indicated by the increased percentage of Oil Red O-positive cells ($P < 0.0001$) (Figure 5C). Accordingly, we measured upregulated expression of the adipogenic markers in response to FGF21 (Figure 5D), supporting the notion that FGF21 boosts adipogenic differentiation of dystrophic FAPs.

Notably, rFGF21, at any concentrations tested, did not affect dystrophic satellite cell differentiation under either low- or high-density culture conditions (Figure 5E), indicating that these cells are not directly responsive to FGF21. Therefore, these findings underscore a cell-type-specific effect of this myokine, selectively targeting FAPs.

Mdivi-1 reduces FGF21 levels and fibro-adipogenic degeneration of dystrophic muscle.

To provide pre-clinical insights regarding a therapeutic approach relevant for DMD patients, we set up a pharmacological protocol based on Drp1 inhibitor mdivi-1 (or vehicle) administered systemically by daily intraperitoneal injection at 12.5 mg/kg for 8 weeks (18).

The treatment was well tolerated, as mice maintained normal body weight throughout the experimental period (Figure S4A). Although mdivi-1 did not alter Drp1 expression (Figure S4B), it effectively reduced its pro-fission activity, leading to a more connected and organized

mitochondrial network (Figure 6A). This improvement was confirmed by spatial analysis of fluorescence intensity, which showed an increase in the horizontal asymptote (Figure 6A, box plot), consistent with enhanced mitochondrial network organization. This was further accompanied by enhanced activity of OxPhos complexes in isolated mitochondria (Figure S4C), without changes in their overall expression (Figure S4D).

Particularly, mdivi-1 enhanced Complex I activity, excluding any potential interference with N- and Q-respirasome assembly, as previously demonstrated (49, 50).

Moreover, the Drp1-dependent activation of ISR was significantly attenuated, as shown by reduced levels of both CHOP and FGF21 (Figure 6B). However, mdivi-1 did not change the expression of the ER stress markers (Figure S4E), suggesting that ER stress is independent of Drp1 activity.

At the histological level, these molecular changes were mirrored by decreased fatty infiltration in the diaphragm of *mdx* mice, as shown by lower perilipin accumulation (Figure 6C) and confirmed by reduced expression of the adipogenic markers (Figure 6D). Strikingly, the mdivi-1-treated *mdx* diaphragm showed reduced collagen deposition (Figure 7A), as well as lower expression of fibrotic markers (Figure 7B). These data highlight that mdivi-1 limits aberrant fibroadipogenic differentiation associated with Drp1-dependent increases of FGF21.

Accordingly, upon mdivi-1 exposure, *mdx*-treated diaphragms were morphologically preserved (Figure S4F), showing reduced inflammatory infiltrate as well (Figure 7C). At functional levels, treated *mdx* mice exhibited improved physical performance compared to untreated mice, achieving a recovery score toward WT levels of 21.3% and 30.23%. (Figure 7D).

Collectively, our data show that mdivi-1 treatment confers marked benefits in *mdx* mice. Through the attenuation of maladaptive ISR activation and the reduction of FGF21 production, Drp1 inhibition limits the detrimental impact on FAPs and contributes to the overall improvement of dystrophic muscle pathology.

DISCUSSION

In this study, we reported elevated Drp1 levels in both *mdx* muscles and muscle biopsies from DMD patients. As proof of principle of its functional relevance, genetic knockdown of Drp1 reduced susceptibility to contraction-induced injury and improved dystrophic muscle robustness. We also identified a hitherto undescribed axis in DMD pathophysiology, whereby changes in mitochondrial dynamics triggered by Drp1 led to a complex response shaped by FGF21 that contributed to muscle damage. Aberrant Drp1-dependent mitochondrial fission drove a maladaptive stress response involving p-eIF2 α and CHOP, leading to prolonged UPR^{mt} activation and FGF21 induction. Moreover, this myokine exerted a selective effect on FAPs, enhancing fibroadipogenic degeneration of *mdx* muscles. Notably, pharmacological inhibition of Drp1 with mdivi-1 curtailed pro-fission activity, improved mitochondrial metabolic function, and dampened ISR activation and FGF21 induction, resulting in a marked attenuation of fibroadipogenic degeneration of dystrophic muscle. By simultaneously improving mitochondrial energy metabolism and limiting FAP-driven fibro-adipogenic remodeling, mdivi-1 emerges as a strategy capable of targeting two central pathological hallmarks of DMD.

Of note, *mdx* muscles did not show an imbalance between mitochondrial fission and fusion protein expression. Instead, from 12 weeks of age onward, both machineries were upregulated. Structural analysis of the mitochondrial network revealed a less interconnected and disorganized architecture in myofibers, accompanied by increased mitochondrial Drp1 bound to its receptor Fis1. Two different modes of fission have been reported depending on the positioning of the fission site, and both require Drp1 (16, 17). Peripheral divisions depend on Fis1 and mitochondria-lysosome contacts to excise small, depolarized mitochondria with high levels of ROS and Ca²⁺, which are often depleted of mtDNA and destined for mitophagy (17). Midzone fissions, instead, mainly rely on ER and INF2-recruited actin as constriction machinery, severing mitochondria for biogenesis (17). In adult *mdx* muscles, Drp1 was markedly bound to Fis1, indicating that peripheral fission was occurring. This finding could also explain the decrease in mtDNA content

observed in adult *mdx* fibers (9) and is consistent with the inhibition of mitochondrial biogenesis (9), which, conversely, depends on midzone fission events.

Beyond their metabolic and energetic roles, mitochondria act as signaling hubs (16). In the *mdx* muscle, ISR activation occurred alongside Drp1 activation, leading to preferential translation of ATF4-dependent genes and activation of UPR^{mt}, supporting mitochondrial proteostasis, energy production, and FGF21 expression (51). Although this pathway can tackle transient stress, its chronic activation is associated with maladaptive outcomes (19, 24, 32, 34, 52, 53). Consistently, in *mdx* mice, mitochondrial function remained compromised despite the upregulation of ClpP and HSP60; while FGF21 promoted the adipogenic differentiation of dystrophic FAPs, enhancing lipid accumulation and exacerbating muscle damage.

In contrast to a previous report (48), FGF21 exerted no detectable effects on dystrophic satellite cells, revealing a cell-type-specific action of this myokine, selectively targeting FAPs.

The central role of Drp1 was further supported by the benefits observed in dystrophic muscle upon its pharmacological inhibition with mdivi-1. Prolonged administration of a low dose of mdivi-1 restored mitochondrial network architecture and improved mitochondrial function, thereby alleviating Drp1-driven mitochondrial stress. In parallel, mdivi-1 suppressed ISR activation, as reflected by reduced CHOP expression and decreased FGF21 levels, linking Drp1-dependent mitochondrial dynamics to downstream stress signaling. Importantly, attenuation of this axis led to a marked reduction of fibro-adipogenic degeneration in dystrophic muscle, as evidenced by decreased fibrotic and lipid deposition. These effects were also accompanied by decreased inflammation and improved muscle performance. Our findings are consistent with those reported in a recent study in which D2-*mdx* mice were treated with mdivi-1 (40). Notably, we employed a lower dose for a longer period than Rosen and colleagues, and we now provide molecular insights into mdivi-1 efficacy, elucidating the signaling mechanisms triggered by Drp1 activation. Indeed, we identified the activation of the p-eIF2 α –ATF4 axis as a key event leading to FGF21 induction.

Notably, mdivi-1 did not affect the expression of ER stress markers, indicating that ER stress persists in treated mice and that FGF21 levels can therefore be only partially reduced. This

observation further suggests that ER stress occurs independently of Drp1 and may instead involve dysregulated Ca^{2+} exchange between the ER and mitochondria (54).

Nevertheless, it is evident that Drp1 contributes to FGF21 induction, whose decrease achieved with mdivi-1 is sufficient to ameliorate the dystrophic phenotype, underscoring the relevance of this myokine in dystrophic pathology.

Collectively, our findings identified a critical axis in which Drp1-driven mitochondrial fission is the trigger of ISR activation and FGF21 production in DMD muscle, promoting FAP adipogenic degeneration and contributing to dystrophic muscle damage. Strikingly, the limitation of FGF21-induced fibrosis, observed upon mdivi-1 treatment, reveals the therapeutic potential of restoring the altered mitochondrial dynamics in DMD and possibly in other muscle diseases with such pathological hallmarks.

Data and code availability

The uncropped western blots used to generate figures are included as a supplementary figure.

Further information and requests for resources or reagents should be directed to corresponding author, Clara De Palma (clara.depalma@unimi.it).

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Author contributions

All contributing authors have agreed to the submission of this manuscript for publication.

Conceptualization: M.G., S.F., and C.D.P. Investigation: S.R.C., S.Z., C.G., O.G., L.L., D.B., P.O., H.D.M., E.M. Images acquisition and analysis: M.C., Z.L., T.S., D.P. Visualization: S.R.C., S.Z., C.G., O.G., L.L., D.B., P.O., H.D.M., E.M., D.C., F.P.R., C.P., M.G., S.F., and C.D.P. Supervision: M.G., S.F., and C.D.P. Funding acquisition: E.C., D.C., M.G., S.F., and C.D.P. Project administration: C.D.P. Writing - original draft: E.C., F.P.R., C.P., M.G., S.F., and C.D.P. Writing – review and editing: all authors.

Conflict of interest

The authors declare no competing interests.

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Figure Legends

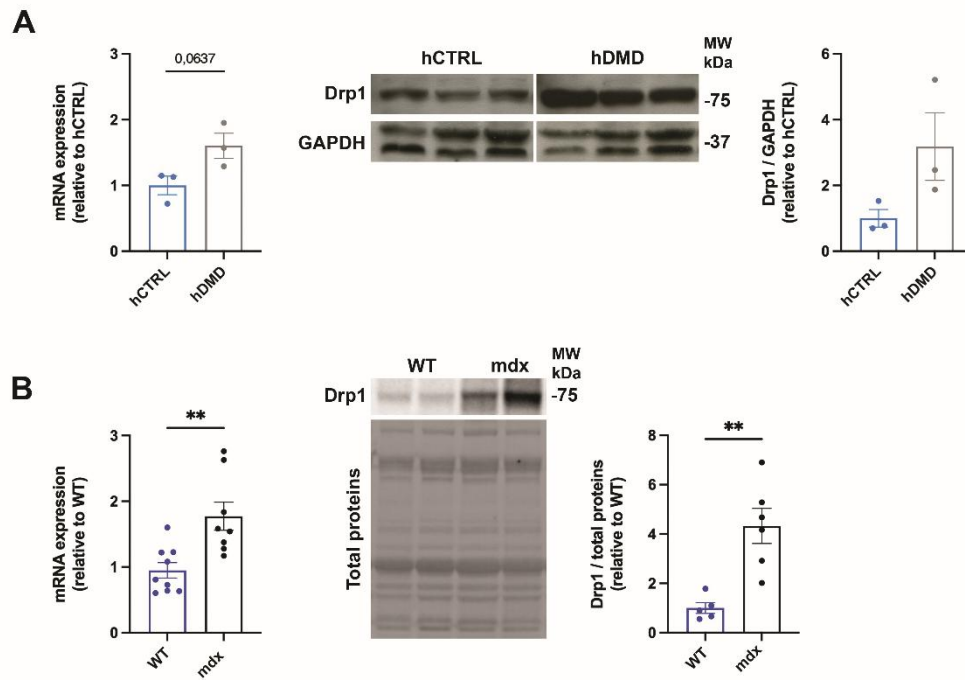


Figure 1. Drp1 is enhanced in both DMD patients and *mdx* mice

(A) mRNA expression levels of *Drp1* in human muscle biopsies from Duchenne muscular dystrophy patients (hDMD) and healthy controls (hCTRL) (n = 3 per group) (left panel).

Representative western blot images and quantification of Drp1 protein levels in hCTRL and hDMD samples (n = 3 per group). GAPDH was used for normalization (right panel).

(B) mRNA expression levels of *Drp1* in tibialis anterior (TA) muscles from 12-week-old WT and *mdx* mice (n ≥ 8 per group) (left panel). Representative western blot images and quantification of

Drp1 protein levels in TA muscles from 12-week-old WT and *mdx* mice (n ≥ 5 per group). Total protein staining was used for normalization (right panel).

Data are represented as mean ± SEM, **p < 0.01. (A, B) Unpaired t-test.

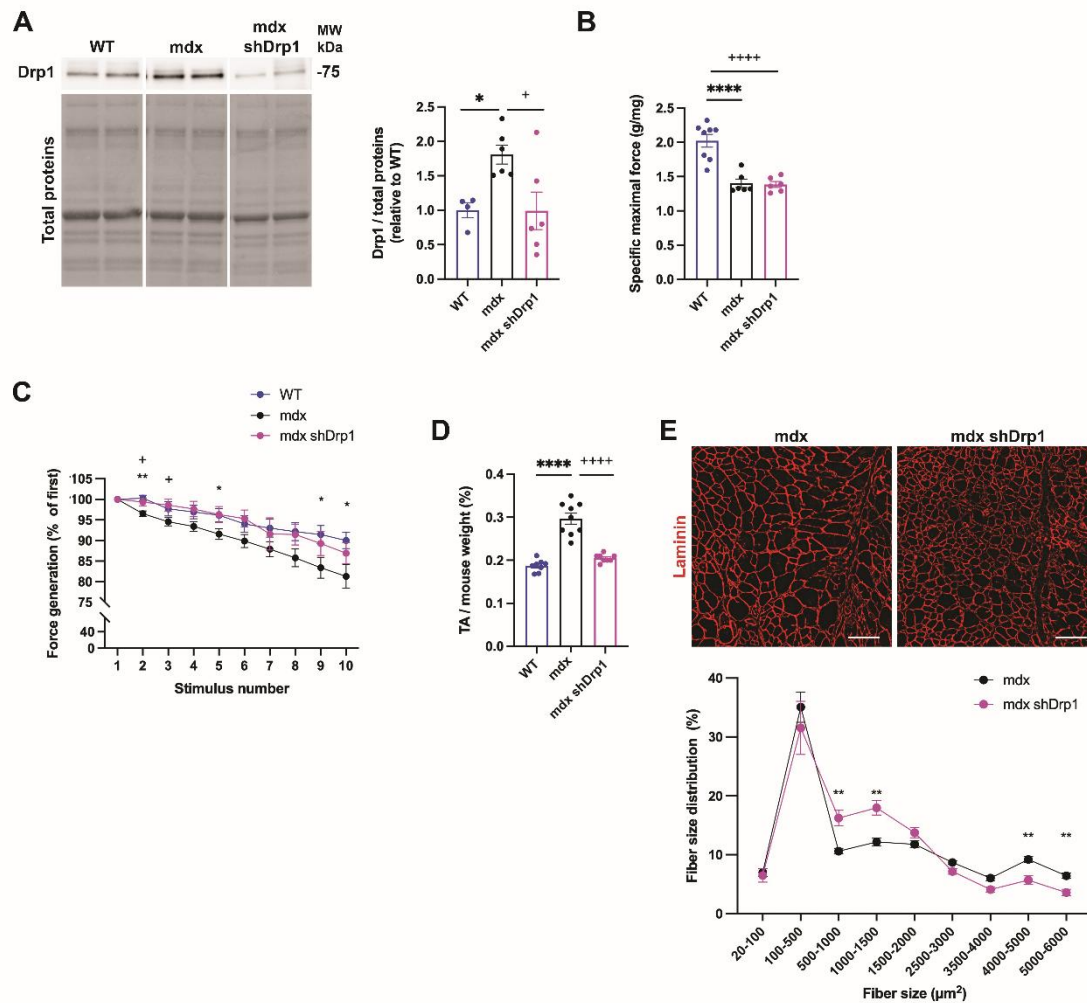


Figure 2. Drp1 knockdown protects from contraction-induced damage.

(A) Representative western blot images and quantification of Drp1 protein levels in TA muscles from *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1) or PBS (*mdx*) and from age-matched WT mice ($n \geq 4$ per group). Total protein staining was used for normalization.

(B) Specific maximal force of TA muscles from *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1) or PBS (*mdx*) and from age-matched WT mice ($n \geq 6$ per group).

(C) Force drop following ten lengthening contractions (in situ fragility test) in TA muscles from *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1, $n = 5$) or PBS (*mdx*, $n = 13$) and from age-matched WT mice ($n = 8$).

(D) TA muscles' weight of *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1) or PBS (*mdx*) and from age-matched WT mice, normalized to body weight. TA muscle weight is expressed as a percentage of total body weight ($n \geq 8$ per group).

(E) Representative laminin immunostaining (red) of TA muscle sections from *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1) or PBS (*mdx*). Scale bars: 200 μm (upper panel). Quantification of fiber size distribution is provided (lower panel). Data are expressed as a percentage of fibers per cross-sectional area (CSA) range ($n = 6$ per group).

Data are represented as mean $\pm$ SEM, $^{*}/^{+}p < 0.05$, $^{**}/^{++}p < 0.01$, $^{***}/^{+++}p < 0.001$, $^{****}/^{++++}p < 0.0001$. (A, D) Ordinary one-way ANOVA (* *mdx* versus WT, $^{+}$ *mdx* shDrp1 versus *mdx*). (B) Ordinary one-way ANOVA (* *mdx* versus WT, $^{+}$ *mdx* shDrp1 versus WT). (C) Mixed-effects analysis (* *mdx* versus WT, $^{+}$ *mdx* shDrp1 versus *mdx*). (E) Multiple unpaired t-test.

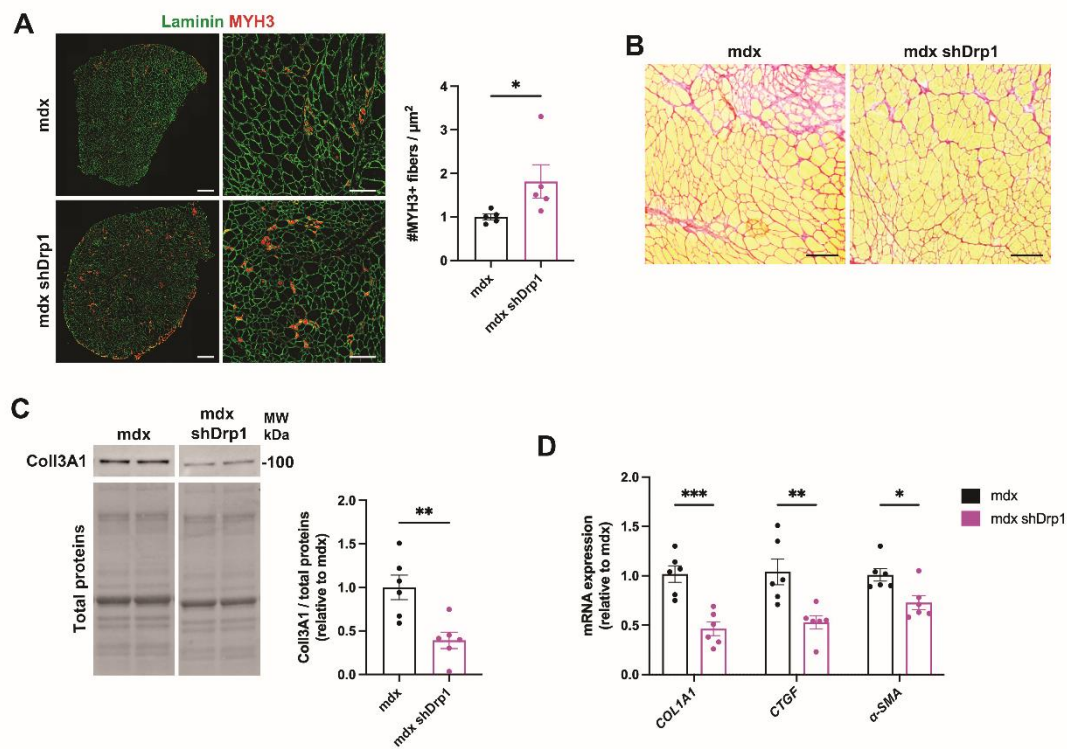


Figure 3. Drp1 knockdown improves the DMD phenotype

(A) Representative laminin (green) and embryonal myosin-MYH3 (red) immunostaining of TA muscle sections from *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1) or PBS (*mdx*).

Scale bars: 500 μm (left) and 200 μm (right). Quantification of regenerating fibers, expressed as the number of MYH3-positive fibers per μm^2 , is provided ($n = 5$ per group).

(B) Representative images of Sirius red-stained TA muscle sections from *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1) or PBS (*mdx*) showing collagen deposition. Scale bars: 200 μm .

(C) Representative western blot images and quantification of Collagen3A1 protein levels in TA muscles from *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1) or PBS (*mdx*) ($n = 6$ per group). Total protein staining was used for normalization.

(D) mRNA expression levels of fibrosis marker genes (*COL1A1*, *CTGF*, *α -SMA*) in TA muscles from *mdx* mice injected with either AAV-shDrp1 (*mdx* shDrp1) or PBS (*mdx*) ($n = 6$ per group).

Data are represented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (A) Mann-Whitney test. (C) Unpaired t-test. (D) Multiple unpaired t-test.

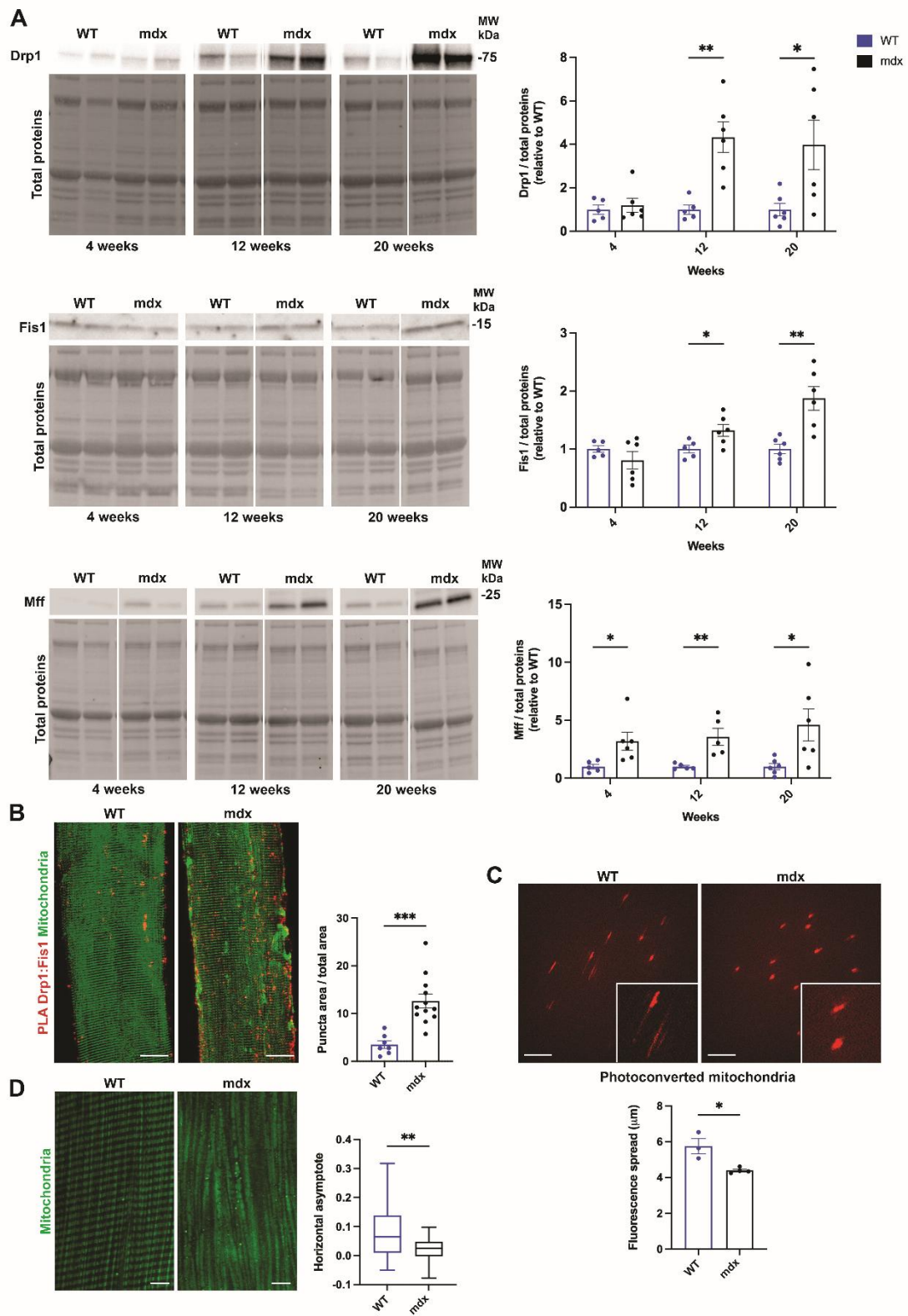


Figure 4. Mitochondrial fission proteins are dysregulated during DMD progression, and the network appears less interconnected

(A) Representative western blot images and quantification of mitochondrial fission protein levels (Drp1, Fis1, and Mff) in GS muscles from *mdx* and WT mice at different disease stages (4, 12, and 20 weeks of age) ($n \geq 5$ per group). Total protein staining was used for normalization.

(B) Representative images of Drp1:Fis1 PLA (red puncta) in GS single fibers from 20-week-old WT and *mdx* mice, with green mitochondria due to mito-Dendra2 protein expression (PhAM mice). Scale bars: 20 μm (left panel). Quantification of Drp1:Fis1 puncta-positive area normalized to the total area is provided ($n \geq 7$ fibers per group).

(C) Representative images of photoconverted mitochondria (red) in soleus fiber bundles from 20-week-old WT and *mdx* mice. Scale bars: 10 μm . (upper panel) Quantification of the red signal spread 2 minutes after photoconversion (green to red) of mito-Dendra2 protein is provided ($n \geq 3$ per group).

(D) Quantification of mitochondrial network organization based on spatial analysis of fluorescence intensity. The horizontal asymptote reflects the persistence of signal oscillations and thus the degree of network organization. Scale bars = 5 μm .

Data are represented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (A) Multiple unpaired t-test. (B, C) Unpaired t-test. (D) Mann-Whitney test.

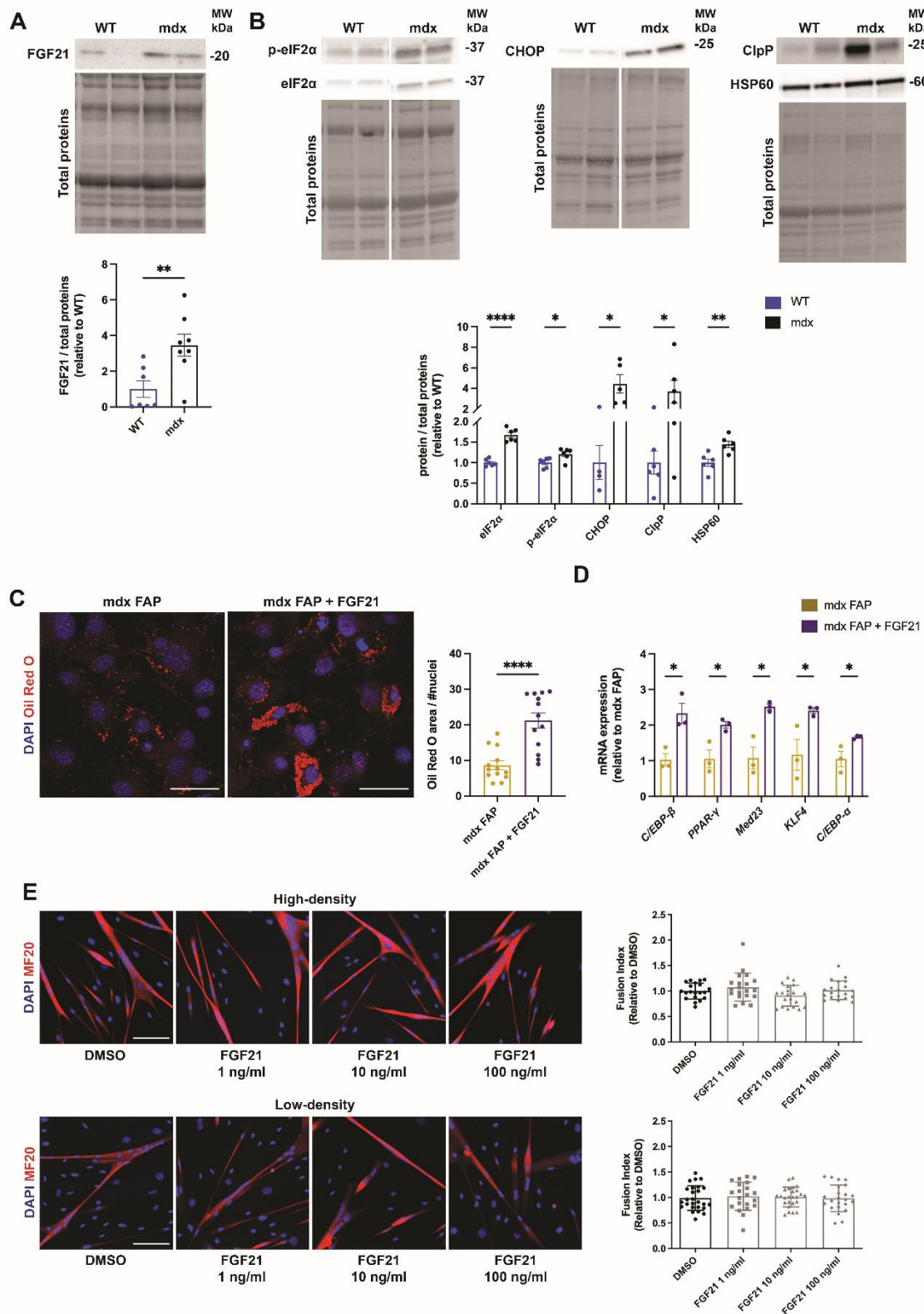


Figure 5. Mitochondrial fission activates ISR and increases FGF21 levels, specifically promoting FAPs' adipogenic differentiation

(A) Representative western blot images and quantification of FGF21 protein levels in GS muscles from 20-week-old WT and *mdx* mice ($n \geq 7$ per group). Total protein staining was used for normalization.

(B) Representative western blot images and quantification of ISR and UPR^{mt} protein levels (eif2 α , p-eif2 α , CHOP, ClpP, and HSP60) in GS muscles from 20-week-old WT and *mdx* mice ($n \geq 7$ per group). Total protein staining was used for normalization.

(C) Representative images and quantification of *mdx* FAPs cultured for 72 h with either 1 ng/mL recombinant FGF21 (*mdx* FAP + FGF21) or DMSO vehicle (*mdx* FAP), stained with Oil Red O (red) and DAPI (nuclei, blue). Scale bars: 50 μ m (left panel). Quantification of Oil Red O-positive area normalized to the total number of nuclei is provided ($n \geq 13$ images per group).

(D) mRNA expression levels of adipogenic marker genes (*C/EBP- β* , *PPAR- γ* , *Med23*, *KLF4*, *C/EBP- α*) in *mdx* FAPs cultured for 72 h with either 1 ng/mL recombinant FGF21 (*mdx* FAP + FGF21) or DMSO vehicle (*mdx* FAP) ($n = 3$ per group)

(E) Representative images of *mdx* satellite cells cultured with either different concentrations of recombinant FGF21 (1, 10, and 100 ng/mL) or DMSO vehicle stained with MF20 (myosin heavy chain, red) and DAPI (nuclei, blue) (left panel). Two culture conditions are shown: high-density (upper panel) and low-density (lower panel). Scale bars: 100 μ m. Quantification of the fusion index, expressed as the ratio of fused nuclei to total nuclei, is provided ($n \geq 20$ images per group) (right panel).

Data are represented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (A) Mann-Whitney test. (B, D) Multiple unpaired t-test. (C) Unpaired t-test. (E) Ordinary one-way ANOVA.

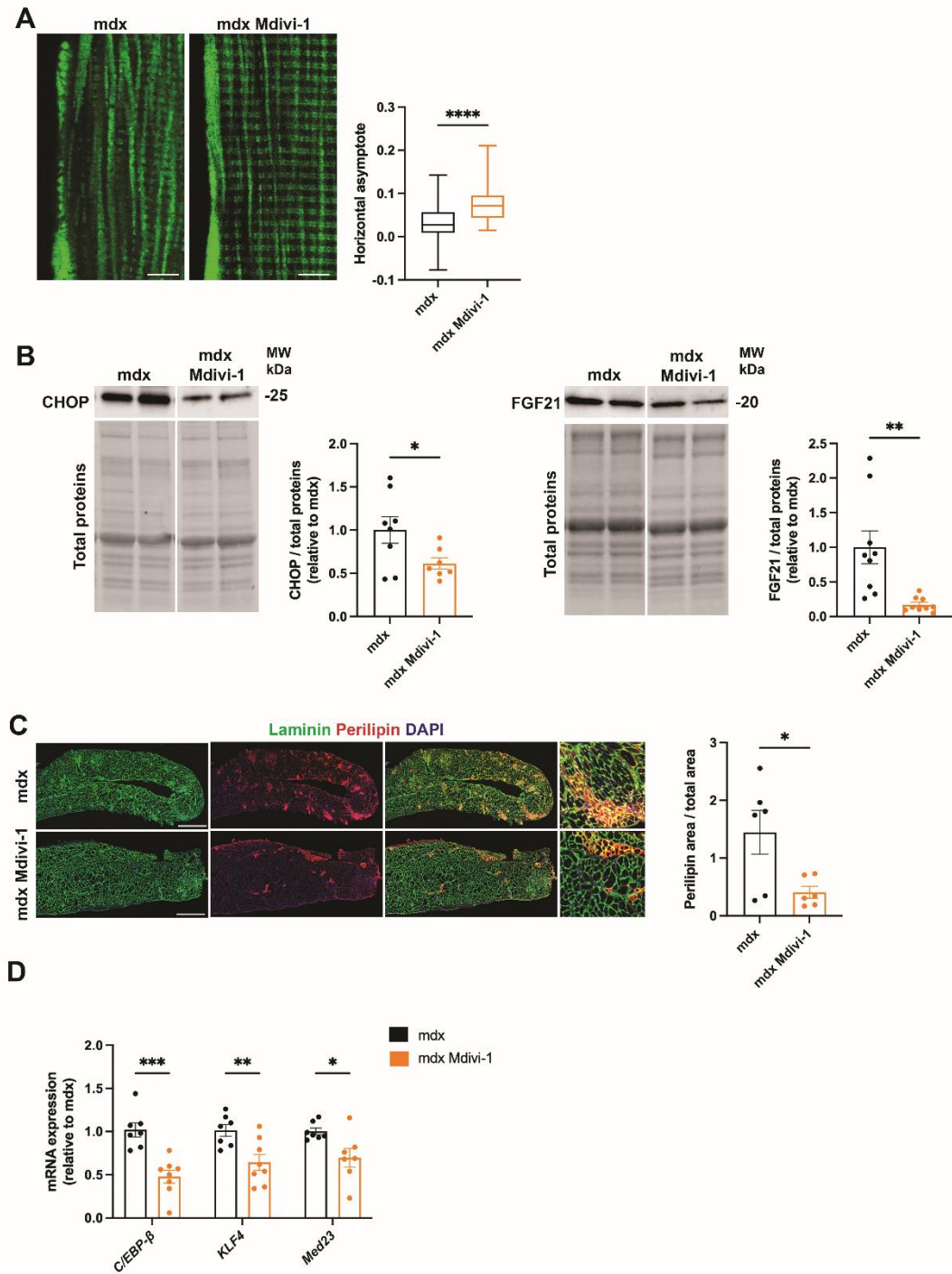


Figure 6. Drp1 inhibition by mdivi-1 improves mitochondrial network architecture, reduces FGF21 levels, and limits adipogenic infiltration in *mdx* muscles.

(A) Quantification of mitochondrial network organization based on spatial analysis of fluorescence intensity. The horizontal asymptote reflects the persistence of signal oscillations and thus the degree of network organization. Scale bars: 5 μ m

(B) Representative western blot images and quantifications of CHOP and FGF21 protein levels in GS muscles from *mdx* mice treated with either mdivi-1 (*mdx* Mdivi-1) or vehicle (*mdx*) ($n \geq 8$ per group). Total protein staining was used for normalization.

(C) Representative perilipin (red) and laminin (green) immunostaining of diaphragm (DP) muscle sections from *mdx* mice treated with either mdivi-1 (*mdx* Mdivi-1) or vehicle (*mdx*). Scale bars: 400 μ m. Quantification of perilipin-positive area normalized to the total area is provided ($n = 6$ per group).

(D) mRNA expression levels of adipogenic marker genes (*C/EBP- β* , *KLF4*, *Med23*) in DP muscle from *mdx* mice treated with either mdivi-1 (*mdx* Mdivi-1) or vehicle (*mdx*) ($n \geq 7$ per group).

Data are represented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (A) Mann-Whitney test. (B, C) Unpaired t-test. (D) Multiple unpaired t-test.

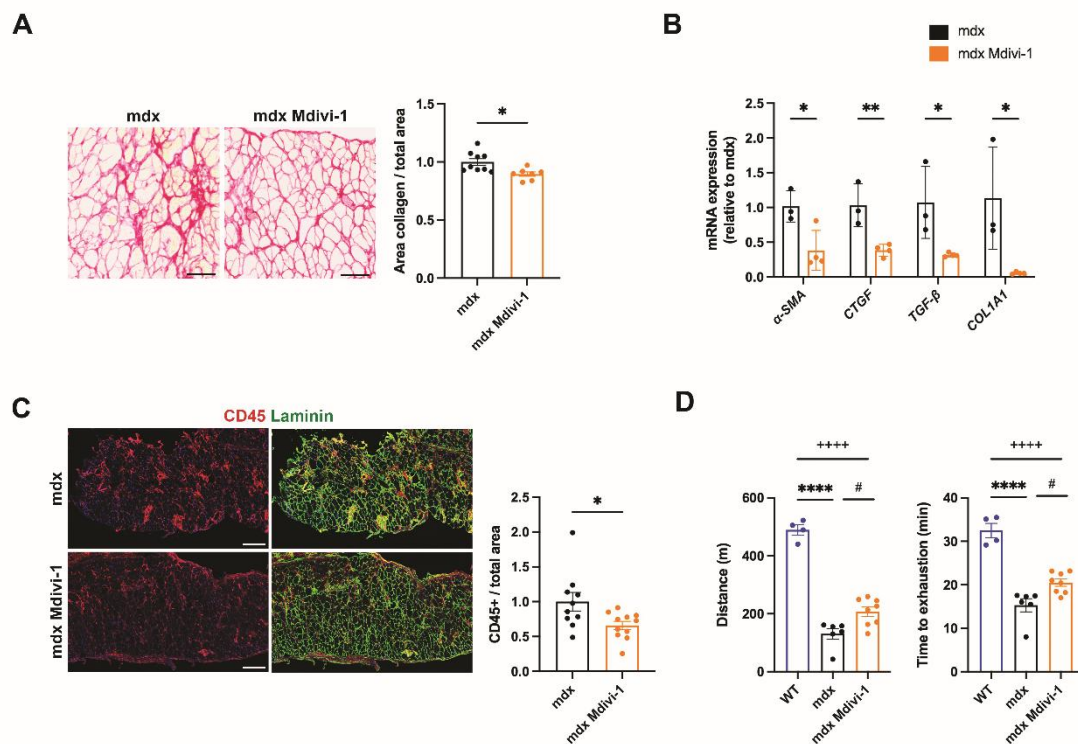


Figure 7. Drp1 inhibition by mdivi-1 restrains fibrosis and inflammation in DMD muscle and ameliorates muscle performance.

(A) Representative images of Sirius red-stained DP muscle sections from *mdx* mice treated with either mdivi-1 (*mdx* Mdivi-1) or vehicle (*mdx*). Scale bars: 100 μ m. Quantification of Sirius red-positive area was normalized to the total area is provided ($n \geq 7$ per group).

(B) mRNA expression levels of fibrosis marker genes (*α -SMA*, *CTGF*, *TGF- β* , *COL1A*) in DP muscle from *mdx* mice treated with either mdivi-1 (*mdx* Mdivi-1) or vehicle (*mdx*) ($n = 4$ per group).

(C) Representative CD45 (red) and laminin (green) immunostaining of DP muscle sections from *mdx* mice treated with either mdivi-1 (*mdx* Mdivi-1) or vehicle (*mdx*). Scale bars: 200 μ m. Quantification of CD45-positive area normalized to the total area is provided ($n \geq 10$ per group).

(D) Exhaustion treadmill running test, measuring distance run and time to exhaustion ($n \geq 4$ per group).

Data are represented as mean $\pm$ SEM, */# $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ****/++++ $p < 0.0001$.

(A, C) Unpaired t-test. (B) Multiple unpaired t-test. (D) Ordinary one-way ANOVA (* *mdx* versus WT, + *mdx* Mdivi-1 versus WT, # *mdx* Mdivi-1 versus *mdx*).

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