BETs inhibition attenuates oxidative stress and preserves muscle integrity in Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) affects 1 in 3500 live male births. To date, there is no effective cure for DMD, and the identification of novel molecular targets involved in disease progression is important to design more effective treatments and therapies to alleviate DMD symptoms. Here, we show that protein levels of the Bromodomain and extra-terminal domain (BET) protein BRD4 are significantly increased in the muscle of the mouse model of DMD, the mdx mouse, and that pharmacological inhibition of the BET proteins has a beneficial outcome, tempering oxidative stress and muscle damage. Alterations in reactive oxygen species (ROS) metabolism are an early event in DMD onset and they are tightly linked to inflammation, fibrosis, and necrosis in skeletal muscle. By restoring ROS metabolism, BET inhibition ameliorates these hallmarks of the dystrophic muscle, translating to a beneficial effect on muscle function. BRD4 direct association to chromatin regulatory regions of the NADPH oxidase subunits increases in the mdx muscle and JQ1 administration reduces BRD4 and BRD2 recruitment at these regions. JQ1 treatment reduces NADPH subunit transcript levels in mdx muscles, isolated myofibers and DMD immortalized myoblasts. Our data highlight novel functions of the BET proteins in dystrophic skeletal muscle and suggest that BET inhibitors may ameliorate the pathophysiology of DMD.

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Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy. This X-linked recessive disorder is caused by mutations in the dystrophin gene, and it affects approximately 1 in 3500 male births worldwide. A decisive therapy for DMD treatment is not available yet. Genome editing approaches hold extensive promise for a future resolutive strategy to pursue, but current clinical approaches are still not effective in reversing the phenotype. Thus, pre-clinical and clinical studies are focusing on pharmacological therapies targeting downstream events of the genetic mutation, including inflammation, fibrosis, adipocyte infiltration, and metabolism.

Dystrophin is a large structural protein located at the sarcolemma that mechanically links the internal cytoskeleton to the extracellular matrix, thus conferring membrane stability during contraction. Lack of dystrophin dramatically increases the sarcolemma susceptibility to contraction-induced injury, leading to myofiber necrosis and triggering secondary events, such as inflammation and fibrosis.

The direct molecular mechanisms for loss of muscle function in mouse models and DMD patients are still under active investigation. Several aberrant processes (e.g., intracellular calcium homeostasis, inflammation and ROS metabolism) are indeed implicated as early events in the disease pathophysiology, since they result in the activation of calcium-dependent degradative pathways, myofibril damage and necrosis, incomplete regeneration cycles, autophagy impairment, increased fibrosis and adipose tissue accumulation. In particular, muscle biopsies from DMD patients show increased oxidative stress compared to controls, and increased NADPH oxidase (Nox2) activity as an early event in the disease onset. Preceding immune cells infiltration and necrosis. Increased oxidative stress has been recently causally linked to autophagy impairment in the mdx dystrophic muscle and the genetic elimination of Nox2-mediated ROS production has been reported to reduce inflammation and fibrosis.

Gene expression profiles of skeletal muscle are altered in muscular dystrophies and the epigenetic regulation of muscle stem cells plays a crucial role for their regenerative potential in the mdx model. In addition, dystrophin loss leads to alteration in signaling pathways that eventually translate in transcriptional reprogramming. For example, histone deacetylase (HDAC) activity is perturbed by dystrophin deficiency and this contributes to transcriptional alteration in mdx mice. Furthermore, epigenetic drugs targeting HDACs are in clinical trial for DMD and showed promising results in the histological progression of the disease.

We have recently shown that the BET protein BRD4 promotes muscle atrophy in an in vitro model of glucocorticoid-induced atrophy and in experimental models of cancer cachexia. Because of this evidence and the well-established role played by BRD4 in inflammation, we aimed to characterize BRD4 contribution in skeletal muscle pathophysiology of a mouse model of DMD. In this study, we show that BRD4 influences ROS metabolism by regulating the transcriptional activation of different subunits of the NADPH oxidase complex in the mdx muscle. Furthermore, administration of the BET inhibitor JQ1 reduces oxidative stress and ameliorates skeletal muscle morphology and muscle function. JQ1 treatment rescues autophagy and dramatically restricts muscle damage, preventing muscle inflammation and fibrosis, and tempering muscle regeneration.

Results
BRD4 levels increase in the muscle of DMD patients and of the mdx mouse. The involvement of BET proteins in inflammatory processes and in skeletal muscle homeostasis prompted us to study their role in the mdx muscle. First, we examined their protein levels in tibialis anterior (TA) of control and mdx mice. BRD2 and BRD3 abundance was comparable in muscles from control and mdx mice, whereas BRD4 protein levels were significantly increased in the dystrophic muscle (Fig. 1a). Notably, the BRD4 antibody specificity was ascertained by silencing experiments (Supplementary Fig. 1A). Conversely, BRD2/3/4 transcripts were expressed at a similar rate both in control and mdx TAs, suggesting that post-transcriptional events are involved in BRD4 regulation (Fig. 1b). We next analyzed BRD4 levels in DMD muscle samples, and found that BRD4 protein was higher in muscles of DMD patients than in age-matched controls (Fig. 1c). We, therefore, interrogated RNA-Seq results published by Khairallah et al. and found that BET transcript levels do not significantly change in DMD muscles (Fig. 1d).

Overall, these data show that BRD4 levels are higher in the muscle of Duchenne patients and in the mdx skeletal muscle, prompting us to further characterize BRD4 function in the mouse model for DMD.

JQ1 treatment reduces muscle damage in the mdx mouse. Based on our initial data (Fig. 1) and our previous observation that BRD4 blockade ameliorates glucocorticoid-induced atrophy in C2C12 myotubes, as well as taking into account the well-documented anti-inflammatory effect of BET inhibitors, we hypothesized that JQ1 treatment may ameliorate the dystrophic phenotype in the mdx mouse model of DMD.

We daily treated 10-week-old mdx mice with JQ1 (20 mg/kg per day) by intraperitoneal injection for two weeks and performed morphological studies on TA muscle sections to examine the effects of BET inhibition on the histopathology of dystrophic mdx muscle fibers. Hematoxylin/eosin staining confirmed the presence of a distinctive pattern of dystrophic muscle pathology in vehicle-treated mdx mice, evidenced by mononuclear cell infiltration and centrally located nuclei. Conversely, the number of infiltrating inflammatory cells was reduced in muscles of JQ1-treated mdx mice (Fig. 2a). In addition, cellular membrane permeability and subsequent fiber necrosis was reduced by JQ1 treatment, as revealed by the decreased number of Evans blue positive fluorescent cells (Fig. 2c). While the total number of fibers per area was identical in the mdx and JQ1-treated mdx muscles (Supplementary Fig. 2A), JQ1 administration significantly increased the number of peripherally nucleated fibers and decreased the centrally nucleated fibers (Fig. 2b), further suggesting that muscles from JQ1-treated mdx mice were less vulnerable to mechanical stress. The increased resistance to the dystrophic phenotype was also associated with reduced cell death, as revealed by the decreased levels of cleaved caspase-3 (Fig. 2d). As previously reported, succinate dehydrogenase (SDH) staining decreased in the mdx muscle compared to WT; however the staining intensity was recovered to that of the WT in JQ1-treated mdx mice (Fig. 2e and Supplementary Fig. 2B), indicating an improved energy metabolism following JQ1 administration, in the mdx TA muscle. Overall these data hint for a beneficial effect of JQ1 treatment in skeletal muscle of mdx mice.

JQ1 restores autophagy in the mdx muscle. To elucidate the molecular mechanisms underlying JQ1 beneficial effects in the mdx skeletal muscle, we investigated JQ1 impact on key metabolic processes, which are affected by the mdx physiopathology. Recent reports revealed that autophagy suppression contributes to the symptomatology of different forms of muscular dystrophies and is detrimental for the maintenance of muscle homeostasis. To test whether autophagy recovery was, at least in part, responsible for JQ1-mediated amelioration in muscle...
mitigated the upregulation of Akt/mTOR/p70S6k pathway in the decreased following JQ1 administration (Fig. 3c).

Consistent with this evidence, acetylation on histone H3 lysine 9 Sirt1 protein levels were recovered following JQ1 treatment. AMPK phosphorylation correlated with Sirt1 protein levels42,43, treated mdx mice (Fig. 3b). In agreement with previous reports, autophagy initiation, was also upregulated in TAs from JQ1-phosphorylation of Ulk1 (Ser555), which plays a crucial role in phosphorylation in mdx TAs. Likewise, AMPK-dependent autophagy/lysosomal pathways and tested whether they were morphology, we examined the abundance of proteins involved in autophagy and its activation is known to decrease in mdx muscles47. JQ1 treatment significantly reduced oxidative stress, as indicated by the upregulation of 8-OHdG immunoreactivity, in TAs (Fig. 4a).

To investigate whether JQ1 was able to protect cells from a second source of ROS, we also employed an in vitro model in which oxidative stress was induced by hydrogen peroxide (H2O2) treatment in C2C12 cells. In agreement with our in vivo findings, JQ1 treatment prevented H2O2-induced oxidative stress, as revealed by 8-OHdG staining (Fig. 4b). Moreover, H2O2 treatment was able to impair autophagy in C2C12 myotubes in which the autophagic flux was blocked by chloroquine, as indicated by the pattern of LC3II and p62. Nevertheless, co-administration of JQ1 to H2O2-treated cells restored LC3II abundance and reduced p62 levels, as observed for mdx muscles in Fig. 4c.

JQ1 restrains oxidative stress. To further investigate JQ1 impact on ROS metabolism in the mdx muscle, we asked whether ROS levels were affected by JQ1 treatment in C2C12 cells, in which previously published RNA-seq datasets show that BRD2/3/4 are highly expressed, with BRD2 transcript being the most abundant transcript previously published RNA-seq datasets show that BRD2/3/4 are highly expressed, with BRD2 transcript being the most abundant.

morphology, we examined the abundance of proteins involved in autophagy/lysosomal pathways and tested whether they were influenced by JQ1 treatment. As demonstrated by other reports11,12,39, we found that the ratio between LC3II (the active lipidated LC3 form) and LC3I (the cytosolic inactive LC3 form) was reduced in TA muscles from vehicle-treated mdx mice. JQ1 treatment restored LC3II/LC3I ratios to levels comparable to the ones observed in control animals. Concurrently, p62 levels, which were upregulated in the mdx muscle, were comparable to those of control mice in TAs of JQ1-treated mdx mice (Fig. 3a). We concluded that JQ1 treatment promotes restoration of autophagy in the mdx mouse model. The rescue in autophagy could not be explained by a JQ1-mediated effect on transcriptional regulation of autophagy genes, because mRNA levels of a group of key autophagy genes did not increase following JQ1 treatment (Supplementary Fig. 3A). We, therefore, interrogated the activation state of different signaling pathways known to regulate autophagy. The metabolic sensor AMPK is a potent inducer of autophagy genes, because mRNA levels of a group of key autophagy genes did not increase following JQ1 treatment.

JQ1 restrains oxidative stress. To further investigate JQ1 impact on ROS metabolism in the mdx muscle, we asked whether ROS levels were affected by JQ1 treatment in C2C12 cells, in which previously published RNA-seq datasets show that BRD2/3/4 are highly expressed, with BRD2 transcript being the most abundant followed by BRD4 and then BRD325,44–46. We first performed immunofluorescence experiments with anti-8-OHdG antibody, which confirmed increased oxidative stress in the mdx muscle, when compared to control muscles47. JQ1 treatment significantly reduced 8-OHdG immunoreactivity, in TAs (Fig. 4a).

To investigate whether JQ1 was able to protect cells from a second source of ROS, we also employed an in vitro model in which oxidative stress was induced by hydrogen peroxide (H2O2) treatment in C2C12 cells. In agreement with our in vivo findings, JQ1 treatment prevented H2O2-induced oxidative stress, as revealed by 8-OHdG staining (Fig. 4b). Moreover, H2O2 treatment was able to impair autophagy in C2C12 myotubes in which the autophagic flux was blocked by chloroquine, as indicated by the pattern of LC3II and p62. Nevertheless, co-administration of JQ1 to H2O2-treated cells restored LC3II abundance and reduced p62 levels, as observed for mdx muscles in Fig. 4c.
In several cultured cell lines, defined doses of H2O2 were able to induce a reduction in p-AMPK levels48–51. Similarly, in C2C12 myotubes, oxidative stress generated by H2O2 administration led to a decrease in AMPK and AMPK-dependent Ulk1 phosphorylation, and was associated with an increase in Akt phosphorylation (Ser473); however, all these events were prevented by JQ1 co-treatment (Fig. 4d).

Oxidative stress was previously shown to induce Sirt1 carbonylation and proteasomal degradation52. H2O2 treatment reduced Sirt1 abundance in C2C12 myotubes, as reported53 (Fig. 4d). However, JQ1 co-treatment prevented H2O2-induced Sirt1 protein reduction, as revealed by a degradation assay (Fig. 4d, Supplementary Fig. 4A). Moreover, to link Sirt1 activity with p-AMPK levels, we challenged Sirt1 function with nicotinamide (NAM) and we observed that JQ1-mediated recovery in AMPK phosphorylation was dependent on Sirt1 activity, in H2O2-treated myotubes. Coherently, AMPK-dependent phosphorylation of Ulk1 was also influenced by Sirt1 inhibition (Supplementary Fig. 4B). To test whether JQ1 is effective when oxidative stress is already established, we administered JQ1 after treating C2C12 myotubes with H2O2 for 2 hours, a sufficient time to induce oxidative stress in C2C12 cells (Supplementary Fig. 5A). The modulation of Sirt1, p62, LC3 as well as of phosphorylated AKT, AMPK and Ulk1 were similar to the one obtained when cells were pretreated with JQ1, followed by H2O2 stimulation ( Supplementary Fig. 5B, C). In addition, JQ1 treatment alone did not affect p-AKT and p62 levels, but it increased Sirt1, lipidated LC3 levels, AMPK and Ulk1 phosphorylation (Supplementary Fig. 5B–D). In this experimental setting, we confirmed that NAM treatment prevented AMPK activation and Ulk1 phosphorylation (Supplementary Fig. 5C). Moreover, Sirt1 pharmacological blockade hindered LC3 accumulation and...
p62 downregulation in cells in which oxidative stress was induced by H2O2 followed by JQ1 treatment (Supplementary Fig. 5D), further supporting the idea that Sirt1 plays a key role in AMPK activation and autophagy regulation.

Collectively, these data suggest that JQ1 treatment prevented and tempered perturbation in ROS metabolism both in the dystrophic muscle and in an in vitro model of oxidative stress, thus restoring Sirt1 levels, AMPK and Akt phosphorylation state, and autophagy.

**BETs pharmacological blockade prevents NADPH subunit transcription upregulation.** NADPH oxidase subunits are over-expressed in the adult mdx muscle and they represent a major source of ROS production in the mdx muscle.4,34,35 Accordingly, we observed that mRNA levels of the Nox2, Nox4, p67-phox, and p47-phox subunits were higher in TAs from mdx mice, when compared to those of control mice. JQ1 treatment in mdx mice restored the transcripts of NADPH oxidase subunits to the level of control mice (Fig. 5a). Similarly, Nox2 and p67-phox protein levels were higher in TAs from mdx mice when compared to control mice, but their levels decreased in muscles from JQ1-treated mdx mice (Fig. 5b and Supplementary Fig. 6A). Likewise, when we treated isolated mdx myofibers with JQ1, we observed a downregulation in transcription levels of the NADPH oxidase subunits, suggesting that JQ1-mediated transcriptional regulation can occur in myofibers, once depleted of infiltrating mononuclear cells (Fig. 5c).

We also analyzed transcripts of the NADPH oxidase subunits in immortalized myoblasts from young DMD donors and observed that their levels significantly decreased following JQ1 treatment (Fig. 5d). Likewise, interrogation of RNA-seq datasets, revealed that Nox2, p47-phox, and p67-phox transcript levels increased in skeletal muscles from DMD patients36, extending the relevance of our findings to the human pathology (Fig. 5e).

In the mdx muscle, JQ1 administration did not affect mRNA and protein levels of Nrf2, a transcription factor that plays a key role in the antioxidant response pathway (Supplementary Fig. 7A, B). Likewise, JQ1 treatment did not alter the transcriptional...
regulation of Nrf2 targets, Hmox1, Gclm and Gclc (Supplementary Fig. 7C), suggesting that restoration of ROS metabolism was not ascribed to the transcriptional activation of anti-oxidant genes.

Next, we mimicked ROS induction in C2C12 myotubes, by treating the cells with 250 μM H$_2$O$_2$ for 24 h. H$_2$O$_2$ is a pro-oxidant that contributes to the generation of a vicious cycle of ROS production by upregulating NADPH oxidase subunits through the activation of redox-sensitive transcription factors.$^{30,36}$ H$_2$O$_2$ treatment promoted p67-phox, p47-phox, Nox2 and Nox4 transcription in C2C12 myotubes, whereas JQ1 co-treatment prevented their transcriptional activation (Fig. 5f). H$_2$O$_2$-induced upregulation of Nox2 and p67-phox proteins was also detectable and it was abrogated by JQ1 pre-treatment (Fig. 5g and Supplementary Fig. 6B). Similarly, NADPH oxidase subunits transcriptional upregulation was hindered when JQ1 administration followed H$_2$O$_2$ treatment (Supplementary Fig. 8A).

JQ1 binds the BET proteins (BRD2, BRD3, BRD4, BRDT) with different affinity and it is potentially capable of displacing BRD2/BRD3/BRD4 from chromatin.$^{57}$ To define which BET protein plays a major role in NADPH oxidase subunits modulation, we employed a siRNA approach in C2C12 myoblasts. BRD2 knockdown reduced the transcript levels of Nox2, Nox4, p47-phox, and p67-phox, while BRD4 depletion did not affect Nox4 mRNA but decreased Nox2, p47-phox, and p67-phox expression. BRD3 did not influence NADPH subunits expression levels (Fig. 5h and Supplementary Fig. 9A, B). Because of BRD2 and BRD4 ability to modulate NADPH oxidase subunits, we performed ChIP assays for these two BET proteins in skeletal...
mRNAs in immortalized DMD myoblast cells treated with 200 nM JQ1 for 24 h. Data are normalized against GAPDH and expressed as the mean ± SD, n = 3 immortalized cell lines. e RPKM expression levels of NADPH oxidase subunit mRNAs in published RNA-Seq dataset for DMD (n = 6) and healthy donors (n = 6). Data are expressed as a mean ± SD. f qRT-PCR analysis of Nox2, Nox4, p47-phox (n = 4 for each experimental group) and p67-phox (n = 3 for each experimental group) mRNAs in C2C12 myotubes cells were pretreated with 200 nM JQ1 and then co-treated with 250 μM H2O2 for 8 h. Data are normalized against GAPDH and expressed as the mean ± SD. g Representative images of immunoblot analysis for Nox2 and p67-phox in TA muscles from control, vehicle-, and JQ1-treated mice. Data are normalized against GAPDH and expressed as the mean ± SD. h Representative images of immunoblot analysis for Nox2 and p67-phox in TAs from control, vehicle-, JQ1-treated mice. Data represent mean ± SD, normalized against GAPDH and expressed as the mean ± SD.

Fig. 5 JQ1 reduces the transcriptional upregulation of NADPH oxidase subunits. a qRT-PCR analysis of Nox2 (WT, n = 4; mdx, n = 6; mdx+JQ1, n = 8), Nox4 (WT, n = 4; mdx, n = 7; mdx+JQ1, n = 8), p47-phox (WT, n = 3; mdx, n = 6; mdx+JQ1, n = 8) and p67-phox (WT, n = 4; mdx, n = 5; mdx+JQ1, n = 8) mRNAs in TAs from control, vehicle- and JQ1-treated mice. Data are normalized against GAPDH and expressed as the mean ± SD. b Representative images of immunoblot analysis for recruitment at regulatory regions of Nox2, Nox4, p47-phox and p67-phox genes. Data represent mean ± SD, normalized against GAPDH and expressed as the mean ± SD. c qRT-PCR analysis of NADPH oxidase subunit mRNAs in myofibers isolated from mdx EDL muscles and treated with 200 nM JQ1 for 16 h. Data are normalized against HPRT and expressed as the mean ± SD, n = 3 animals. d qRT-PCR analysis of NADPH oxidase subunit mRNAs in immortalized DMD myoblasts treated with 200 nM JQ1 for 24 h. Data are normalized against GAPDH and expressed as the mean ± SD, n = 3 immortalized cell lines. e RPKM expression levels of NADPH oxidase subunit transcripts in published RNA-Seq dataset for DMD (n = 6) and healthy donors (n = 6). Data are expressed as a mean ± SD. f qRT-PCR analysis of Nox2, Nox4, p47-phox (n = 4 for each experimental group) and p67-phox (n = 3 for each experimental group) mRNAs in C2C12 myotubes cells were pretreated with 200 nM JQ1 and then co-treated with 250 μM H2O2 for 8 h. Data are normalized against GAPDH and expressed as the mean ± SD. g Representative images of immunoblot analysis for Nox2 and p67-phox in TA muscles from control, vehicle-, and JQ1-treated mice. Data are normalized against GAPDH and expressed as the mean ± SD. h Representative images of immunoblot analysis for Nox2 and p67-phox in TAs from control, vehicle-, JQ1-treated mice. Data represent mean ± SD, normalized against GAPDH and expressed as the mean ± SD.
JQ1 treatment improves muscle physiopathology in the mdx muscle. We further tested whether JQ1 treatment was accompanied by an overall improvement of the pathological abnormalities of the mdx skeletal muscle. In mdx mice, the p65 subunit of NFκB is activated by phosphorylation at Ser536 (Fig. 6a), as a consequence of increased intracellular Ca²⁺ and altered ROS production. NFκB activation, in turn, promotes the transcription of pro-inflammatory cytokines. Remarkably, 2 weeks of JQ1 treatment resulted in a significant reduction in p65 phosphorylation (Fig. 6a). Inflammation was mitigated by JQ1 treatment, as shown by immunostainings for the leukocyte antigen CD45 and the macrophage marker F4/80, which signals were attenuated following BET blockade (Fig. 6b). These findings were supported by the JQ1-dependent decrease in F4/80 and CD45 protein levels in TAs of mdx mice (Fig. 6c). The reduction of inflammatory infiltrate in TA muscles of JQ1-treated animals was coupled to the suppression of TNFα (Supplementary Fig. 10A) and of IL6 pathways (Supplementary Fig. 10B–D), two crucial pro-inflammatory signaling events involved in the pathogenesis of DMD. Reduction in Fibronectin transcript levels and decreased collagen fibers deposition in TAs, revealed by Sirius red staining, was observed following JQ1 administration (Fig. 6d, e).

**Fig. 6 JQ1 treatment ameliorates pathological phenotypes in dystrophic muscle.** a JQ1 administration decreases NFκB p65-Ser536 phosphorylation in TA muscles from mdx mice (n = 5 for each experimental group). Lower panel: quantification of signals was performed with ImageJ. Data are expressed as the mean ± SD. ***P < 0.001. Statistical significance was determined by using one-way ANOVA followed by Tukey’s post hoc test. b Immunohistochimical evaluation of F4/80 and CD45 (n = 3 for each experimental group). Scale bar: 50 µm. c Immunoblot analysis of F4/80 (WT, n = 6; mdx, n = 7; mdx+JQ1, n = 7) and CD45 (WT, n = 6; mdx, n = 8; mdx+JQ1, n = 8) in muscles from control, vehicle- and mdx-treated mice. Vinculin serves as a loading control. Lower panel: quantification of signals was performed with ImageJ. Data are expressed as the mean ± SD. **P < 0.01, ***P < 0.001. Statistical significance was determined by using one-way ANOVA followed by Tukey’s post hoc test. d Sirius red staining shows attenuation of fibrosis in JQ1-treated mice (n = 2 sections examined from n = 3 WT animals; mdx mice, n = 5 sections examined from n = 3 mdx-veh and mdx-JQ1 animals). Scale bar: 50 µm. Right panel: quantification of staining. **P < 0.01, ***P < 0.001. Statistical significance was determined by using one-way ANOVA followed by Tukey’s post hoc test. e qRT-PCR analysis of Fibronectin mRNA in TA muscles from control (n = 4), vehicle- (n = 3) and mdx-treated (n = 3) mice. Data are normalized against HPRT and expressed as the mean ± SD. *P < 0.05, **P < 0.001. Statistical significance was determined by using one-way ANOVA followed by Tukey’s post hoc test. f Immunofluorescence analysis with an antibody raised against the regeneration marker Pax7 in TA cross-sectional section of WT and vehicle- or JQ1-treated mdx mice. n = 3 animals for each experimental group. Scale bar: 25 µm. g Immunoblot analysis of Pax7 (WT, n = 7; mdx, n = 6; mdx+JQ1, n = 7) and eMyHC (WT, n = 3; mdx, n = 3; mdx+JQ1, n = 3) levels in TA of WT, vehicle- and JQ1-treated mdx mice. h Immunoblot analysis of alpha tubulin (WT, n = 4; mdx, n = 5; mdx+JQ1, n = 5), dY tubulin (WT, n = 6; mdx, n = 5; mdx+JQ1, n = 5) and Tubulin6 (WT, n = 4; mdx, n = 4; mdx+JQ1, n = 3) levels in TA of WT, vehicle- and JQ1-treated mdx mice. Right panel: quantification of signals was performed with ImageJ. Data represent mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. Statistical significance was determined by using one-way ANOVA followed by Tukey’s post hoc test.
Amelioration of muscle functional performance following JQ1 treatment in mdx mice. In agreement with the overall reduced muscle damage, JQ1-treated mdx mice significantly increased resistance to fatigue in the treadmill test, and they showed a substantial amelioration in endurance. In addition, overall evaluation of muscle force employing the inverted screen and the wire tests also showed improvements in muscle performance and in vivo force, as early as 2 and 1 week of treatment, respectively (Fig. 7a). Significant amelioration was maintained for one week in the treadmill, wire and inverted screen tests after JQ1 treatment withdrawal and overall motor function of JQ1-treated mdx mice returned to levels comparable to vehicle mdx-mice only three weeks after JQ1 withdrawal (Fig. 7b).

Taken together, our findings demonstrate that BET inhibition ameliorates the patho-physiological defects of dystrophic skeletal muscle, suggesting that BET targeting may be beneficial for patients with muscular dystrophies.

**(a)** Treadmill (WT: n = 3; mdx veh: n ≥ 7; mdx JQ1: n ≥ 8), inverted screen (n = 9 for each experimental group) and wire (wt animals: n = 9; mdx mice: n = 18 for each group) tests were performed on control, vehicle- and mdx-treated mice. Data are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 were determined by one-way ANOVA followed by Tukey’s post hoc test for the treadmill test and with Kruskal-Wallis test followed by Dunns post hoc for wire and inverted screen tests. a indicates statistical significance compared to Control group; b indicates statistical significance compared to the mdx mouse animal group.

**(b)** Mice were treated with JQ1 or vehicle for 3 weeks and treadmill (n = 3 for each experimental group), inverted screen (n = 9 for each group) and wire (n ≥ 10 for each group) tests were performed once a week, and for additional 3 weeks after JQ1 withdrawal. Data are expressed as the mean ± SEM. *P < 0.05 and **P < 0.01 indicate statistical significance versus mdx-vehicle group, and were determined by one-way ANOVA followed by Tukey’s post hoc test.
Discussion
Alteration in ROS metabolism has been identified as an early event in Duchenne muscular dystrophy, leading to myonecrosis, muscle damage and inflammatory cell infiltration\(^7\). Increased oxidative stress results from an unbalance between increased production of reactive oxygen/nitrogen species and an insufficient antioxidant response, leading to myofiber damage and tissue degeneration. Muscle biopsies from DMD patients show increased oxidative stress compared to controls\(^12\), in mdx muscles, increased NADPH oxidase Nox2 activity and Src kinase activation cause an increase in oxidative stress\(^13\). Here we show that BRD4 is involved in transcriptional activation of different subunits of the NADPH oxidase complex in the mdx muscle and that BETs pharmacological inhibition dramatically reduces oxidative stress and ameliorates skeletal muscle homeostasis and muscle function. Increased Nox2 activity and oxidative stress has been recently casually linked to autophagy impairment in the mdx muscle. Furthermore, Nox2 genetic ablation ameliorates pathological and functional phenotypes in dystrophic muscle\(^13\).

Accordingly, we show that JQ1 administration rescues autophagy and restricts muscle inflammation and fibrosis (Fig. 8). In Duchenne muscular dystrophy several processes are deregulated and concur to exacerbate the dystrophic phenotype. Moreover, phagocytic inflammatory cells are a significant source of ROS and the reciprocal stimulation between oxidative stress and inflammation rapidly amplifies the axis leading to muscle degeneration, throughout disease progress. In this scenario, the transcription factor p65 plays a pivotal role, since it is a redox-sensing transcription factor, activated by ROS increase\(^68\) and it concurrently regulates activation of inflammatory transcripts. BRD4 has been shown to play a key role as a cofactor in promoting transcriptional activation of inflammatory genes, in sepsis as well as atherogenesis\(^33\)-\(^35\). Therefore, BRD4 may not only regulate p65-regulated genes indirectly through p65 ROS-mediated activation, but also directly tempering p65 mediated transcriptional activation at certain target genes such as inflammatory cytokines. BET inhibitors ability to concurrently counteract different processes, such as oxidative stress and inflammatory pathways (Fig. 8), may be a key advantage to pharmacologically target different aspects of the DMD pathology, through an epigenetic approach. For instance, IL6 transcript levels may be influenced both by p65 activation and by direct BRD4 regulation. Restoration of SIRT1 levels by JQ1 treatment may also potentially contribute to NFkB inactivation via p65 subunit deacetylation\(^69\).

As shown by our in vitro model of oxidative stress induced by H\(_2\)O\(_2\) treatment, Sirt1 protein levels are also vulnerable to ROS, which lead to Sirt1 post-translational modifications and protein degradation\(^69\). Notably, Sirt1 protein degradation is prevented by JQ1 treatment. Modulation of ROS metabolism by JQ1 administration also leads to Sirt1-dependent AMPK activation (Supplementary Figs. S4 and S5), which is consistent with data observed in the mdx skeletal muscle (Fig. 4d) and may provide a link to autophagy restoration, together with changes observed in Src/Akt axis activation.

Experiments in C2C12 myoblasts, in which oxidative stress was induced by H\(_2\)O\(_2\) treatment, suggest that JQ1 ability to decrease ROS levels represents a more general capability, which can be extended to different sources of ROS production. Similar results were also recently reported in chondrocytes\(^70\).

BRD4 level is enhanced in skeletal muscle of DMD patients and of mdx mice. Other changes in epigenetic factors have been observed in dystrophic muscle, such as augmented HDAC2 levels\(^26\) and increased histone acetylation marks\(^27\). BRD4 stabilization may lead to altered transcription at specific chromatin domain characterized by acetylated histones, thus taking part to the altered genome-wide transcriptional program observed in the mdx muscle. Several factors, such as SPOT\(^27\), DUB\(^37\), PIN\(^74\), and BRD4 post-translational modifications\(^75\) have been involved in BRD4 protein stabilization in cancer models, and further investigation is warranted to understand the dynamics of BRD4 stabilization in the DMD and mdx muscle.

BET inhibitors appear to have different effects on C2C12 differentiation, according to dosage, selected chemical compound and timing of treatment\(^31\),\(^45\),\(^76\). The evidence indicating that in certain experimental conditions BET inhibitors can block differentiation has to be taken into careful account, particularly when considering potential translational applications with human experienced BET inhibitors. Nevertheless, our data suggest that decreased regeneration is associated to reduced damage and necrosis in the mdx muscle, and precedes the repeated cycles of degeneration and regeneration triggered by muscle necrosis. Thus, the positive effects observed in vivo are ascribed to a beneficial effect on myofiber integrity, which may delay satellite cell activation and the need for regeneration. In addition, we employed a relatively low dose of the BET inhibitor, which may preserve satellite cell proliferation and regeneration potential, as suggested by our in vitro data on isolated satellite cells.

Overall, our data demonstrate that BET inhibition holds the potential to counterbalance alterations of ROS metabolism in the dystrophic skeletal muscle, ameliorating myofibers physiology and muscle function. Preserving muscle integrity and improving performance by tuning ROS metabolism may also represent a promising approach in other conditions in which oxidative stress plays a pivotal role in skeletal muscle, such as in sarcopenia.

Methods
Animal study experimental design. All procedures involving animal care or treatments were approved by the Italian Ministry of Health and performed in compliance with the guidelines of the Italian Ministry of Health (according to Legislative Decree 116/92), the Directive 2010/63/EU of the European Parliament and the Council on the protection of animals used for scientific purposes (Protocols 3/2014 and 791/2018). C57BL/10ScSn- Dmd-mdx/j and control C57BL/10ScSnj mice (Charles River, Italy) were housed in groups of five and maintained.
under controlled temperature (20 ± 1 °C), humidity (55 ± 10%), and illumination (12/12 h light cycle with lights on at 07:30 am). Food and water were provided ad libitum. Tubes for tunneling and nesting materials (paper towels) were routinely placed in cages as environmental enrichment. Treatment was performed as in Segato et al.22. For each litter, half of the mice were randomly allocated in the control group and half to the treatment group.

**Muscle morphological analysis and immunofluorescence.** In vivo morphological evaluation was performed on OCT frozen TA muscles. Transverse, 10-μm thick sections were cut by a cryostat and collected on SuperFrost Plus slides (BioOptica). For each TA muscle (n = 3 per experimental group), a minimum of 20 sections were processed for hematoxylin-eosin (H&E) staining or Sirius red and dehydrated and mounted with Eukitt (Kindler GmbH & Co., Germany). Peripheral and centrally nucleated fibers imaging and analysis was not performed blinded to the treatment group. For immunofluorescence analysis of TA muscles, 10-μm frozen sections were blocked with 10% Normal Goat Serum (NGS, Vector Laboratories) in PBS with 2.5% Triton-X 100, incubated with HCl for 20 min at RT and then incubated overnight at 4 °C with 8-OHdA antibodies. Negative controls were performed omitting the primary antibodies. Sections were rinsed with PBS, incubated for 1 h at room temperature with Alexa 488 conjugated goat anti-rabbit IgG in 5% NGS PBS (1:500, Invitrogen Life Technologies, Carlsbad, CA, USA). Slides were mounted with Fluoroshield mounting medium with DAPI (Sigma Aldrich, Italy). Muscle slices were analyzed with a Leica TCS SP8 microscope (Leica, Germany) equipped with a DCM600 camera (immunofluorescence visualization) and Leica DFC480 (bright field visualization). Images were captured using Leica Application Suite System and files were converted in Adobe Photoshop CS5 format.

**Evans blue.** Evans blue dye (EBD) incorporation into necrotic/damaged fibers was assessed as previously reported77. Briefly, EBD (10 mg/ml in PBS) was dissolved and sterilized by using a 0.2-μm pore size filters. Subsequently, 0.05 ml/10 g body weight of dye solution was intraperitoneally administered to WT, vehicle- and QJ1-treated animals. The mice were then sacrificed 24 h after injection, and TA muscles were collected and immediately frozen in liquid nitrogen-cooled isopentane. Muscles were cut with a cryostat and analysis of EBD uptake was performed on 10-μm muscle cryosections. Muscles sections were fixed in cold acetone at −30 °C for 10 min, washed three times with PBS, oversupplied with aqueous mounting medium and evaluated by fluorescence microscopy. Imaging and analysis was not performed blinded to the treatment group.

**Treadmill test.** Mice were acclimatized to treadmill running with a 10-min run at a constant speed of 6 m/min. During the test sessions, mice were run at an initial speed of 6 m/min, and every 2 min speed was increased by 2 m/min until exhaustion. The first exercise test was used to set the baseline of each experimental group. QJ1 administration was started at the end of the training phase and was continued for the entire period of the exercise test (four weeks).

**Wire and inverted screen tests.** For wire test (or wire grip test) mice were tested at 1, 2, 3, and 4 weeks from the beginning of QJ1 administration. The animals were allowed to grasp by their four paws a 2-mm diameter metal wire, which was horizontally positioned 35 cm above a padded surface. The observers recorded the time spent on the wire, until the mice fell on the soft bedding. After each fall, the mice were allowed to rest for 1 minute. Each test consisted of three trials, and values derived from each trial were then averaged85. For the inverted screen test, the animals were tested at 1, 2, 3, and 4 weeks starting from the first day of treatment. Mice were placed in the center of a wire mesh screen, which was subsequently rotated to an inverted position over 2 s. The screen was held steadily 40 cm above thick soft bedding. The length of time until the animal fell on the padded surface was recorded. 60 s was considered the cut-off time in each trial97. Results derived from inverted screen tests were then analyzed by assigning the following scores: Falling between 1 and 10 s = 1; Falling between 11 and 25 s = 2; Falling between 26 and 60 s = 3; Falling after 60 s = 4.

**Isolation of EDL fibers and treatment.** EDL muscles were surgically isolated and incubated in DMEM containing 0.4% Collagenase type I at 37 °C for 1 h to release single fibers. After 1 h in DMEM with 1% penicillin/streptomycin, fibers were transferred to a new dish in DMEM with 20% FBS, 1% chicken embryo extract and 1% penicillin/streptomycin and treated with JQ1 (200 μM) overnight and Trizol was added for RNA extraction.

**C2C12 treatments and immunofluorescence.** C2C12 cells (ATCC) were grown in DMEM high glucose with 20% FBS. Cells were pretreated with JQ1 (0.2 μM) for 24 h and H2O2 (250 μM) was added for 8 h or 24 h. For Sir1 blockade, C2C12 myotubes treated with JQ1 (0.2 μM) and Nocodazole (10 μM) for 24 h prior to H2O2 stimulation. 24 h after H2O2 administration, C2C12 myotubes were harvested and processed for subsequent Western blot analysis. For 8OHdG staining, immunofluorescence was performed as in Feniuzia et al.81, with one additional step of incubation with 2 N HCl for 20 min at RT, after cell fixation. The samples were examined with a Leica TCS SP8 microscope (Leica, Germany) equipped with Leica DFC360 camera (immunofluorescence visualization) and Leica DFC480 (bright field visualization). Images were captured using Leica Application Suite System and files were converted in Adobe Photoshop CS5 format. DAPI was used for nuclear staining.

**Degradation assay.** Protein degradation assay was performed as in Segato et al.,201422. Briefly, C2C12 myotubes were treated with vehicle, H2O2 (250 μM) or co-treated with H2O2 (250 μM) and JQ1 (0.2 μM) as already reported. 24 h after H2O2 stimulation, cells were lysed in ice cold 0.1 M Tris-HCl (pH 7.4), 0.150 M sucrose. 30 μg of protein extraction was employed for each reaction. Samples were incubated at 37 °C, and the reaction was stopped by the addition of an equal volume of cold 10% TCA (Sigma Fluka, Italy) and 10% Trichloroacetic acid (Merck, Italy) containing 2% of buffered 3-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CMH2DCFDA) (ThermoFisher, Italy) for 30 min at 37 °C and washed with PBS (pH 6.8), at different time points (2 h, 4 h, and 8 h). Samples were then boiled for 3 min and used for western blot analysis.

**siRNA C2C12 transfection.** C2C12 myoblasts were transfected in suspension with siBrd2 (0.1 μM), siBrd3 (0.1 μM), siBrd4 (0.1 μM) e siScribble (0.1 μM) (Supplementary Table 4), with Lipofectamine 2000 (Bio-Rad Laboratories, Italy) with CFX Connect detection system (Bio-Rad Laboratories, Italy).

**Satellite cells isolation.** Satellite cells were isolated as in Proserpio et al.13 and growth curve was performed as in Feniuzia et al.81.

**Analysis of intracellular ROS levels in C2C12 cells.** Cells were incubated with 5-((and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CMH2DCFDA) (ThermoFisher, Italy) for 30 min at 37 °C and washed with PBS (pH 7.4). The green fluorescence intensity of the oxidized DCF probe per cell was quantified by Image J software and compared at the different time points.

**ChIp assay.** Chromatin isolated from muscles was subjected to ChIP assay as in Segato et al.32. 100 mg of starting tissue was used for each antibody. Chromatin was sonicated to fragments length of approximately 0.5 Kb and immunoprecipitated with 3.5 μg rabbit IgG or antibodies listed in Supplementary Table 1. ChIP primers are listed in Supplementary Table 3. Quantitation of Real Time PCR was performed using SYBR green I Q reagent (Bio-Rad Laboratories, Italy) with CFX Connect detection system (Bio-Rad Laboratories, Italy).

**DMD immortalized cells and human tissues.** Approval from Ethics committee was obtained by the University of Milan. Human myoblast cells were immortalized by the Institute of Myologie (Pitié-Salpêtrière Hospital, Paris, France) (Supplementary Table 5). Cells were expanded in Skeletal muscle cell growth medium (Promocell C-23060) with Supplement mix (Promocell C-39365), 15% FBS, 1% L-glutamine, 1% gentamicine (Sigma G-1272) and 1% penicillin/streptomycin. Myoblast cells were treated with QJ1 (200 nm) for 24 h. DMD and control tissues were obtained from the AFlM-Myobank (Paris) and tissues were processed as described for mouse muscles (Supplementary Table 6). The study design and conduct complied with all relevant regulations regarding the use of human study participants and was conducted in accordance to the criteria set by the Declaration of Helsinki. The participants, or their legal guardians, provided written informed consent.

**Statistics and reproducibility.** Data obtained from functional, morphological, western blot, and mRNA analysis are expressed as means ± SD (standard deviation). Measurements were taken from distinct samples, except for morphological analysis. All the biological replicates were checked for their normal distribution by using Shapiro–Wilk Test. When we compared 2 experimental groups we used unpaired t test and when we compared 3 or more experimental groups we used one-way analysis of variance (ANOVA) followed by the Tukey’s post hoc test. Statistical analysis was performed using Graphpad Instat3 (GraphPad, La Jolla, CA, USA) for Windows.

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**ARTICLE**

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Author contributions
M.S. performed animal treatments, immunoblotts, immunohistochemical, morphological, and functional analysis, helped with C2C12 experiments, designed experiments, analyzed the data and wrote the manuscript. T.S. performed single fiber isolation experiments, L.N. performed immunoblotts, M.C. generated DMD immortalized cell lines, P.F. provided JQ1 and contributed in experimental design and manuscript preparation. G.C. conceived the study and designed experiments, analyzed data and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Competing interests
The authors declare no competing interests.

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