CHRONIC EXERCISE AND MYOTONIC DYSTROPHY TYPE 1

SKELETAL MUSCLE ADAPTATIONS TO CHRONIC EXERCISE IN A PRE-CLINICAL MODEL OF MYOTONIC DYSTROPHY TYPE 1

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Lay abstract

Myotonic dystrophy type 1 (DM1) is the second most common muscular dystrophy and most prevalent adult form. Muscle weakness, wasting, and myotonia most prominently characterize DM1. A microsatellite repeat expansion mutation in the dystrophia myotonica protein kinase gene, which results in RNA toxicity and dysregulation of mRNA processing, are the root causes of the disorder. Recent studies with DM1 participants demonstrate that exercise is safe, enjoyable, and elicits benefits in muscle strength and function. However, the molecular mechanisms of exercise adaptation in DM1 are largely unknown. Understanding the cellular processes that drive exercise-induced remodelling may assist in the discovery of effective lifestyle interventions to mitigate DM1. In this thesis, three groups of mice were utilized: i) sedentary DM1 animals (SED-DM1), ii) DM1 mice who volitionally exercised daily on a home cage running wheel for 7 weeks (EX-DM1), and iii) sedentary healthy, wild-type mice (WT). Post-exercise functional tests demonstrated that chronic exercise significantly improved motor performance, muscle strength and endurance, as well as reduced myotonia. At the cellular level, we found that chronic physical activity attenuated RNA toxicity and improved mRNA processing. Our data indicate that physical activity improves DM1 at the molecular and physiological levels and lays the foundation for future work to optimize the exercise prescription.

Abstract

Myotonic dystrophy type 1 (DM1) is the second most common muscular dystrophy and most prevalent adult form. A microsatellite expansion comprised of CTG repetitions in the dystrophia myotonica protein kinase (DMPK) gene, DM1 is characterized by muscle weakness, wasting, and myotonia. The expanded nucleotide sequence of the DMPK mRNA results in the misregulation of important RNA-binding proteins (RNABPs), Muscleblind-like 1 (MBNL1) in particular. MBNL1 becomes trapped in myonuclei within the repeating CUG transcript, which reduces the RNABPs ability to process newly synthesized mRNAs that are important for the maintenance of healthy muscle function. Recent studies with DM1 participants demonstrate that exercise is safe, enjoyable, and elicits benefits in muscle strength and function. However, the molecular mechanisms of exercise adaptation in DM1 are largely unknown. Understanding the cellular processes that drive exercise-induced remodelling may assist in the discovery of effective lifestyle interventions to mitigate DM1. In this thesis, three groups of mice were utilized: i) sedentary DM1 animals (SED-DM1), ii) DM1 mice who volitionally exercised daily on a home cage running wheel (EX-DM1), and iii) sedentary healthy, wild-type mice (WT). EX-DM1 animals ran 5.6 km/day during the 7-week experimental time course, a volume of volitional physical activity that is lower than that observed in WT animals. Post-exercise functional tests demonstrated that chronic exercise significantly improved motor performance, muscle strength and endurance. Electromyography revealed that chronic exercise mitigated myotonia. At the cellular and molecular levels, we found that chronic physical activity attenuated RNA toxicity, liberated MBNL1 from myonuclear sequestration, and selectively normalized the spliceopathy of bridging integrator 1 and muscle-specific chloride channel mRNAs. Collectively, our data indicate that chronic exercise improves DM1 at the molecular, cellular and physiological levels.

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Review of the Literature

List of acronyms

AICAR - 5-aminoimidazole4-carboxamide 1-β-D-ribofuranoside, Acadesine, N1-(β-Dribofuranosyl)-5-aminoimidazole-4-carboxamide

AMPK - 5' AMP-activated protein kinase

ASO - Antisense oligonucleotides

BIN1 - Bridging integrator 1

Ca^{2+} - calcium ion

CELF1 - CUG-Binding Protein, Elav-like family member 1

CLC-1 - muscle-specific chloride channel

Cn - calcineurin

CRISPR/Cas9 - Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9

CTCF - CCCTC-binding factor

DM1 - Myotonic Dystrophy Type 1

DM2 - Myotonic Dystrophy Type 2

DMD - Duchenne Muscular Dystrophy

DMPK - dystrophia myotonica protein kinase

DMSXL - Mice carrying over 1000 CTG repeats

DNA - deoxyribonucleic acid

hnRNP H - heterogeneous nuclear ribonucleoprotein H

HSA-LR - human skeletal actin, long-repeats

INSR - Insulin receptor

IR-A - Insulin receptor alpha isoform

IR-B - Insulin receptor beta isoform

LC3 - Microtubule-associated proteins 1A/1B light chain 3B

MBNL - Muscleblind-like protein

MET- metformin

MMR - Mismatch repair

mRNA - messenger ribonucleic acid

mTORC1 - mammalian target of rapamycin complex 1

NF-ATc1- Nuclear factor of activated T-cells, cytoplasmic 1

NMD - Neuromuscular disorders

NRF-1 - Nuclear respiratory factor 1

NRF-2/GABP - Nuclear respiratory factor 1

PGC-1α - Peroxisome proliferator-activated receptor gamma coactivator 1-α

PI3K/Akt - Phosphatidylinositol-4,5-bisphosphate 3-kinase/Protein kinase B

RCT - Randomized control trial

siRNA/shRNA - small (or short) interfering RNA

TNNT3 - troponin T3

UTR - untranslated region

Introduction to DM1

Neuromuscular disease (NMD)s are a heterogenous group of disorders either inherited or developed that cause abnormalities to components of lower motor neurons, the neuromuscular junction, or skeletal muscle (Cup et al., 2007; McDonald, 2012; Ng et al., 2018). NMDs can be isolated to the anatomic region associated with the affected lower motor neuron or multisystemic, targeting tissues such as cardiac and smooth muscle in addition to lower motor neurons and skeletal muscle (McDonald, 2012). The manifestation, progression and loss of bodily functions and processes vary depending on the particular NMD. In diseases such as Duchenne Muscular Dystrophy (DMD), symptoms such as delay in motor milestones and abnormalities in gait movement appear in the first decade of life while NMDs with a later onset may result in muscle strength loss and wasting during adulthood (McDonald, 2012).

Currently, there are almost 600 different NMDs (Cup et al., 2007). Myotonic Dystrophy type I (DM1) affects approximately 1 in 8,000 individuals, with a prevalence as high as 1 in 600 in parts of Quebec (Chau & Kalsotra, 2015; Yum et al., 2017). DM1 is the most common form of myotonic dystrophy, most prevalent adult form of muscular dystrophy as well as the second most commonly inherited NMD after DMD (Chau & Kalsotra, 2015). It arises from a CTG microsatellite repeat expansion mutation in the 3' untranslated region (UTR) of the Dystrophia myotonica protein kinase (DMPK) gene on chromosome 19q13.3 (Smith & Gutmann, 2016; Yum et al., 2017). It is an autosomal dominant trinucleotide repeat disease affecting multiple systems leading to symptoms such as skeletal muscle weakness, wasting, myotonia, and insulin resistance, as well as cardiac conduction defects and hypersomnolence (Cho & Tapscott, 2007).

Genetic factors influencing the onset and severity of DM1

Healthy individuals may have between 5-37 CTG repetitions in the 3' UTR of the DMPK gene, whereas pre-mutation symptoms will manifest with 38-49 repeats and individuals with 50 repeats or more are diagnosed with DM1 (Cho & Tapscott, 2007). At least two genetic phenomena account for the variability in repeat length size not only across the DM1 population but amongst different somatic tissues. The first is known as anticipation, where the mutation rate increases with repeat length size leading to successive offspring developing a more severe phenotype with an earlier disease-onset (Brouwer et al., 2013; Barbé et al., 2017). This gives rise to varying disease phenotypes: congenital, juvenile- and adult-onset DM1, a feature exclusive to DM1 despite sharing similar features and etiology to Myotonic dystrophy type II (DM2; McDonald, 2012). Genetic instability accounts for variation amongst somatic tissue. Repeat sizes above 400 repeats are mitotically unstable leading to the increase in repeat size in some tissues by 50-80 repeats each year (Turner & Hilton-Jones, 2010; Veyckemans & Scholtes, 2013). The pathological increase in repeat size has been attributed to both replication-dependent and -independent mechanisms (Cleary et al., 2010).

Interestingly, repeat length variability and size is much greater in non-dividing cells such as skeletal muscle than in dividing cells such as hematopoietic cells (Thornton, 2014). The dramatic differences and growth in repeat size in post-mitotic cells is believed to be a manifestation of dysregulated DNA repair (Pearson et al., 2005). DNA mismatch repair (MMR), a system designed for repairing erroneous insertion, deletion and mis-incorporation of bases during replication, has been implicated as a driving factor of DM1-specific instability (Tomé et al., 2009). Due to the repetitive sequence in trinucleotide repeat mutations such as DM1, replication slippage is a common phenomenon (Cleary et al., 2010). DNA slip-outs arise when

DNA polymerase encounters a repeating sequence in the template strand. During moments when the complex temporarily releases, it may reassemble at a position upstream leading to the expansion in the newly synthesized strand (Cleary et al., 2010). When DNA slip-outs begin to accumulate and cluster, this will dysregulate MMR function, leading to an increase in the number of slip-outs, ultimately advancing CTG/CAG expansion in the affected locus (Panigrahi et al., 2010). This notion is further supported by the high correlation between levels of slipped DNA and CTG instability; defined as heterogenous CTG size and increased length in the same tissue (Axford et al., 2013).

Moreover, CCCTC-binding factor (CTCF)-binding sites flanking CTG repeats in the DMPK gene regulate instability (Dion & Wilson, 2009; Cleary et al., 2010; Nakamori et al., 2017). CTCF is a protein that regulates gene expression at the specific loci by altering chromatin packaging (Nakamori et al., 2017). Cleary et al. (2010) examined the influence of CTCF binding on instability by utilizing a plasmid replication model with a fragment of the human DM1 locus bearing either wild-type CTCF binding sites or a mutant to abrogate the signal. Blocking of CTCF binding led to an increase in replication efficiency, enabling genetic instability (Tomé et al., 2009; Cleary et al., 2010). The effect on replication efficiency was further augmented when the plasmid was transfected into cells allowing the template to be compacted into chromatin (Cleary et al., 2010). Heterochromatization, the process of repressing transcription through packaging DNA, has been shown to spread across the gene with increases in repeat size length, further implicating chromatin dynamics in the disease phenotype variability (Cho et al., 2005; Brouwer et al., 2013).

Methylation at the CTCF-binding sites, specifically the CpG islands upstream of the repeats, inhibits CTCF-binding and augments genetic instability (Cho et al., 2005; Dion &

Wilson, 2009; Cleary et al., 2010; Brouwer et al., 2013; Barbé et al., 2017; Nakamori et al., 2017). While CpG methylation is correlated with repeat size length, it does vary between tissues (Cleary et al., 2010; Brouwer et al., 2013; Barbé et al., 2017). More methylation is observed in tissues such as the pancreas and cardiac muscle which exhibit high CTG repeat instability while stable tissues such as blood will have a fixed number of repeats and low amount of CpG methylation (Cleary et al., 2010; Turner & Hilton-Jones, 2010).

Elucidating the contribution of CpG methylation has provided an explanation for pioneering genealogical work reporting intergenerational CTG-contraction, where the offspring has a smaller repeat length than the affected parent (Ashizawa et al., 1994). Contemporary research has suggested that the biological significance of aberrant upstream CpG methylation is the reduction in antisense transcription of the CTG repeats. Expanded CAG repeats are subjected to Dicer cleavage and are processed into short CAG RNA repeats. Short CAG RNA repeats can downregulate the CTG repeat-induced pathogenesis similar to small interfering RNA and correlate negatively with the associated histopathology and markers of congenital DM1 (Nakamori et al., 2017). Whether induction of antisense transcription has therapeutic potential remains to be elucidated but collectively it is now understood that aberrant CpG methylation in the CTCF-binding site upstream of the toxic repeats affects replication efficiency, antisense transcription and chromatin dynamics leading to genetic instability and anticipation. Recent work from Christopher Pearson’s laboratory has elucidated the almost exclusive maternal transmission of the congenital disease phenotype (Thornton, 2014; Barbé et al., 2017). By examining patients with higher repeat length (> 100 repeats), the authors were able to disprove the notion from early work suggesting a strong positive correlation between paternal transmission and offspring repeat length (Brunner et al., 1993). Dr. Pearson’s laboratory has demonstrated and postulated that larger allele size in sperm may promote germline contraction frequency, reducing methylation, thus promoting CTCF-binding-induced stability (Cleary et al., 2010; Barbé et al., 2017). The increased stability in the shorter CTG sizes gives rise to the increased bias for paternal transmission for a contracted repeat length in the offspring and contribute for the maternal bias of congenital DM1 (Barbé et al., 2017). The research done elucidating the genetic signature and pathogenesis of DM1 has allowed for a better predictive model, definition of the differing phenotypes and understanding of the natural progression of the disease.

Pathophysiology

Early work with DMPK-knockout mice disproved the lack of normal DMPK protein as the cause for this pathology (Jansen et al., 1996). The expanded DMPK transcripts form stable, double-stranded hairpin structures that aggregate in myonuclei. These CUG expansions result in the dysregulation of several important RNA-binding proteins (RNABPs), namely the nuclear sequestration of the Muscleblind-like protein family (MBNL1-3), as well as protein kinase C-mediated hyperphosphorylation and stabilization of CUGBP Elav-Like Family Member 1 (CELF1 also known as CUGBP1; Chau & Kalsotra, 2015). The imbalance in MBNL versus CELF1 mRNA splicing activities result in the clinical presentation of the disease. Models lacking functional MBNL1 protein recapitulates 80% of the DM1-related alternative splicing events (Wang et al., 2012). MBNL1 has been shown to regulate mRNA stability and membrane-localization while CELF1 regulates mRNA decay (Masuda et al., 2012; Wang et al., 2012; Batra et al., 2014). Indeed, the dysregulation of these RNABPs results in an adult-to-fetal switch in pre-mRNA splicing patterns characterizing the misregulated mRNA processing events associated with the DM1 myopathy. These immature isoforms are unable to meet the requirements of adult skeletal muscle, which then manifest into DM1 symptoms. For example, the myotonia of DM1 is largely attributed to the fetal isoform splicing pattern of the muscle-specific chloride channel (CLC-1; Chau & Kalsotra, 2015). The loss- and gain-of-function of MBNL1 and CELF1, respectively, contribute to this aberrant splicing pattern of CLC-1 mRNA, which includes exon 7a. CLC-1 exon 7a, which is normally excluded from the mature transcript in adult skeletal muscle, contains a premature stop codon resulting in nonsense-mediated decay of the mRNA (Lueck & Wheeler, 2007). Similarly, the insulin resistance associated with DM1 can be attributed to the adult-to-fetal switch in the mRNA splicing pattern of the insulin receptor alpha isoform (IR-A). IR-A is the alpha subunit isoform without exon 11 that has a higher affinity for insulin, a higher internalization rate, and a lower signalling ability, as compared to insulin receptor beta isoform (IR-B; Guiraud-Dogan et al., 2007; Savkur, Philips, & Cooper, 2001). IR-B is the beta subunit isoform containing exon 11, which demonstrates a higher kinase activity level, while transmitting the insulin signal more effectively versus the IR-A (Savkur et al., 2001; Guiraud-Dogan et al., 2007). In healthy individuals, there is a significantly greater ratio of IR-B:IR-A in skeletal muscle, allowing for efficient insulin signalling (Savkur et al., 2001). In contrast, IR-A is the main isoform in DM1 skeletal muscle, resulting in decreased insulin signalling because of impaired insulin-related kinetics (Savkur et al., 2001). Much like other alternatively spliced mRNAs, the expression of the fetal isoform of the IR transcript is unable to meet the demand of adult tissue, which manifests into the clinical symptom of insulin-resistance.

There is evidence to suggest that while the expansion length of the CUG repeats in skeletal muscle does not correlate with splicing misregulation nor muscle weakness, alternative splicing events can serve as a biomarker for DM1 severity (Nakamori et al., 2013). Several splicing events have been implicated in DM1-associated muscle weakness including a reduction of bridging integrator 1 (BIN1) exon 11 inclusion, which correlates most strongly with muscle weakness (Fugier et al., 2011; Nakamori et al., 2013). BIN1 exon 11 inclusion is necessary for proper T-tubule formation, which is critical for excitation-contraction coupling (Fugier et al., 2011). Other alternative splicing events that regulate excitation-contraction coupling also correlate highly with muscle weakness, including mRNAs for the ryanodine and dihydropyridine receptors (Nakamori et al., 2013; Ravel-Chapuis et al., 2017).

Calcium ion (Ca^{2+}) signalling plays a crucial role in regulating skeletal muscle development, maintenance and function (Semsarian et al., 1999; Tu et al., 2016). Intramuscular Ca^{2+} flux and homeostasis are achieved through orchestration of several Ca^{2+}-handling proteins that collectively translate T-tubule membrane depolarizations, release Ca^{2+} from the sarcoplasmic reticulum, and pump the ions back for subsequent releases (Tavi & Westerblad, 2011). Through transient intramuscular Ca^{2+} fluctuations, key enzymes are activated and able to regulate acute processes, such as muscle contraction, as well as chronic adaptations, including a shift in muscle phenotype (Semsarian et al., 1999; Tavi & Westerblad, 2011; Ravel-Chapuis et al., 2017). Recent work has shown that Ca^{2+}-signalling is hyperactive in DM1 mice, leading to an increase in calcineurin (Cn) activity (Ravel-Chapuis et al., 2017). Cn has garnered a great deal of research attention as it has been shown to be pivotal for physiological muscle adaptations (Semsarian et al., 1999). Cn activity leads to an increase in the levels and nuclear translocation of the transcription factor Nuclear factor of activated T-cells, cytoplasmic 1 (NF-ATc1), which regulates the transcription of proteins involved in the slow-oxidative myogenic program (Semsarian et al., 1999). In DM1 mice, basal levels and activity of Cn are increased, coinciding with the missplicing of calcium-handling mRNAs, aberrant corresponding protein levels and increased intramuscular Ca^{2+} concentration (Ravel-Chapuis et al., 2017). As a prominent characteristic of the DM1-phenotype is the increase in type 1 oxidative fibers, the authors suggest a novel mechanism involving the pathological increase in Cn signalling (Thornton, 2014; Ravel-Chapuis et al., 2017; Morriss et al., 2018). While the authors also observed other hallmarks of the DM1-related myopathy, they did not provide measures of muscle wasting and weakness.

Skeletal muscle loss accounts for 60% of mortality associated with DM1 however the underlying mechanism directly underlying skeletal muscle wasting remains largely unknown (Thornton, 2014; Bargiela et al., 2015; Morriss et al., 2018). Utilizing a tetracycline-inducible, skeletal muscle-specific mouse model of DM1, recent work from Thomas Cooper's laboratory has implicated perturbed metabolic processes rather than the spliceopathy as a mechanism for this manifestation (Morriss et al., 2018). Despite carrying 960 CUG repeats, these mice (CUG960) only demonstrated a mild spliceopathy and severe muscle wasting and weakness. As muscle loss and MBNL1-sequestration were rescued when the gene was "turned off, alternative pathomechanisms resulting in these clinical symptoms were explored. Examining the activation of key kinases and proteins in CUG960 ¬mice, Morriss and colleagues observed that the top canonical pathways involved were the Phosphatidylinositol-4,5-bisphosphate 3-kinase/Protein kinase B (PI3K/Akt) pathway and glucocorticoid receptor signalling. The former is primarily anabolic and regulates muscle hypertrophy, while the latter can indirectly activate the autophagic/lysosomal pathway, resulting in muscle atrophy. As there is evidence to support crosstalk between these anabolic and catabolic processes, these data support that maintenance of skeletal muscle mass in DM1 is dysregulated.

Autophagy is the coordinated, intracellular degradation process of unnecessary or dysfunctional cellular components. Autophagy is required for the maintenance of skeletal muscle homeostasis, as evidenced by the myopathy observed in transgenic animals that lack autophagy-related genes. Autophagic flux is governed by various stress stimuli, including fasting and exercise, and has been documented to mediate chronic exercise-induced health benefits (Vainshtein & Hood, 2016). Recent work strongly suggests that in DM1 mice and in DM1 patient cells, the 5' AMP-activated protein kinase (AMPK) and the mechanistic target of rapamycin complex 1 (mTORC1) pathways are dysregulated and that autophagic flux is perturbed in skeletal muscle (Brockhoff et al., 2017; Dial et al., 2018). For example, in response to both physiological and pharmacological cues for autophagy induction, muscles from the human skeletal actin-long repeat (HSA-LR) mouse model (Mankodi et al., 2000) demonstrated reduced microtubule-associated proteins 1A/1B light chain 3B (LC3) lipidation and puncta formation, as compared to wild-type (WT) mice. Earlier work in cultured myoblasts collected from DM1 fetuses shows dysregulated autophagic signalling concomitant with aberrant skeletal muscle differentiation, potentially shedding light on disease pathogenesis (Beffy et al., 2010). Other models have suggested that the basal autophagic flux is pathologically increased contributing to the progression of the disease pathology (Bargiela et al., 2015; Morriss et al., 2018). Moreover, work in drosophila models of DM1 demonstrate a 35% decrease in mean muscle area, which was rescued when genes related to autophagy were silenced (Bargiela et al., 2015). Thus, the literature shows that while the CUG repeat-foci may contribute to aberrant protein levels and correlate with clinical symptoms, the perturbations in key metabolic processes such as Ca^{2+} homeostasis, PI3K/Akt signalling, and autophagy, account for the progressive nature of DM1 (Figure 1).

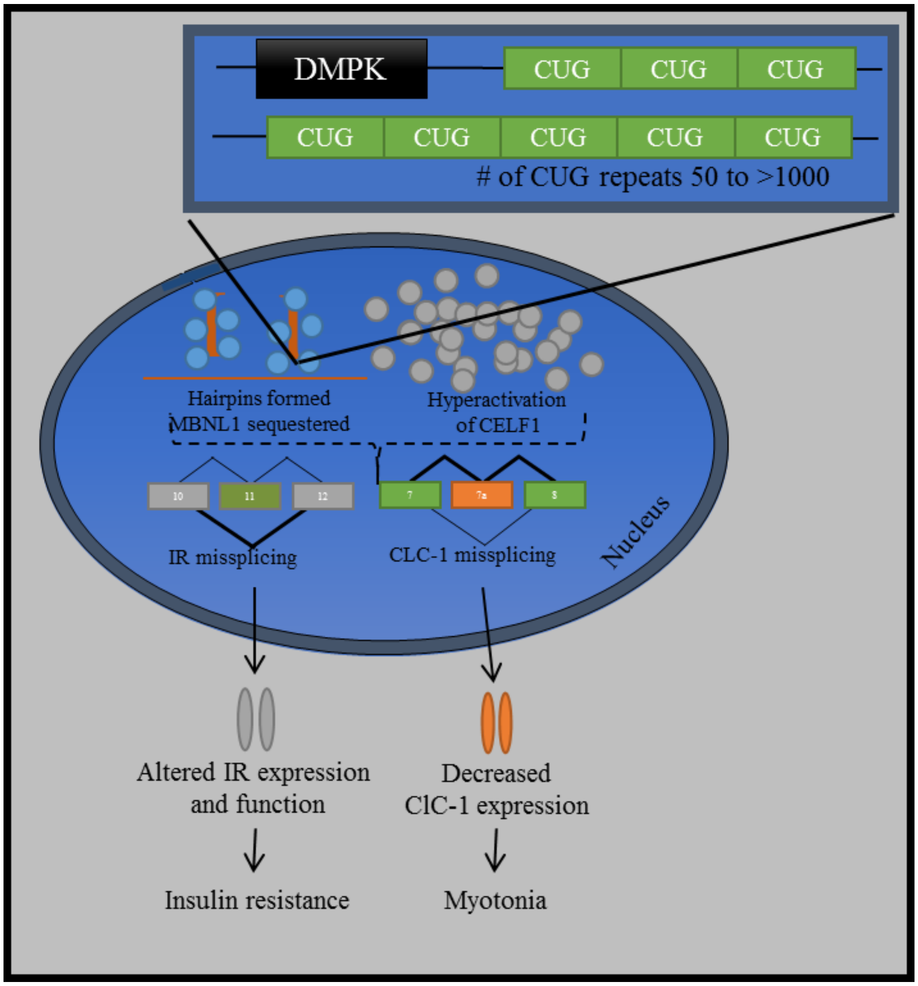
DMPK
CUG
CUG
CUG
CUG
CUG
CUG
CUG
CUG
# of CUG repeats 50 to >1000
Hairpins formed
MBNL1 sequestered
Hyperactivation
of CELF1
10
11
12
7
7a
8
IR missplicing
CLC-1 missplicing
Nucleus
Altered IR expression
and function
Decreased
ClC-1 expression
Insulin resistance
Myotonia

Figure 1. Overview of pathogenesis of DM1. In DM1, the DMPK gene contains >50 CTG repeats that cause its transcripts to form stable arrays of CUG hairpins, which aggregate as myonuclear foci. These hairpins sequester MBNL1 causing its loss of function, while CELF1 exhibits an aberrant gain of function. Collectively, the alterations to these RNABPs lead to the aberrant alternative splicing of transcripts including the exclusion of IR exon 11 that corresponds to reduced IR expression and insulin sensitivity, as well as the inclusion of ClC-1 exon 7a that causes myotonia. Abbreviations: CLC-1, muscle-specific chloride channels; DM1, myotonic dystrophy type 1; DMPK, dystrophia myotonica protein kinase; IR, insulin receptor; RNABP, RNA-binding protein

Current treatments

Currently there is no cure for DM1, however there is promising evidence in pre-clinical models that the use of small (or short) interfering RNA (siRNA/shRNA) technologies or small molecules may ameliorate the disease phenotype by targeting DMPK transcript degradation or disrupting the MBNL1 and CUG repeat-foci interaction, respectively (Wheeler et al., 2007, 2012; Warf et al., 2009; Thornton et al., 2017). Transcription silencing is a strategy that is typically not robust enough to treat gain-of-function mutations (Thornton et al., 2017). However, CTG repeats are known to cause RNA polymerase II stalling, perhaps increasing its susceptibility to interventions that target transcription inhibition. Recent work has highlighted the ability of pentamidine as a potent inhibitor of CTG repeat transcription through direct interaction with the repeats. Treatment with pentamidine, as well as other analogs, selectively rescued certain missplicing events, namely Fast skeletal muscle troponin T (TNNT3) and CLC-1, in a cell model of DM1 and in the HSA-LR murine model, respectively (Coonrod et al., 2013). Future work is required to see if this molecule has the same potency at the DMPK locus. While other analogs have been synthesized to reduce the toxicity of this class of small molecules, experiments on its safety and efficacy in DM1 patients are needed to assess its full therapeutic potential (Siboni et al., 2015; Thornton et al., 2017).

The use of antisense oligonucleotides (ASOs) is an effective genetic strategy to knockdown toxic RNA post-transcriptionally (Mulders et al., 2009; Jauvin et al., 2017). ASOs targeting MBNL-CUG repeat expansion binding, CLC-1 exon 7a exclusion, and CUG reduction via binding outside the repeat tract, have been demonstrated to effectively attenuate MBNL-myonuclear sequestration and splicing errors in cell cultures from DM1 patients, as well as in pre-clinical murine models of the disorder (Wheeler et al., 2007, 2012; Mulders et al., 2009). For many ASOs, the limitation still remains elucidating an effective systemic delivery to all affected tissues, particularly cardiac muscle and the central nervous system (Havens & Hastings, 2016; Khorkova & Wahlestedt, 2017). Most recently, Jauvin et al. tested systemic delivery of two ASOs that previously have shown strong efficacy, pharmacokinetics and pharmacodynamics (Pandey et al., 2015). Four weeks of biweekly subcutaneous injections were able to reduce DMPK expression in six different muscles and the heart in the DMSXL murine model. Concomitantly, there was a 43% reduction in the proportion of myonuclei with CUG repeat-foci. This model expresses 1,000-1,600 CTG repeats downstream of the human DMPK gene under mouse-regulated transcriptional machinery (Huguet et al., 2012). Utilizing this model allows for screening of ASOs targeting the repeats within the DMPK locus. A previous study also demonstrated the alleviation of the DM1-molecular signature with systemic administration of an ASO (Wheeler et al., 2012). However, this study is the first to show that reduction in physiological, histopathologic, and transcriptomic features of the disease coincided with alleviation of clinical symptoms such as myotonia and muscle weakness. Moreover, the effect on DMPK transcript reduction was seen in the heart suggesting that the ASOs displays tissue sensitivity. While there was no effect on the brain, as to be expected with ASOs, future studies should examine the effects of intrathecal injections, like the administration of the SPINRAZA ASO in patients with spinal muscular atrophy.

There is also evidence for the therapeutic potential of genomic editing via the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9-mediated cleavage. Using complimentary RNA to the mutant CUG repeats, the use of this system effectively degraded the expansion, alleviated the MBNL1 loss-of-function, and normalized many alternative splicing events (van Agtmaal et al., 2017; Batra et al., 2017). Though these results are promising to not only DM1, but also other diseases linked to microsatellite nuclear expansions, further work assessing the viability using in-vivo models is required. While these obstacles will very likely be surmounted eventually, it would be advantageous to identify lifestyle-based interventions that are safe, effective, and immediately deployable to improve muscle function and increase quality of life in DM1 patients.

Exercise and DM1

Exercise is a safe, cost-effective and beneficial strategy to not only combat the progressive nature of the disease such as muscle weakness and wasting but to potentially attenuate the DM1 molecular signature. Though a large randomized control trial RCT assessing the effect of exercise on the DM1-molecular signature as not yet been conducted, there is evidence to suggest that exercise in DM1 is well received and results in improvements in muscle functionality and measures of quality of life (Ng et al., 2018). Pioneering work from Lindeman and colleagues (1995) sought to assess strength training in patients with DM1. This was the first clinical trial that included a separate, non-exercise control group of DM1 patients. Previous work investigating exercise training in slow, progressive NMDs utilized either the contralateral untrained muscle group or a healthy control group (Milner-Brown & Miller, 1988; Kilmer et al., 1994). Following 24-weeks of free weight training targeting knee movements, knee torque measures did not change between the training and control group. However, the authors did report an increase in functional abilities. DM1 participants in the training group reported improvements on standing, getting into and out of the car and putting on socks. No patients, including those with the lowest baseline strength measures, showed any signs of overwork damage. Specifically, serum myoglobin levels, a marker of muscle fiber permeability due to damage, did not differ from the control, non-exercise training group.

Though this field was further explored in several other studies (See Table 1 for summary of all exercise studies on DM1 patients; Tollbäck et al., 1999; Aldehag et al., 2005), the first RCT, was not conducted until nearly a decade later (Aldehag et al., 2013). This study implemented 12 weeks of specific hand training. The participants did show marginal, albeit significant, improvements in muscle force and fine motor control but not in grip force, recapitulating the previous findings from their cohort study (Aldehag et al., 2005).

Aerobic exercise in DM1 has not been studied as heavily as resistance training. Early evidence evaluating light aerobic exercise in a variety of NMDs showed no difference in heart rate, power output and blood pressure. The training consisted of walking at 50-60% of max heart rate for 15-30 minutes for 12 weeks. It is worth noting that only 5 participants had DM, not specifying the subtype (Wright et al., 1996). The first study to exclusively examine aerobic exercise in DM1 patients was conducted almost a decade later. 12 weeks of ergometer cycling at 65% maximal oxygen uptake demonstrated positive adaptations in 12 patients with DM1. Improved workload was observed as well as patients were able to achieve this workload with a lower heart rate. No signs of damage or discomfort were observed and myofibre cross-sectional area increased, indicating beneficial physiological adaptations (Ørngreen et al., 2005).

Acknowledging the observed, albeit inconsistent, beneficial adaptations with both resistance and aerobic training, recent evidence attempted to use a training protocol that utilized strength, aerobic and balance exercises in DM1 patients. (Kierkegaard et al., 2011). While this study showed no difference in any of their outcome measures, the protocol was well-received with no signs of training-related muscle damage.

In total, it is difficult to summarize the limited research done with exercise due to differences in training regiments, small sample sizes and variability among participants.

Understanding the difficulty in reproducibility with short-term, controlled exercise trials and in order to ascertain the longer term benefits, Brady and colleagues (2014) retrospectively analyzed clinical information of DM1 patients to determine whether there was an association between long-term habitual exercise and muscular improvements. Their results suggest that those individuals who chronically exercised outperformed in grip strength, as well as in knee extension and elbow extension torques, as compared to DM1 patients who were habitually sedentary. Moreover, a marked decrease in knee extension strength was observed in those who stopped exercising over the course of the investigation (Brady et al., 2014). Those who began exercising improved their strength, suggesting that sedentary DM1 patients have a lot to gain from beginning to exercise (Aldehag et al., 2013; Brady et al., 2014). This notion is extremely important as recent survey data suggests that of the 200 included DM1 patients (158 with the classical phenotype and 42 with the mild phenotype), 68.5% wish to do more exercise and 76% currently exercise less than three times per week (Gagnon et al., 2013). Developing an effective training protocol may have great benefits since the literature suggests that compliance in DM1 patients is typically high.

Potential mechanism mediating exercise adaptations

Of the studies examining exercise in DM1 that were discussed above, the only molecular analyses implemented are those pertaining to assessing muscular damage and muscle phenotype (Lindeman et al., 1995; Ørngreen et al., 2005; Tollbäck et al., 1999). This presents a major gap in knowledge, as the effects of exercise on the molecular signature of DM1. Recently, Ravel-Chapuis and colleagues (2018) demonstrated that voluntary running for 8 weeks can rescue select missplicing events in HSA-LR mice including TNNT3, CLC-1 and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA or ATP2A1). While the direct cellular mechanism was not elucidated, this provides great support for the therapeutic potential of exercise in DM1.Since it has been shown that as many as 20 different missplicing events correlate with muscle weakness, further understanding the adaptations to exercise, as well as the effect on DM1-specific symptoms can highlight the endogenous molecular modifiers involved in the exercise-induced cellular response. This has the potential to provide further insight into perturbed pathways mediating the progression of the disease (Nakamori et al., 2013; Thornton et al., 2017).

AMPK is a key cellular sensor critical for energy homeostasis. It is activated during times of cellular stress with the purpose inhibiting energy-demanding anabolic processes while activating energy-producing catabolic processes. Physiologically, it is activated by a depletion in ATP, the body's main source for energy, and an increase in its by-product AMP, otherwise known as an increase in the AMP:ATP ratio. Various stimuli can activate this molecule including starvation, stress and exercise. AMPK is a key molecule in driving the slow-oxidative myogenic program associated with exercise adaptations (Dial et al., 2018; Ng et al., 2018; Steinberg & Kemp, 2009). Through a plethora of literature utilizing either pharmacological inhibition or genetic knockdown of AMPK subunits, the kinase has been shown to govern mitochondrial biogenesis, angiogenesis, fiber-type plasticity, autophagy and glucose uptake (O'Neill et al., 2013; Laker et al., 2017). Knocking-out both the β1 and β2 subunits in skeletal muscle produced transgenic mice that were exercise intolerant. These mice displayed reduced mitochondrial content, impaired contraction-stimulated glucose uptake and drastic reduction in treadmill running (O'Neill et al., 2011). While there are other key regulators of these processes such as Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), as well as nuclear respiratory factor 1 (NRF-1) and nuclear respiratory factor 2 (NRF-2)/GA-binding protein, the levels and activity of these molecules are regulated upstream by the activation of

AMPK (Zong et al., 2002; Ljubicic et al., 2014). For example, in response to starvation, wildtype mice demonstrated increased levels of PGC-1α and enhanced binding of PGC-1α to NRF-1, indicative of its activity and enhanced mitochondrial gene expression. However, this stimulus was unable to evoke these responses in mutant AMPK mice (Zong et al., 2002). Concomitantly, pharmacological activation of AMPK increases mitochondrial protein content and promotes a slower, more oxidative fiber type, in part, by stimulating PGC-1α (Suwa et al., 2003; Dial et al., 2018). AMPK has also been suggested to mediate the plasticity of the neuromuscular junction and α-motor neurons (Dial et al., 2018). Though its role is not fully understood, there is evidence to suggest that its regulation of α-motor neurons may have therapeutic potential in amyotrophic lateral sclerosis, since pharmacological treatment preserved the α-motor neurons and increased the lifespan of a murine model of the disease (Mancuso et al., 2014).

Work once again from Dr. Thomas Cooper's laboratory was the first to suggest the potential disease-modifying role of AMPK in DM1 biology. Savkur and colleagues assessed the insulin sensitivity of DM1 cell-lines as compared to healthy controls. The authors noted that although DM1 cultures had lower insulin responsiveness, as explained by the higher proportion of IR-A, stimulation of glucose uptake by metformin (MET) was similar between both cell lines (Savkur et al., 2001). MET can stimulate insulin-independent glucose uptake via AMPK activation (Zhou et al., 2001). These data were complimented with individual case reports of DM1 patients who take MET to combat insulin resistance (Kouki et al., 2005).

Laustriat and colleagues (2015) demonstrated that MET can modulate alternative splicing in both in vitro and in vivo DM1 models. MET increase IR exon 11 by ~90%, TNNT2 exon 5 exclusion by ~70% and CLC-1 exon 7a exclusion by approximately 36%. In fact, high-throughput analysis of the transcriptome suggested that 416 splicing events were regulated by

MET (Laustriat et al., 2015). More recently, seven days of daily 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) administration to chronically activate AMPK was effective at either fully or partially correcting several abnormal characteristics of skeletal muscle at the physiological, cellular, and molecular levels (Brockhoff et al., 2017; Ravel-Chapuis et al., 2018). For example, myotonia was reduced, which was caused, in part, by increased skeletal muscle CLC-1 protein content due, in turn, to a normalization of CLC-1 mRNA splicing that occurred coincident with a reduction in myonuclear CUG-repeat foci (Brockhoff et al., 2017). It is reasonable to suspect therefore that via an AMPK-mediated pathway the DM1-molecular signature can be alleviated (Dial et al., 2018).

AMPK can potentially disperse the CUG-repeat foci through its interaction with heterogeneous nuclear ribonucleoprotein H (hnRNP H). There is evidence to suggest that AMPK can translocate to the nucleus as early as 60 minutes after exercise to increase the expression of key metabolic genes (da Silva Xavier et al., 2000; McGee et al., 2003). These data were complimented with an AICAR- and MET-induced nuclear translocation (Kim et al., 2014). In the nucleus, AMPK phosphorylates hnRNP H, mediating glucose uptake (Kim et al., 2014). hnRNP H has also been suggested to contribute to the stability of the CUG-repeat foci in DM1 perhaps elucidating the disease-modifying mechanism by which AMPK exerts its function. Knockdown of this RNA binding protein in a cell line expressing a gene tag upstream of CUG repeats, demonstrate no nuclear aggregation of the toxic repeats and sequestration of the corresponding gene (Kim et al., 2005).

Alternatively, there is evidence to suggest that AMPK might control alternative splicing of mRNAs via a mechanism involving its downstream target PGC-1α. Though as described above, PGC-1α is traditionally viewed as a transcriptional coactivator and a key regulator of mitochondrial biogenesis through its interaction with NRF-1, PGC-1α also regulates mRNA processing. Monsalve and colleagues observed in skeletal muscle cells that PGC-1α associates with, and alters the activities of, splicing factors and RNA polymerase II during both the mRNA initiation and elongation phases, while Martinez-Redondo et al. engineered skeletal muscle-specific PGC-1α transgenic animals that demonstrated alternative splicing reactions mediated by the coactivator in vivo (Monsalve et al., 2000; Martínez-Redondo et al., 2016).

Additional investigations that expand our knowledge of the mechanisms by which AMPK positively affects insulin sensitivity and glucose metabolism, mRNA processing and alternative splicing, as well as autophagy in DM1 muscle are certainly warranted. Moreover, the stimuli by which AMPK activation is achieved may differentially affect some of the aforementioned processes. For example, while AICAR was able to recapitulate rescuing of the spliceopathy in most alternative splicing events as MET, it was unable to improve INSR exon 11 inclusion (Laustriat et al., 2015). Conversely, AICAR-treatment improved late relaxation time and rescued CLC-1 missplicing in the HSA-LR model but MET-treatment was unable to have these effects (Brockhoff et al., 2017). To best understand the therapeutic potential of targeting this metabolic pathway, endogenous stimuli of AMPK, such as exercise, in DM1 must be examined (Figure 2; Dial et al., 2018).

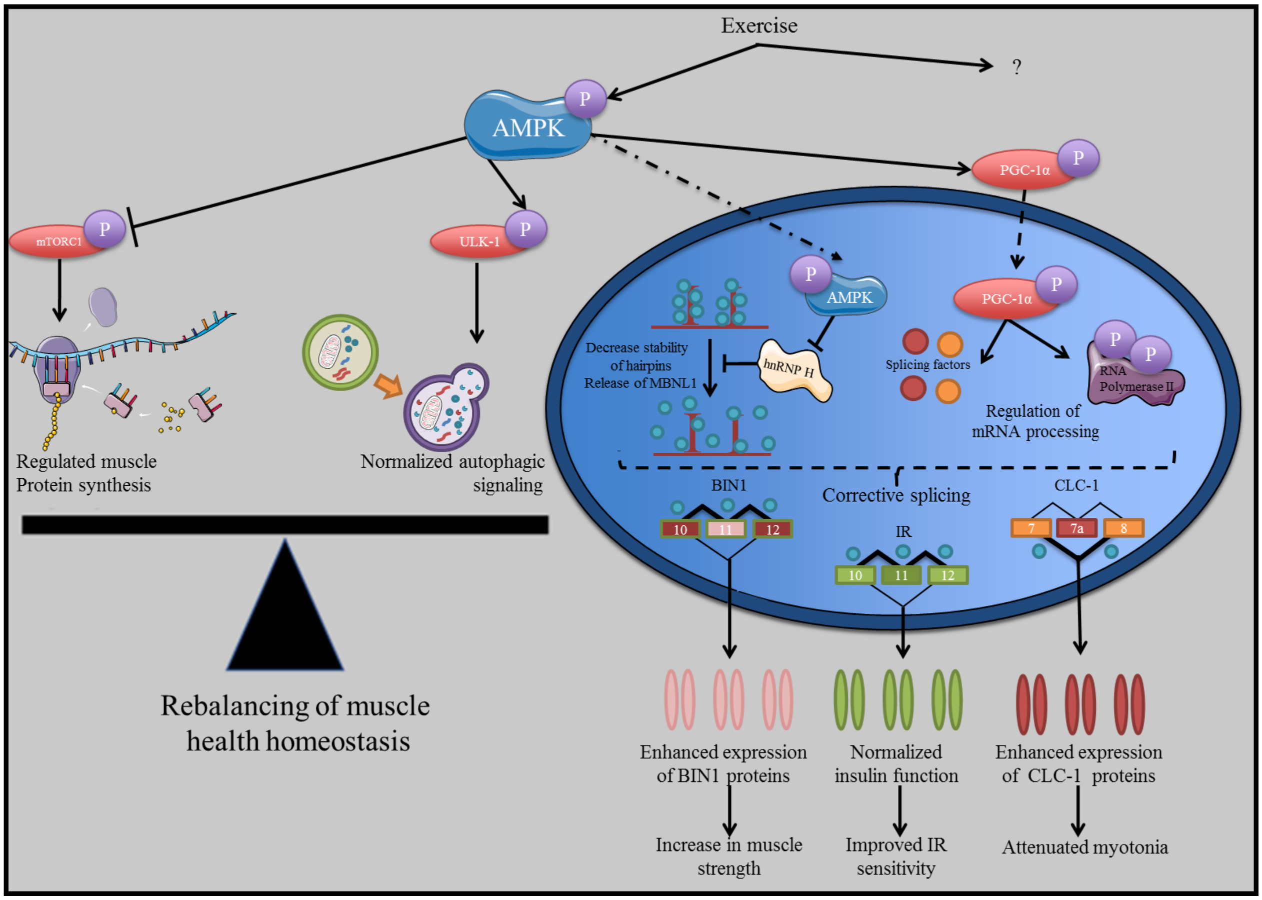
Exercise
?
AMPK
P
mTORC1
P
ULK-1
P
PGC-1α
P
AMPK
P
PGC-1α
P
Decrease stability of hairpins Release of MBNL1
hnRNP H
Splicing factors
P
P
RNA Polymerase II
Regulation of mRNA processing
Regulated muscle Protein synthesis
Normalized autophagic signaling
BIN1
10
11
12
Corrective splicing
IR
10
11
12
CLC-1
7
7a
8
Rebalancing of muscle health homeostasis
Enhanced expression of BIN1 proteins
Normalized insulin function
Enhanced expression of CLC-1 proteins
Increase in muscle strength
Improved IR sensitivity
Attenuated myotonia

Figure 2. Potential therapeutic mechanism of AMPK in DM1. There is evidence to suggest that AMPK can alleviate hallmark features of DM1, namely the excessive muscle wasting as well as the missplicing of genes necessary for muscle health and function. AMPK can normalize muscle homeostasis by regulating the turnover of dysfunctional cellular constituents via phosphorylation of ULK-1, a master regulator of autophagy and muscle protein synthesis through phosphorylation of mTORC1. The normalization in aberrant ASE and subsequent rescuing of protein content of proteins such as BIN1 and CLC-1 as well as signalling of IR is hypothesized to occur via two mechanisms. 1) Evidence demonstrates that upon the translocation of AMPK in the nucleus, it interacts with hnRNP H, a stabilizing protein for the myonuclear foci. Disrupting this interaction may liberate sequestered MBNL1. Additionally, AMPK-mediated PGC-1α activation may regulate a splicing correction cascade via PGC-1α exon splicing. We acknowledge that there may be alternative mechanisms by which exercise can alleviate the molecular signature of DM1 independent of AMPK. Unbroken lines represent an interaction between the two connected molecules while broken lines denote translocation. Abbreviations: AMPK, AMP-activated protein kinase; BIN1, bridging integrator 1; CLC-1, skeletal muscle-specific chloride channels; DM1, myotonic dystrophy type 1; hnRNP, heterogeneous nuclear ribonucleoprotein H; IR, Insulin receptor; MBNL1, muscleblind-like 1; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α; ULK-1, unc-51-like autophagy activating kinase 1; UTR, untranslated region.

Thesis Purpose, Objectives, and Hypothesis

There is no cure for DM1, which signifies a critically unmet clinical need. Exercise is a safe, effective, accessible, and therefore practical lifestyle intervention that reduces all-cause mortality and improves quality of life, in part by evoking favourable systemic adaptations via the stimulation of myriad gene expression programs (Ng et al., 2018). Studies examining the efficacy of exercise training in DM1 participants clearly demonstrate that physical activity can elicit modest, but significant physiological benefits, such as improved strength, endurance, function, and quality of life metrics (Lindeman et al., 1995; Wright et al., 1996; Aldehag et al., 2005; Ørngreen et al., 2005; Kierkegaard & Tollbäck, 2007; Aldehag et al., 2013; Ng et al., 2018; Ravel-Chapuis et al., 2018)). However, the molecular mechanisms of exercise adaptation in DM1 are unknown. Understanding the cellular processes that drive exercise-induced remodelling in DM1 is important because it 1) will increase our knowledge of the basic biological mechanisms of the disorder, and 2) may assist in the discovery of more effective lifestyle and/or pharmacological interventions to mitigate the disease.

Thus, the Purpose of this thesis is to examine the cellular and molecular mechanisms of exercise-induced neuromuscular plasticity in a pre-clinical model of DM1. The Specific Objectives are to:

1) Explore the impact of chronic exercise on metrics of muscle performance, strength and function utilizing both gross- and in-situ measures;
2) Utilizing intramuscular electromyographic measures, determine if exercise has an impact on attenuating the hallmark symptom of DM1, myotonia
3) Examine the molecular signature germane to DM1, MBNL1 sequestration using combination fluorescence in-situ hybridization and immunofluorescence. We will

explore the potential effect of exercise on the liberation of this RNABP and downstream effect on normalization of MBNL1-mediated splicing events such as SERCA, CLC-1 and BIN1 using both endpoint and Realtime polymerize chain reaction techniques.

We hypothesize that chronic exercise will ameliorate the disease phenotype at the physiological, cellular, and molecular levels.

Table 1: Summary of exercise studies with DM1 participants **(Ng et al., 2018)**

Exercise mode	*Participant cohort size*	*DM type*	*Exercise Protocol*	*Safety*	*Adherence*	*Effects*	*Reference*
Habitual physical activity	63	DM1	Retrospective study; participants were habitually active > 2x per week	Not stated	Not stated	Mean handgrip strength, knee extension and elbow flexion torques higher in active cohort versus sedentary	Brady, MacNeil, Tarnopolsky, 2014
Hand resistance training	35	DM1	12 week training program; 3x per week	Not stated	Ten participants dropped out; 13 participants had acceptable adherence (> 75%)	Isometric wrist flexor force improved post-training; no change in handgrip force; satisfaction and change in self-perception of occupational performance increased	Aldehag et al., 2013
Resistance and endurance exercise	35 (n = 17 trained; n = 18 sedentary)	DM1	14 week training program; 2x per week; 60 minute group-training programme at 60% MHR	One participant demonstrated abnormal ECG, otherwise no adverse events reported	11/18 participants ≥ 75% attendance	Mean 6MWT increased ($P > 0.05$) in the trained group by 9m	Kierkegaard et al., 2011
Leg cycle-	12	DM1	12 week training program; 5x per week;	Plasma creatine kinase was	76% adherence to training	Training improved VO_{2max} by 14%, maximal workload	Ørngreen et

ergometer			intensity of 65% of VO_{2max}; 35 minute sessions	unchanged	program	by 11%; increased type I and IIa fibre cross sectional area; no change in capillary density	al., 2005
Hand resistance training	5	DM1	12 week training program; 3x per week	Not stated	Not stated	Increase in muscle force of wrist and finger extensors and flexors of the dominant hand; no difference in grip force; improved fine motor control in both hands; participants rated high occupational performance and satisfaction	Aldehag et al., 2005
Resistance training	9	DM	12 week training program; 3x per week; progressive resistance training with 3 x 10 repetitions 80% 1RM	No histological evidence of increased muscle damage with training	6/9 participants completed training program	1 RM increased post-training; no change in peak isokinetic torque; no difference in muscle cross sectional area; no effect on fiber type distribution	Tollbäck et al., 1999

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Skeletal muscle adaptations to chronic exercise in a pre-clinical model of myotonic dystrophy type 1

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Abstract

Myotonic dystrophy type 1 (DM1) is a trinucleotide repeat expansion neuromuscular disorder that is most prominently characterized by skeletal muscle weakness, wasting, and myotonia. Chronic physical activity is safe, satisfying, and can elicit functional benefits such as improved strength and endurance in DM1 patients, however the underlying cellular basis of exercise adaptation is undefined. Our purpose was to examine the mechanisms of exercise biology in DM1. Healthy, sedentary wild-type (SED-WT) mice, as well as sedentary human skeletal actin long repeat animals, a murine model of DM1 myopathy (SED-DM1), and DM1 mice with volitional access to a running wheel for 7 weeks (EX-DM1), were utilized. Chronic exercise augmented strength and endurance in vivo and in situ in DM1 mice. These alterations coincided with normalized histological measures of myopathy, as well as increased mitochondrial content. Electromyography revealed a 70-85% decrease in the duration of myotonic discharges in muscles from EX-DM1 compared to SED-DM1 animals. The exercise-induced enhancements in muscle function corresponded at the molecular level with mitigated spliceopathy, specifically the processing of bridging integrator 1 and muscle-specific chloride channel (CLC-1) transcripts. ClC-1 protein content and sarcolemmal expression were lower in SED-DM1 versus SED-WT animals, however they were similar between SED-WT and EX-DM1 groups. Chronic exercise also attenuated RNA toxicity, as indicated by reduced CUG)$_n$ foci-positive myonuclei and sequestered muscleblind-like 1 (MBNL1). Our data indicate that chronic exercise-induced physiological improvements in DM1 occur in concert with mitigated primary downstream disease mechanisms, including RNA toxicity, MBNL1 loss-of-function, and alternative mRNA splicing.

Introduction

Myotonic dystrophy type 1 (DM1) affects ~1/8,000 individuals, making it the most common form of myotonic dystrophy (Chau & Kalsotra, 2015). It is also the most prevalent adult form of muscular dystrophy, as well as the second most common type of muscular dystrophy after Duchenne muscular dystrophy (DMD; Chau & Kalsotra, 2015). DM1 is an autosomal dominant trinucleotide repeat neuromuscular disorder with multisystem involvement, which is most prominently characterized by skeletal muscle weakness, wasting, myotonia, and insulin resistance. DM1 is caused by a CTG microsatellite repeat expansion mutation in the 3' untranslated region (UTR) of the dystrophia myotonica protein kinase (DMPK) gene (Cho & Tapscott, 2007). Healthy individuals have between 5-37 repetitions of the CTG trinucleotide sequence, whereas pre-mutation symptoms will manifest with 38-49 repeats and individuals with 50 repeats or more are diagnosed with DM1 (Cho & Tapscott, 2007). Generally, the greater the number of repeats portends an earlier age of symptom onset, as well as a more severe phenotype (Brook *et al.*, 1992). Furthermore, the appearance and severity of the disorder is also related to the extent and pattern of epigenetic methylation of CpG islands upstream of the repeats (Barbé *et al.*, 2017).

The DM1 mutation results in expanded DMPK transcripts, which form stable, double-stranded hairpin structures that aggregate as foci within nuclei. Via a toxic gain-of-function mechanism, these CUG expansions result in the dysregulation of several important RNA-binding proteins (RNABPs), namely Muscleblind-like 1 (MBNL1; Chau & Kalsotra, 2015). MBNL1 becomes sequestered by the expanded CUG nuclear aggregates, which leads to MBNL1 loss-of-function (Kalsotra *et al.*, 2014; Chau & Kalsotra, 2015). MBNL1 plays critical roles in many steps of RNA metabolism, including primarily pre-mRNA processing, as well as in the stability and transport of newly synthesized transcripts. Defects in these events due to MBNL1 loss-of-function contribute significantly to the clinical presentation of the disorder. Thus, the downstream functional consequences of the DM1 mutation are ultimately due to the toxic gain-of-function of DMPK mRNA within nuclei that alters RNABP function. For example, the myotonia of DM1 is largely attributed to the fetal isoform splicing pattern of the muscle-specific chloride channel (CLC-1; Chau & Kalsotra, 2015). The MBNL1 loss-of-function contributes to the aberrant splicing pattern of CLC-1 mRNA, which includes exon 7a. CLC-1 exon 7a, which is normally excluded from the mature transcript in adult skeletal muscle, contains a premature stop codon resulting in nonsense-mediated decay of the mRNA (Wheeler *et al.*, 2007). An adult-to-fetal switch in pre-mRNA splicing patterns characterizes the misregulated mRNA processing events associated with the DM1 myopathy. These immature isoforms are unable to meet the requirements of adult skeletal muscle, which then manifest into DM1 symptoms.

There is no cure for DM1, which signifies a critically unmet clinical need. Exercise is a safe, effective, accessible, and therefore practical lifestyle intervention that reduces all-cause mortality and improves quality of life, in part by evoking favourable systemic adaptations via the stimulation of myriad gene expression programs (Egan & Zierath, 2013). Studies examining the efficacy of exercise training in DM1 participants clearly demonstrate that physical activity can elicit modest, but significant physiological benefits, such as improved strength, endurance, function, and quality of life metrics (Lindeman *et al.*, 1995; Tollbäck *et al.*, 1999; Ørngreen *et al.*, 2005*a*; Aldehag *et al.*, 2005, 2013; Kierkegaard & Tollbäck, 2007; Brady *et al.*, 2014; Dial *et al.*, 2018*a*; Ng *et al.*, 2018). Most recently, work in a pre-clinical murine model of DM1 demonstrated that chronic exercise improved the spliceopathy of select genes germane to the disorder, such as CLC-1, sarco/endoplasmic reticulum Ca^{2+}-ATPase 1 (SERCA) and ryanodine receptor 1 (RYR1; Ravel-Chapuis *et al.*, 2018). Other than these data, the molecular mechanisms of exercise adaptation in DM1 are unknown. Understanding the cellular processes that drive exercise-induced remodelling in DM1 is important because it 1) will increase our knowledge of the basic biological mechanisms of the disorder, and 2) may assist in the discovery of more effective lifestyle and/or pharmacological interventions to mitigate the disease. Therefore, the purpose of this study was to examine the cellular and molecular mechanisms of exercise-induced neuromuscular plasticity in a pre-clinical model of DM1. We hypothesized that chronic exercise would ameliorate the disease phenotype at the physiological, cellular, and molecular levels.

Methods

Animals. Three-to-six month old human skeletal actin-long repeat (HSA^{LR} ; DM1) mice and wild-type (WT) FVB/N animals (The Jackson Laboratory, Bar Harbor, USA) were utilized in this study. DM1 mice were a kind gift from Dr. Charles Thornton at the University of Rochester (Mankodi *et al.*, 2000). Three groups were defined: 1) sedentary WT mice (SED-WT), 2) SED-DM1, and 3) DM1 mice with access to a home cage running wheel for 7 weeks (EX-DM1). Food and water was provided ad libitum. All experiments were approved by the Animal Research Ethics board of McMaster University operating under the auspices of the Canadian Council for Animal Care.

Chronic exercise. EX-DM1 mice were individually-housed with volitional access to a home cage running wheel (Columbus Instruments, Columbus, USA). The number of revolutions was recorded every 10 minutes (min) daily for 7 weeks. The effect of chronic, volitional exercise in WT mice were not examined in the current study as there is an abundance of literature investigating the impact of exercise training on healthy, non-dystrophic animals (Egan & Zierath, 2013), and our focus here is the effects of habitual physical activity in dystrophic context.

Performance testing. One day prior to tissue collection, pen and grip tests were performed as previously described to assess muscular endurance and balance, as well as strength, respectively (Willmann *et al.*, 2011). Briefly, for the pen test, mice were placed on a pen suspended ~50 cm above a foam mat and the duration that the animal remained on the pen before falling was recorded. The average of three attempts for each mouse was applied. For grip strength, mice grasped a wire mesh with either all limbs, or forelimbs only, and the animal was slowly pulled away horizontally by the base of the tail. A transducer recorded the force exerted against the mesh (Columbus Instruments), and the average of three trials was utilized.

Electromyography. Electromyographic recordings were obtained using two tungsten intramuscular electrodes (6-8 MΩ, FHC, Bowdoinham, USA) with a fixed interelectrode distance of 2 mm. Insulation was removed from the tip of each electrode to expose the bare tungsten as a recording surface. This pair of electrodes was inserted into the muscle belly to rest just beneath the fascia. A ground electrode (a third tungsten electrode with 1 cm of insulation removed) was positioned under the retracted skin in the inguinal region. The intramuscular EMG signal was preamplified at × 35 (EQ Inc., Chalfont, USA) and passed through a second-stage, variable-gain amplifier (x20, Model D423A, York University Electronics Shop, Toronto Canada). The EMG signal was digitized at 1,000 Hz (Micro3 1401, Cambridge Electronics Design, Cambridge, UK), and high-pass filtered offline at 25 Hz (Spike2 version 7, Cambridge Electronics Design). To elicit a myotonic response, a 50-Hz train of twenty 200-μs square-wave pulses (Digitimer constant-current stimulator, model DS7A, Hertfordshire, UK) were applied using a small custom-built probe positioned proximal to the recording electrodes on the surface of the muscle. Five separate trains were applied to the medial gastrocnemius (GAST), tibialis anterior (TA), and triceps brachii (TRI). Muscle activity before and after each train of stimuli was assessed offline (Spike2 version 7). The response to tetanic stimulation was characterized by both burst amplitude and burst duration. Root mean square (RMS) was used as a measure of EMG amplitude for a 1-s period after each train of 50-Hz stimulation (Spike2 version 7). Baseline EMG, recorded over a 1-s period prior to stimulation, was subtracted from the post-stimulus EMG. The burst duration value provides a measure of the time it took for EMG to return to baseline when stimulation led to a burst of EMG activity.

In situ skeletal muscle force production. Separate cohorts of the three experimental groups were anesthetised and their triceps surae complex was distally attached to a force transducer (Grass Instrument, West Warwick, USA) and a fatigue protocol was employed as described earlier (Krause *et al.*, 2008). The protocol consisted of eliciting maximal tetanic contractions at 30 Hz for 333 msec in 1 sec duration for 5 min as previously described (Shortreed *et al.*, 2009). Force output was expressed relative to the tension produced during the initial contraction.

Tissue collection. Animals were anesthetised and the TA, extensor digitorum longus (EDL), soleus (SOL), quadriceps and GAST muscles were harvested and immediately snap frozen in liquid nitrogen. Contralateral EDL and SOL muscles were immersed in Optimal Cutting Temperature compound (OCT; VWR, Mississauga, Canada) and frozen in melting isopentane pre-cooled in liquid nitrogen. Muscles examined in the EMG and in situ force production experiments were not used for further cellular and molecular analyses.

Protein extraction and Western blotting. TA muscles were added to a solution of RIPA buffer (20 μL of RIPA per 1 mg muscle weight; Sigma-Aldrich, Oakville, Canada) with dissolved protease and phosphatase inhibitor cocktail tablets (Roche, Mississauga, Canada). Samples were then homogenized using steel ball bearings and a motorized tissue lyser (Qiagen, Hilden, Germany). Following centrifugation at 14,000 x g for 15 min and aspiration of the supernatant, protein concentration was determined via a bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, USA). Muscle protein content was analyzed via standard Western blotting techniques using SDS-PAGE to separate proteins by size and transferred onto a nitrocellulose membrane (Thermo Fisher Scientific). Commercially available Ponceau S solution (Sigma-Aldrich) was used to assess equal loading between samples. Membranes were blocked for 1 hour in 5% bovine serum albumin (BSA) in Tris-buffered saline with 1% Tween-20 (TBS-T) solution. Membranes were then incubated with a diluted solution of primary antibodies in a 5% BSA in TBS-T buffer. Antibodies and dilutions were as following: mitochondrial oxidative phosphorylation (OXPHOS) complex cocktail (1:1,000, Abcam, Toronto, Canada), AMP-activated protein kinase (AMPK; 1:1,1000, CST, Beverly, USA), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α; 1:1,000, EMD, Etobicoke, Canada), pan Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII: 1:10,000, CST), skeletal muscle-specific chloride channel 1 (CLC-1: 1:200, ADI, San Antonio, USA), and MBNL1 (1:1,000, Santa Cruz Biotechnology, Dallas, USA). Following overnight incubation at 4 ˚C, membranes were washed, incubated with the appropriate horseradish peroxidase-linked secondary antibody (Thermo Fisher Scientific), washed again and visualized via reaction with enhanced chemiluminescence solution (Bio-Rad, Mississauga, Canada) using a FluorChem SP Imaging System (Alpha Innotech Corporation, San Leandro, USA). Densitometry was performed using ImageStudio Lite software (LI-COR Biosciences, Lincoln, USA) or Image Lab (Bio-Rad).

Immunofluorescence (IF) microscopy. EDL muscle samples embedded in OCT were sectioned into 10 μm cross-sections at -20 ˚C using a cryostat (Thermo Fisher Scientific). Microscope slides were blocked in a 10% goat serum in 1% BSA in phosphate-buffered saline with Tween-20 solution (PBS-T). Slides were then incubated with a CLC-1 antibody (1:100 dilution in 1% BSA in PBS-T; ADI) overnight. Following washing, slides were blocked once again and then incubated with a dystrophin antibody (1:1,000 dilution in 1% BSA in PBS-T; Abcam) overnight. Following washing, slides were incubated with an Alexa-conjugated secondary antibody (1:500 in 1% BSA in PBS-T; Thermo Fisher Scientific) and 4',6' Diamidino-2-phenylindole dihydrochloride (DAPI; 1:20,000 in 1% BSA in PBS-T; Thermo Fisher Scientific). After the slides were dried, fluorescent mounting media (Agilent Technologies, Mississauga, Canada) was applied to mount the slide with a cover slip. Slides were imaged by confocal microscopy (60X, 1.4 n.a. oil emersion; Nikon Instruments, Mississauga, Canada). Z-Plane imaging was used to compress 13 images with 0.9 μm into a single plane (Nikon Instruments). Slides were imaged by widefield microscopy (20X, 1.4 n.a.; Nikon Instruments). For each sample, four regions of interest (ROI) were created, each representing approximately 10% of the total cross-sectional area (CSA) of the muscle cross-section. A threshold was applied to dystrophin to define the sarcolemma of each myofiber. The mean intensity of CLC-1 protein localized to this threshold within each ROI was added and compared between the three mice groups. Mean intensity was used to normalize for differences in CSA of individual myofibers within the ROIs.

For IF microscopy analyses of AMPK and PGC-1α, samples were fixed in 4% paraformaldehyde (PFA), washed and blocked as described above and incubated overnight with the respective primary antibody (AMPK: 1:1,000 in 5% BSA in 1xPBST; CST; PGC-1α: 1:1,000 in 5% BSA in 1xPBS-T; SCB). Following incubation with the secondary (1:500 in 1% BSA in PBS-T; Thermo Fisher Scientific), slides were stained with a fluorophore-conjugated wheat-germ agglutinin antibody against laminin (1:500 in 1% BSA in 1xPBS-T; Vector Laboratories, Burlington, Canada) and DAPI (1:20,000 in 1% BSA in PBS-T; Thermo Fisher Scientific) for 15 min. Samples were subsequently washed and dried. Slides were imaged by widefield microscopy (20X, 1.4 n.a.; Nikon Instruments). For analysis, three ROIs were created, each representing 10% of muscle CSA. A threshold was applied to laminin and DAPI in order to assess the cellular localization of AMPK and PGC-1α as either cytosolic or myonuclear, as performed previously (Dial *et al.*, 2018*b*).

Histochemical staining. EDL muscles cross-sections were stained with hematoxylin and eosin, dehydrated with successive 70%, 95% and 100% ethanol exposures, further dried with xylene and mounted with Permount (Thermo Fisher Scientific). Stains were imaged using light microscopy (Nikon) at 20x magnification. For each sample, 100 myofibers were analyzed by quantifying the corresponding CSA and counting the presence of centrally-located nuclei. Centrally-located nuclei were defined as a myonuclei at least one diameter's length away from the dystrophin boundaries.

Fluorescence in-situ hybridization (FISH)-MBNL1 IF. Combination FISH with IF targeting MBNL1 was implemented as described by Mankodi et al. (2001). Briefly, 10 μm cross-sections of EDL muscles embedded in OCT were fixed in 3% PFA for 30 min, washed with PBS and fixed again in chilled 2% acetone. Slides were then incubated in a pre-hybridization solution for 10 min before incubating in the hybridization solution at 45 ˚C for 2 hours. The hybridization solution contained a modified DNA probe complementary to 10 CUG repeats with a 5′ end-labeled Texas Red fluorescein (Integrated DNA Technologies, Coralville, USA), allowing for detection using confocal microscopy. Samples were then washed in a post-hybridization solution and a saline-sodium citrate wash buffer. To probe for MBNL1, slides were blocked in a 1% goat serum in 1% BSA in PBST and then incubated in the antibody solution (1:1,000 in 1% BSA in

PBS-T; a generous gift from Dr. Thornton) overnight. Following overnight incubation, slides were washed and subsequently incubated with an Alexa-conjugated secondary antibody (1:500 in 1% BSA in PBS-T; Thermo Fisher Scientific) and DAPI (1:20,000 in 1% BSA in PBS-T; Thermo Fisher Scientific). After the slides were dried, fluorescent mounting media was applied, and a cover slip added. Slides were imaged by confocal microscopy (60x magnification, 1.4 n.a. oil emersion). Four 60x magnification Z-plane images were taken and used for analysis. Images were taken every 0.9 μm throughout the entire muscle cross-section. Number of myonuclei containing at lease one CUG)$_n$-foci, and CUG)$_n$-foci overlaying a MBNL1 puncta, were counted and expressed as a percentage of total myonuclei in the image.

RNA purification and quantitative real-time (q) and endpoint (EP) polymerase chain reaction (PCR). 5-10 mg of GAST muscle was utilized to extract RNA as described previously(Stouth *et al.*, 2018). All samples were homogenized using 1 ml of TRIzol reagent (Invitrogen, Carlsbad, USA) in Lysing D Matrix tubes (MP BiomedicalsSolon, USA) at a speed of 6 m/sec for 40 sec. Samples were then shaken vigorously for 15 sec with 200 μL of acetone, incubated at room temperature for 5 min and then centrifuged at 12000 x g for 10 min. The aqueous RNA phase was collected and purified using the Total RNA Omega Bio-Tek kit (VWR). RNA concentration was determined using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). Concentrations were normalized, and RNA was reverse transcribed using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

For qPCR, all samples were run in triplicate where each reaction contained GoTaq qPCR Master Mix (Promega, Madison, USA). Data were analyzed using the comparative C_T method (Schmittgen & Livak, 2008) where glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein S11 (RPS11) served as housekeeper genes. Data was analyzed as the ΔΔCT score for the targeted exon relative to the ΔΔCT score for an intron spanning a sequence included in both splicing variants (pan), as previously described (Brockhoff *et al.*, 2017). qPCR primer sequences (Sigma-Aldrich) were as follows: SERCA (also known as ATP2A1) +ex22 forward (F)- GCCCTGGACTTTACCCAGTG, reverse (R)- ACGGTTCAAAGACATGGAGGA: ATP2A1 pan F- GCCCTGGACTTTACCCAGTG, R- CCTCCAGATAGTTCCGAGCA; CAMKIIβ -ex13 F- TTTCTCAGCAGCCAAGAGTTT, R- TTCCTTAATCCCGTCCACTG; CAMKIIβ pan F- GCACGTCATTGGCGAGGAT, R- ACGGGTCTCTTCGGACTGG; CLC-1 +ex7a F- GGGCGTGGGATGCTGCTACTTTG, R- AGGACACGGAACACAAAGGC; CLC-1 pan F- CTGACATCCTGACAGTGGGC, R- AGGACACGGAACACAAAGGC; GAPDH F- AACACTGAGCATCTCCCTCA, R- GTGGGTGCAGCGAACTTTAT; RPS11 F- CGTGACGAACATGAAGATGC, R- GCACATTGAATCGCACAGTC, as previously reported(Lueck & Wheeler, 2007; Nakamori *et al.*, 2013; Brockhoff *et al.*, 2017).

For EPPCR, cDNA was added to a reaction mixture (Thermo Fisher Scientific) containing Taq polymerase (Thermo Fisher Scientific) and primers. EPPCR products were resolved on a 2% agarose gel with electrophoresis at 110 mV for 80 min. The percentage of misspliced mRNA was determined using Image Lab (Biorad), where the intensity of the misspliced product was determined relative to the total band intensities of both the alternatively-spliced and correctly-spliced products. EPPCR primer sequences were as follows: CLC-1 F- GGAATACCTCACACTCAAGGCC, R- CACGGAACACAAAGGCACTGAATGT; ATP2A1 F- ATCTTCAAGCTCCGGGCCCT, R- CAGCTTTGGCTGAAGATGCA; CAMKIIβ F- AAGTCGAGTTCCAGCGTGCA, R- AGGTCCTCATCTTCTGTGGTGG, RYR1 F- GACAATAAGAGCAAAATGGC, R- CTTGGTGCGTTCCTGATCTG; dihydropyridine receptor (DHPR) F- GAGATCCTTGGAATGTGTTTGACTTCCT, R- GGTTCAGCAGCTTGACCAGTCTCAT; bridging integrator 1 (BIN1) F- TCAATGATGTCCTGGTCAGC, R- GCTCATGGTTCACTCTGATC, as previously published (Wheeler *et al.*, 2007; Brockhoff *et al.*, 2017; Ravel-Chapuis *et al.*, 2017; Jauvin *et al.*, 2017).

Citrate synthase assay. Quadriceps muscle citrate synthase (CS) maximal enzyme activity was measured spectrophotometrically using a microplate reader at 37 ˚C and 412 nm, as previously described (Biotek Instruments, Winooski, USA; Srere, 1969).

Statistical analyses. One-way analysis of variance (ANOVA) and Student's t-tests were employed to compare means between experimental groups, as appropriate. Statistical tests were performed on the raw data before any conversion to -fold differences that appear in the graphical summaries. To compare the force-frequency and fatigue curves generated by the in situ skeletal muscle force production experiment, a regression analysis was performed to statistically compare the line of best fit for each group. Statistical analyses were performed with the GraphPad Prism software package (GraphPad Software, La Jolla, USA). Significance was accepted at $p < 0.05$. Data are presented as mean ± SEM.

Results

DM1 mice run less than healthy animals. DM1 mice were given access to a voluntary running wheel for 7 weeks. The mice ran 5.3 ± 1.2 km/day at a consistent rate (Figure 1A, B). This is less than the distance healthy WT mice run, which average over 10 km/day(Lerman *et al.*, 2002; Ghosh *et al.*, 2010; Steiner *et al.*, 2013). Females demonstrated modestly greater daily running distance (~20%) and total distance ran (~20%) versus males, however these differences were not statistically significant (Figure 1C, D).

Chronic exercise improves strength and function in DM1 mice. We first assessed functional adaptations to chronic exercise by examining balance and muscular endurance, as well as strength via the pen and grip strength tests, respectively. As expected, mice in the SED-WT group outperformed the SED-DM1 animals in all three measures of functionality (Figure 2A-C). Both the SED-DM1 and EX-DM1 groups had significantly shorter latencies to fall from the pen as compared to SED-WT mice (Figure 2A). However, EX-DM1 animals increased time on the pen by 118% ($p < 0.05$), as compared to SED-DM1 mice. Strength was 28% ($p < 0.05$) and 29% ($p < 0.05$) greater for SED-WT mice relative to SED-DM1 animals in the forelimb and all limb tests, respectively (Figure 2B, C). While there was a 24% difference ($p < 0.05$) between the SED-WT and EX-DM1 groups when comparing the peak force produced by all limbs, forelimb strength was similar between SED-WT and EX-DM1 animals and 21% greater ($p < 0.05$) in EX-DM1 versus SED-DM1 animals.

Physical activity ameliorates myotonia. We next examined muscle adaptations to exercise by assessing myotonia, a hallmark pathophysiological sign in DM1 patients and pre-clinical murine models. Specifically, in situ needle EMG of the TA, GAST and TRI muscles was employed to reveal muscle electrical activity after a stimulation-induced contraction in anesthetised mice from the three experimental groups. In all muscles examined, action myotonia was virtually non-existent in SED-WT animals, but present in both DM1 groups (Figure 3A-G). However, GAST EMG burst duration was significantly reduced by 80% in the EX-DM1 mice compared to SED-DM1 animals (Figure 3B). In addition, exercise elicited a ~70% decrease in EMG burst duration in the TRI muscle of EX-DM1 versus SED-DM1 mice (Figure 3D). TA muscle action myotonia was 74% lower in the EX-DM1 group relative to SED-DM1 mice, however this did not reach statistical significance ($p = 0.07$; Figure 3F). Examining the RMS amplitude of a 1-second sample of electrical activity following stimulation-induced contraction showed no difference between SED-DM1 and EX-DM1 in any of the muscles (Figure 3C, E, G).

Voluntary running attenuates the DM1-associated myopathy. Here, we first assessed the effects of chronic exercise on in situ muscle fatigue kinetics of the triceps surae complex. EX-DM1 mice demonstrated a greater ($p < 0.05$) resistance to fatigue during repetitive stimulation-induced contractions, as evidenced by a 65% reduction in force production versus a 72% and 75% reduction in SED-WT and SED-DM1 animals, respectively (Figure 4A). We continued to examine the effects of chronic exercise on characteristic features of the DM1 myopathy by examining myofiber CSA, as well as the presence of centrally-located nuclei in EDL muscles (Figure 4B). Volitional exercise in DM1 animals completely normalized the frequency distribution of myofiber CSA by reducing the prevalence of fibers with smaller CSA observed in SED-DM1 mice (Figure 4C). Specifically, the average CSA in the SED-DM1 animals tended ($p = 0.08$) to be less than that in the SED-WT group (Figure 4D), whereas the mean CSA in the EX-DM1 group was significantly greater compared to SED-DM1 mice ($2234 \pm 58\ \mu m^2$ vs. 788 ± 143), and comparable to that in the SED-WT group. Finally, habitual exercise did not alter the presence of centrally-located nuclei, as both DM1 groups had ~3 internalized myonuclei per 10 myofibers ($p < 0.05$ vs. SED-WT; Figure 4E).

Mitochondrial biogenesis in DM1 muscle. Skeletal muscle mitochondrial adaptions are examples of favourable molecular alterations that are evoked by chronic exercise in healthy animals, including humans (Hood, 2001; Egan & Zierath, 2013). We observed that the protein content of representative subunits of mitochondrial OXPHOS complexes I-V in the TA muscles were similar between SED-WT, SED-DM1, and EX-DM1 animals (Figure 5A, B). However, when the data for complexes I, II, III and V were pooled, they revealed that EX-DM1 mice had significantly higher OXPHOS expression than SED-WT (+21%) and SED-DM1 (+30%) animals. We observed a trend towards the normalization of complex IV expression with exercise ($p = 0.06$, Figure 5A, B). Another biomarker associated with mitochondrial density is mitochondrial CS enzyme activity (Larsen *et al.*, 2012). Quadriceps muscle CS activity was 65-90% greater ($p < 0.05$) in the EX-DM1 group compared to the SED-DM1 and SED-WT mice, respectively (Figure 5C).

Physical activity selectively normalizes alternative splicing events associated with DM1. Missplicing of pre-mRNA transcripts such as CLC-1 have been correlated with DM1 symptoms including muscle weakness and myotonia (Nakamori *et al.*, 2013). Using complementary EPPCR and qPCR analyses to examine mRNA splicing(Wheeler *et al.*, 2007; Brockhoff *et al.*, 2017; Ravel-Chapuis *et al.*, 2017; Jauvin *et al.*, 2017), we found that volitional exercise had no effect on the missplicing of CAMKIIβ, ATP2A1, RYR1 and DHPR pre-mRNAs, as the amount of misspliced variant did not differ between the two DM1 groups (Figure 6A-C). However, compared to SED-DM1 mice, the EX-DM1 group demonstrated a significant decrease in the abundance of CLC-1 transcripts containing exon 7a, as revealed by both EPPCR (-35%) and qPCR (-21.3%) assays (Figure 6A-C). Additionally, chronic exercise normalized the inclusion of exon 11 in the BIN1 pre-mRNA, as the % exon 11 inclusion was significantly different between SED-DM1 (77%) and EX-DM1 (90%) groups.

Effect of exercise on proteins indicative of the DM1 pathology. We next sought to investigate the whole cell expression levels of various proteins that are specifically perturbed in DM1. Aligning with the exercise-induced normalization in its alternative splicing, CLC-1 protein content in TA muscles significantly increased (+55%) in the EX-DM1 mice versus their SED-DM1 littermates, reaching levels that were comparable to SED-WT animals (Figure 7A, B). In contrast, CaMKIIα, -β, and pan CaMKII, as well as total MBNL1 content, were similar across all groups (Figure 7A, C, D). To complement the ClC-1 Western blot analysis, quantitative immunofluorescence was employed to determine the localization of CLC-1 at the sarcolemma (Figure 8A, B). SED-DM1 mice displayed significantly less CLC-1 protein in the sarcolemmal rim compared to both SED-WT and EX-DM1 mice. Chronic physical activity augmented sarcolemmal ClC-1 content by 88% ($p < 0.05$) in DM1 animals. As a result, there was no statistical difference ($p = 0.07$) in CLC-1 expression at the sarcolemma between SED-WT and EX-DM1 groups. Expression of the sarcolemmal marker dystrophin was similar between the three experimental cohorts (Figure 8C).

Chronic physical activity attenuates CUG)$_n$ foci and MBNL1 myonuclear sequestration. In an attempt to elucidate a potential mechanism by which chronic volitional exercise normalized CLC-1 and BIN1 alternative splicing, we employed combination FISH/IF to examine the presence of CUG)$_n$-dense myonuclei and MBNL1 myonuclear sequestration (Figure 9A, B), as done previously (Mankodi *et al.*, 2000; Lin *et al.*, 2006; Wheeler *et al.*, 2009; Nakamori *et al.*, 2011; Sobczak *et al.*, 2013). Confocal microscopy analyses of the FISH/IF approach revealed that the prevalence of CUG)$_n$-positive myonuclei in EDL muscles was significantly reduced (-25%) in the EX-DM1 animals, as compared to their SED-DM1 littermates (Figure 9C). Additionally, 7 weeks of volitional running by the DM1 mice reduced sequestered MBNL1 by 45% ($p < 0.05$) versus their sedentary counterparts (Figure 9D).

Figure 1

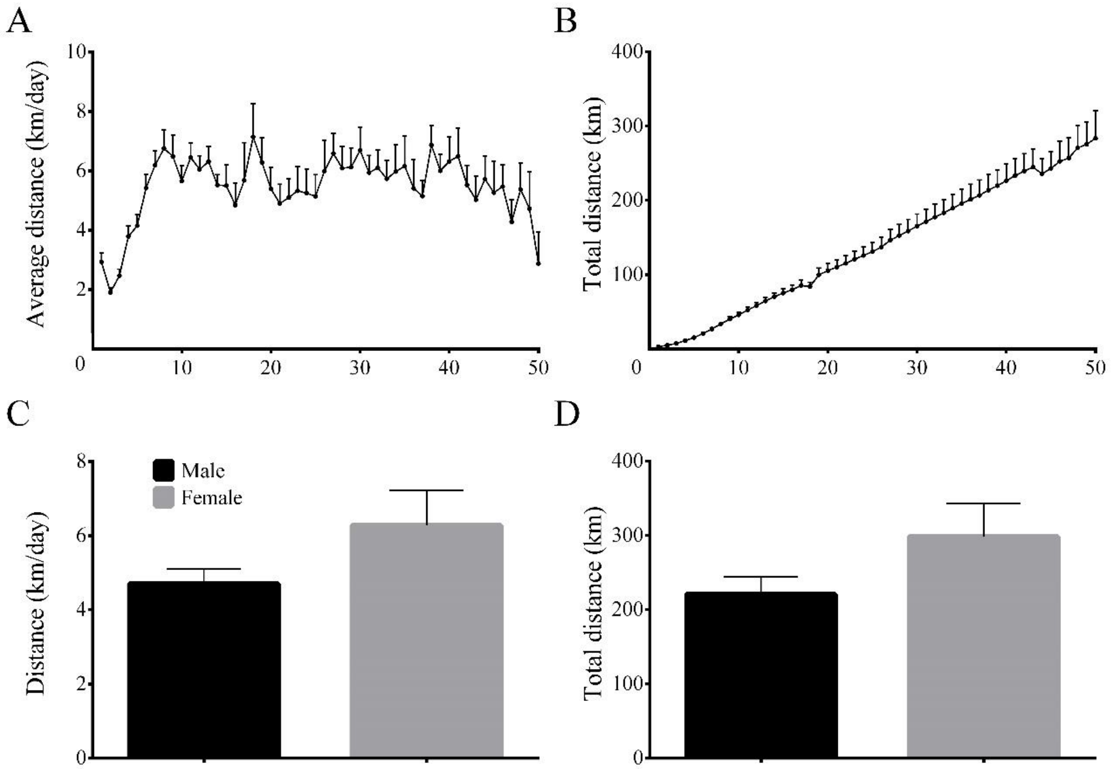

Figure 1. Running behaviour of mice with myotonic dystrophy type 1. Average daily volitional running distance (A) and total run distance (B) of human skeletal actin-long repeat (HSA^{LR}) mice, a murine model of myotonic dystrophy type 1 (DM1), given access to a running wheel for 7 weeks. Average running distance in km/day (C) and total run distance in km (D) of male and female DM1 animals. n = 16 (8 male, 8 female).

Figure 2

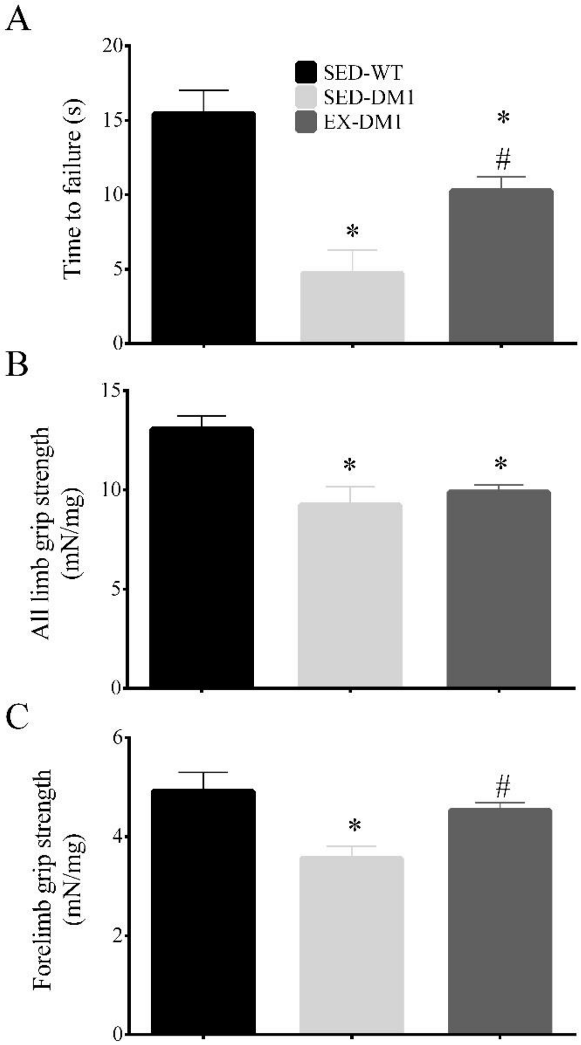

Figure 2. Assessment of muscle strength and endurance. A: Latency to fall during pen test assay for sedentary wild-type (SED-WT), sedentary HSA^{LR} (SED-DM1), and DM1 mice given access to an in-cage running wheel for 7 weeks (EX-DM1) animals. B: Maximal grip strength exerted by all limbs relative to body weight in the three experimental groups. C: Peak forelimb grip strength corrected for body weight in mice from all groups. n = 8-13; *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-DM1.

Figure 3

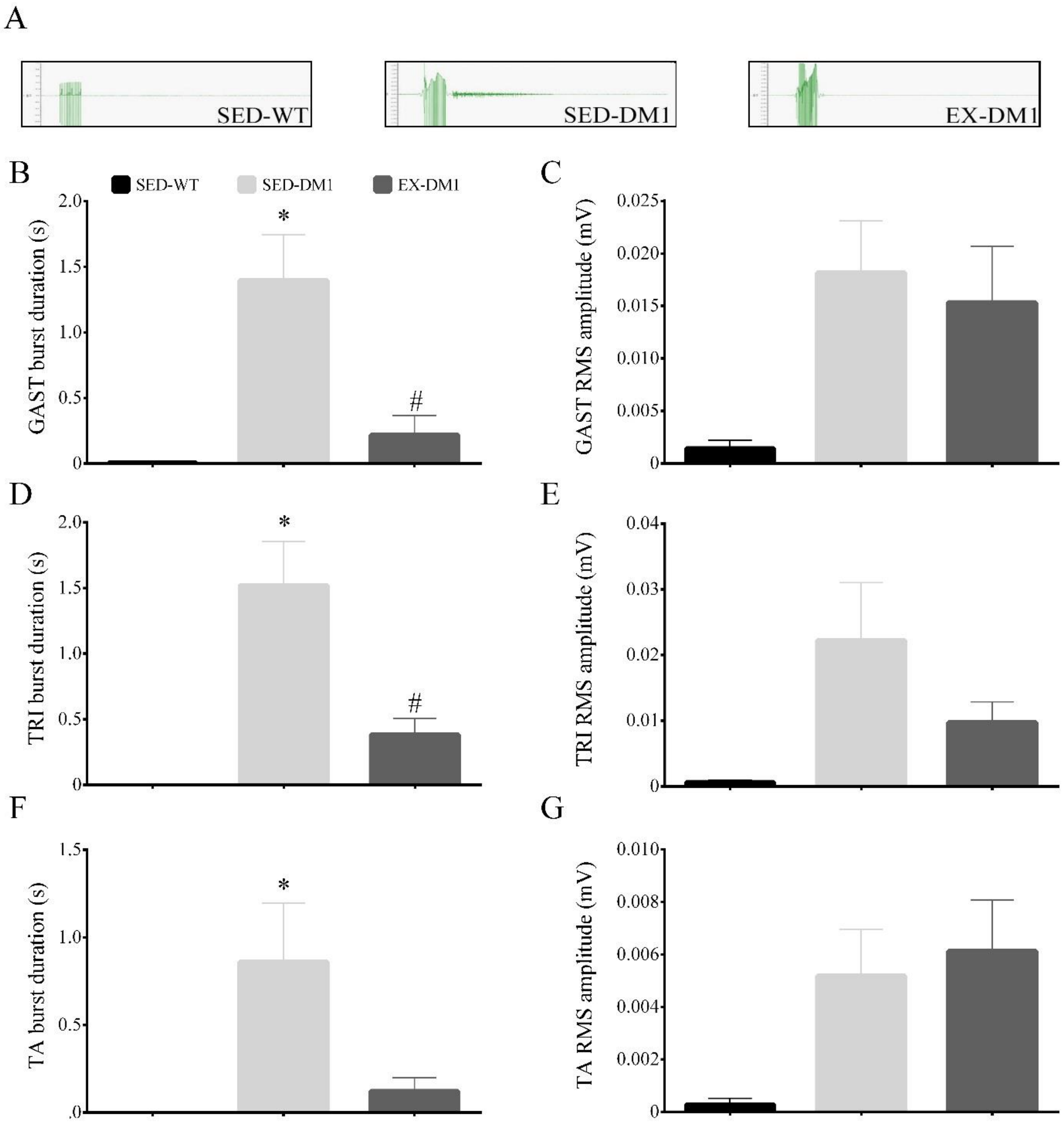
A
SED-WT
SED-DM1
EX-DM1
B
SED-WT
SED-DM1
EX-DM1
GAST burst duration (s)
2.0
1.5
1.0
0.5
0
*
#
C
GAST RMS amplitude (mV)
0.025
0.020
0.015
0.010
0.005
0
D
TRI burst duration (s)
2.0
1.5
1.0
0.5
0
*
#
E
TRI RMS amplitude (mV)
0.04
0.03
0.02
0.01
0
F
TA burst duration (s)
1.5
1.0
0.5
.0
*
G
TA RMS amplitude (mV)
0.010
0.008
0.006
0.004
0.002
0

Figure 3. Effects of chronic exercise on myotonia. A: Representative intramuscular electromyogram (EMG) tracings of gastrocnemius (GAST) muscles in SED-WT, SED-DM1, and EX-DM1 mice. Note the artifact for 50 Hz stimulation present at left of each EMG. Graphical summary of the burst duration and root mean squared (RMS) amplitude of a 1 second stimulation in the GAST (B and C), triceps (TRI; D and E), and tibialis anterior (TA) muscles (F and G). n = 6-11; *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-DM1.

Figure 4

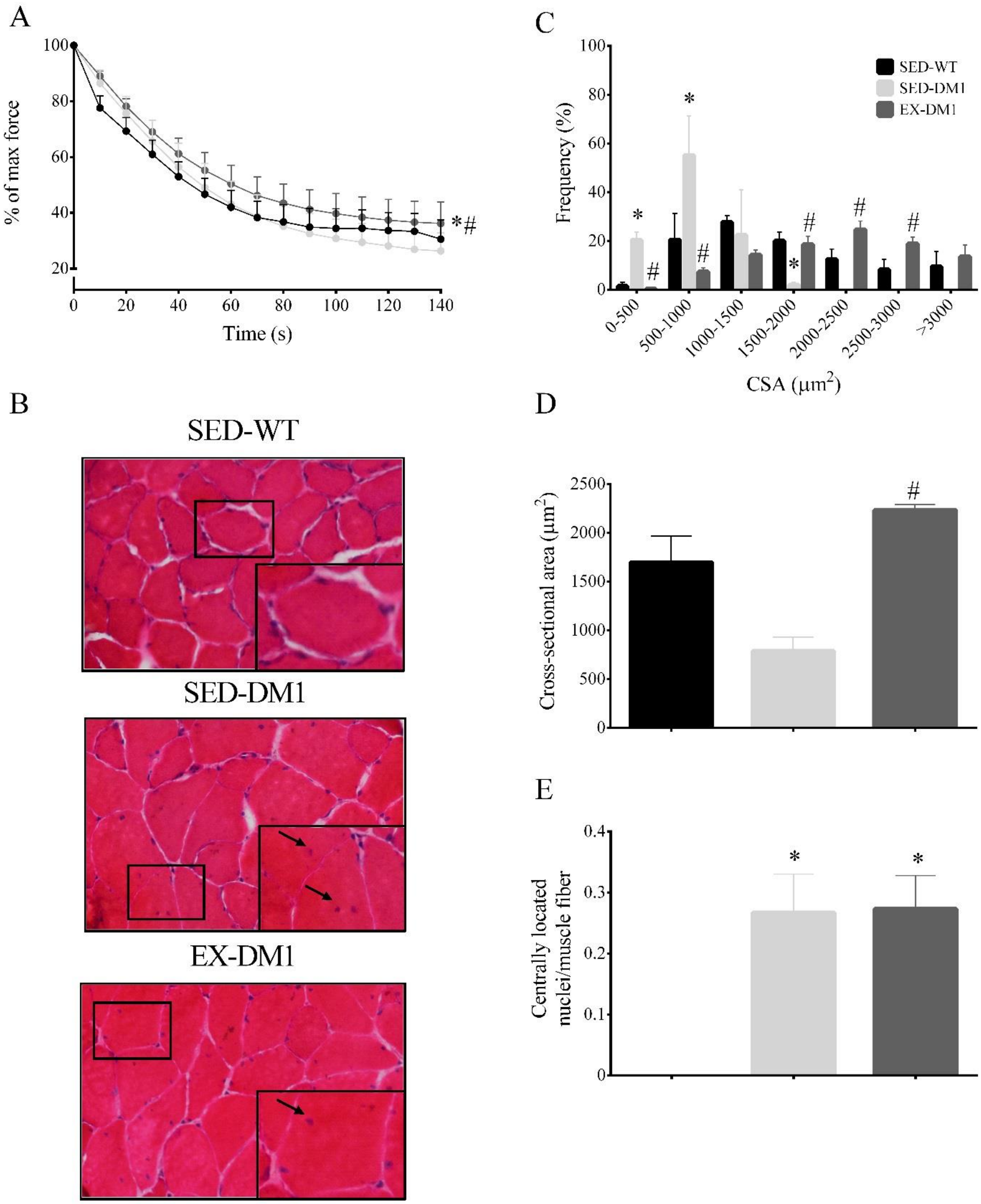
A
100
80
60
40
20
% of max force
0
20
40
60
80
100
120
140
Time (s)
*#
C
Frequency (%)
100
80
60
40
20
0
SED-WT
SED-DM1
EX-DM1
*
#
0-500
500-1000
1000-1500
1500-2000
2000-2500
2500-3000
>3000
CSA (μm^2)
B
SED-WT
SED-DM1
EX-DM1
D
Cross-sectional area (μm^2)
2500
2000
1500
1000
500
0
#
E
Centrally located nuclei/muscle fiber
0.4
0.3
0.2
0.1
0
*
*

Figure 4. Effects of chronic exercise on skeletal muscle fatigability and histological metrics of the DM1 myopathy. A: Fatigue kinetics in triceps surae complex during unilateral in situ stimulation of SED-WT, SED-DM1, and EX-DM1 mice, displayed as a percentage of the force produced during the initial contraction. B: Hematoxylin and eosin (H&E) stain of extensor digitorum longus (EDL) muscle transverse sections from SED-WT, SED-DM1, and EX-DM1 mice. Inset, arrows indicate centrally-located myonuclei. C: Frequency distribution of myofiber cross-sectional area (CSA). D: Graphical summary of average CSA, based on data in panel B. E: Number of internalized myonuclei per muscle fiber. n = 4-6; *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-DM1.

Figure 5

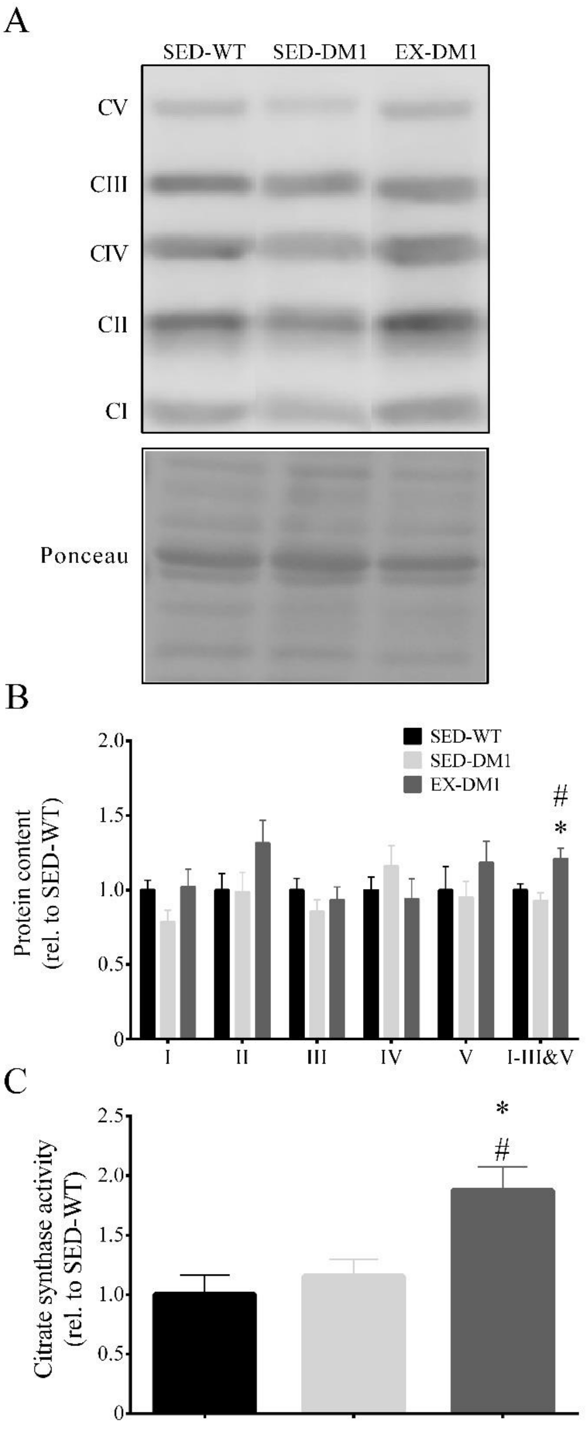
A
SED-WT
SED-DM1
EX-DM1
CV
CIII
CIV
CII
CI
Ponceau
B
Protein content (rel. to SED-WT)
2.0
1.5
1.0
0.5
0
SED-WT
SED-DM1
EX-DM1
#
*
I
II
III
IV
V
I-III&V
C
Citrate synthase activity (rel. to SED-WT)
2.5
2.0
1.5
1.0
0.5
0
*
#

Figure 5. Exercise-induced mitochondrial adaptations in DM1 mice. A: Representative Western blot of mitochondrial oxidative phosphorylation (OXPHOS) complexes I-V (CI-CV) in TA muscles from SED-WT, SED-DM1 and EX-DM1 mice. A typical Ponceau stain is shown below to indicate equal loading between samples. Graphical summaries of CI-CV levels, including pooled CI, II, III and V data (B) and citrate synthase enzyme activity (C). Data is expressed relative to the SED-WT group. n = 8; *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-WT.

Figure 6

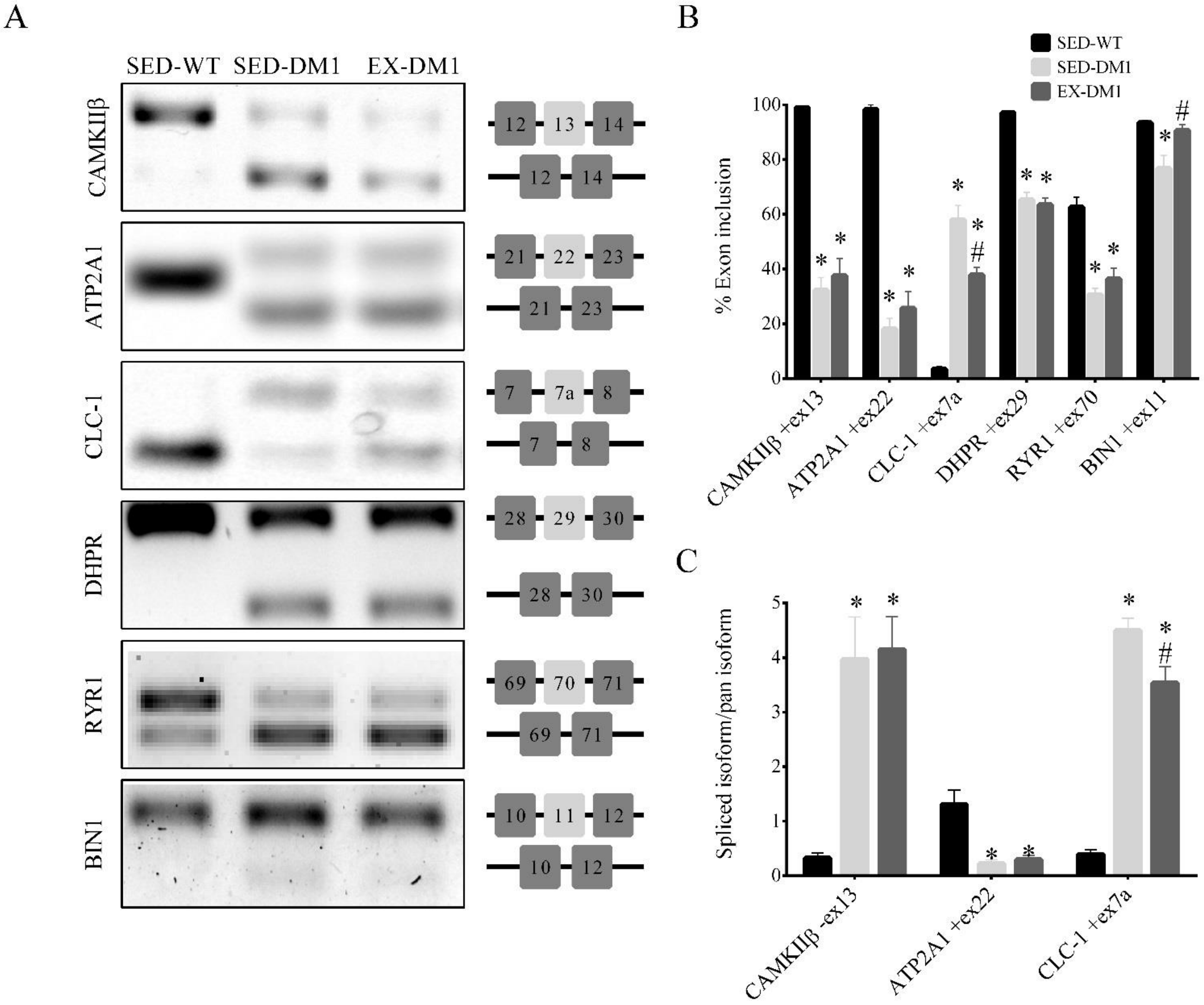
A
SED-WT
SED-DM1
EX-DM1
CAMKIIβ
ATP2A1
CLC-1
DHPR
RYR1
BIN1
12 13 14
12 14
21 22 23
21 23
7 7a 8
7 8
28 29 30
28 30
69 70 71
69 71
10 11 12
10 12
B
SED-WT
SED-DM1
EX-DM1
% Exon inclusion
0
20
40
60
80
100
CAMKIIβ +ex13
ATP2A1 +ex22
CLC-1 +ex7a
DHPR +ex29
RYR1 +ex70
BIN1 +ex11
*
#
C
Spliced isoform/pan isoform
0
1
2
3
4
5
CAMKIIβ -ex13
ATP2A1 +ex22
CLC-1 +ex7a

Figure 6. Effects of daily, volitional physical activity on mRNA splicing. A: Representative endpoint polymerase chain reaction (EPPCR) gel electrophoresis UV images of mRNA splicing assays for Ca^{2+}-calmodulin-dependent protein kinase IIβ (CaMKIIβ), sarco/endoplasmic reticulum Ca^{2+}-ATPase (ATP2A1), muscle-specific voltage-gated chloride channel (ClC-1), dihydropyridine receptor (DHPR), ryanodine receptor 1 (RYR1), and Bridging integrator 1 (BIN1) in the GAST muscles from the three experimental groups. Specific exon alternative splicing diagrams are displayed to the right of each corresponding image. B: Graphical summaries of the % exon inclusion of EPPCR data in panel A. C: Graphical summaries of quantitative, real-time PCR analysis of CaMKIIβ, ATP2A1, and CLC-1 spliced isoform abundance as indicated relative to the amount of the full-length (pan) sequence. $n = 8$; *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-DM1.

Figure 7

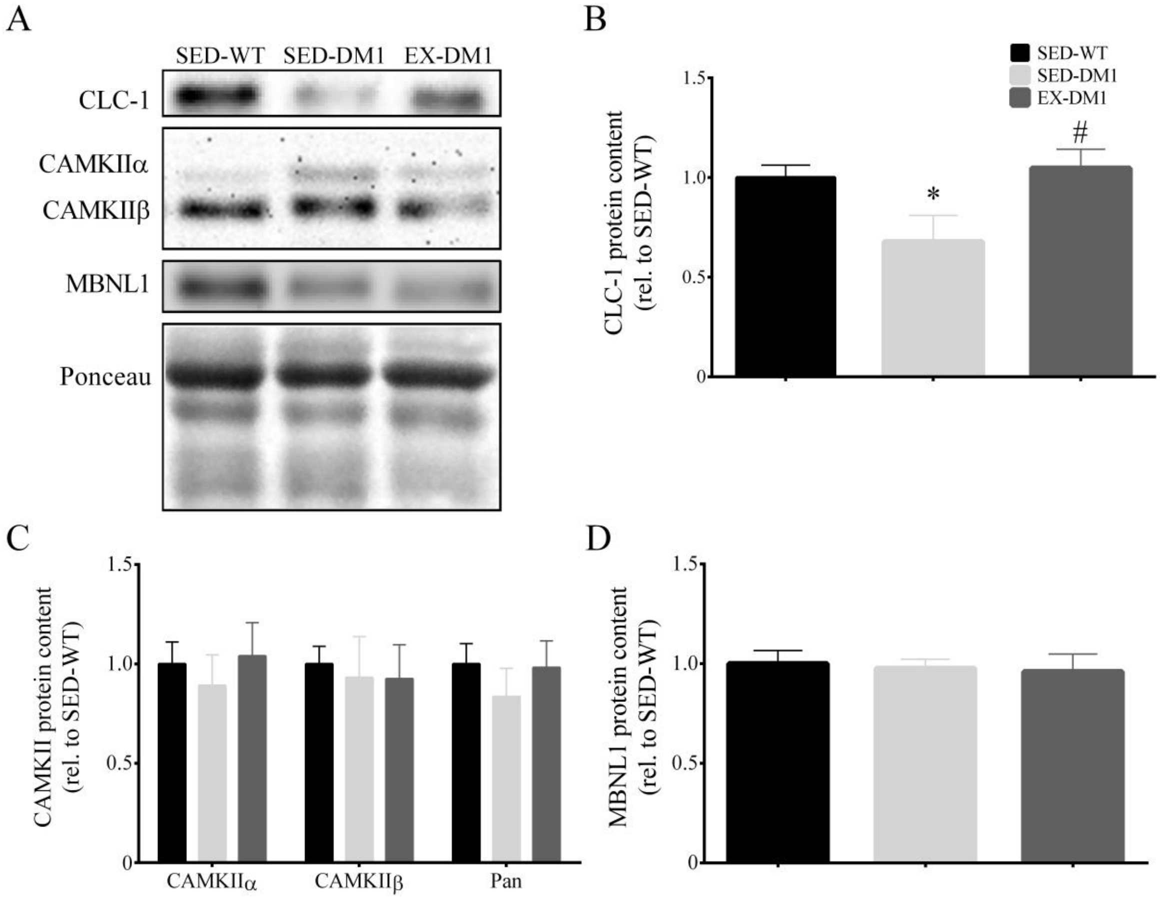

Figure 7. Effects of exercise on the DM1 molecular signature. A: Typical Western blots of CLC-1, CaMKIIα and -β isoforms, as well as Muscleblind-like 1 (MBNL1) protein expression in TA muscles of SED-WT, SED-DM1, and EX-DM1 animals. A representative Ponceau is displayed below. Graphical summaries of CLC-1 (B), CaMKIIα, -β, and pan-CaMKII (C), as well as MBNL1 (D) protein levels. Data is relative to SED-WT. n = 8; *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-DM1.

Figure 8

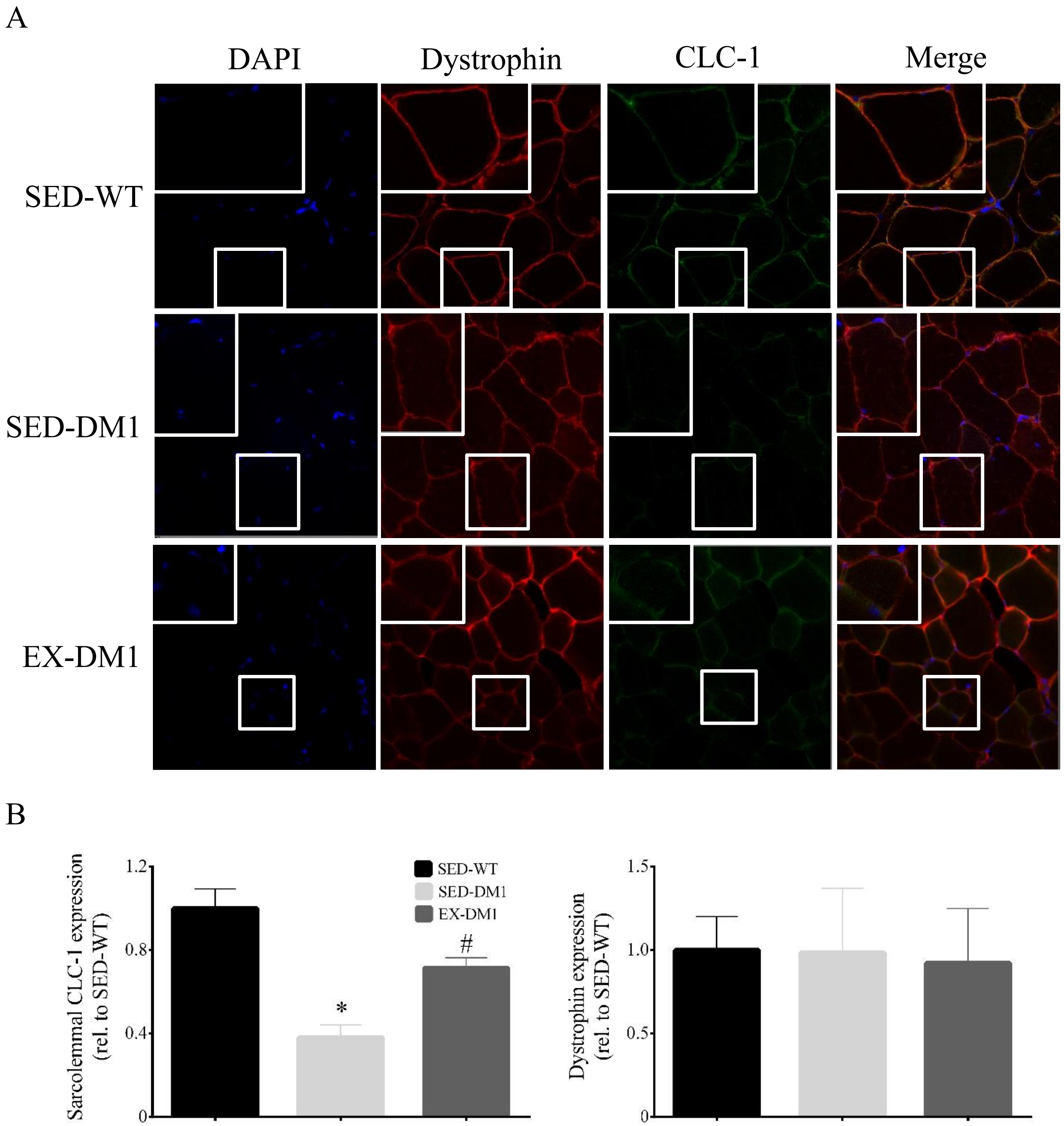
A
DAPI
Dystrophin
CLC-1
Merge
SED-WT
SED-DM1
EX-DM1
B
Sarcolemmal CLC-1 expression (rel. to SED-WT)
1.2
0.8
0.4
0
SED-WT
SED-DM1
EX-DM1
*
#
Dystrophin expression (rel. to SED-WT)
1.5
1.0
0.5
0

Figure 8. Effects of chronic exercise on sarcolemmal CLC-1 expression. A: Representative immunofluorescence (IF) images of EDL muscle cross-sections from SED-WT, SED-DM1 and EX-DM1 mice stained for 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) to identify myonuclei, dystrophin as a marker of the sarcolemma, CLC-1 protein, and merged images. Higher magnifications are inset. B: Graphical summary of the mean fluorescence intensity of CLC-1 protein localized specifically at the sarcolemma as determined by dystrophin overlay. C: Average fluorescence intensity of dystrophin in the three experimental groups. Data is relative to SED-WT. n = 3-4; *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-DM1.

Figure 9

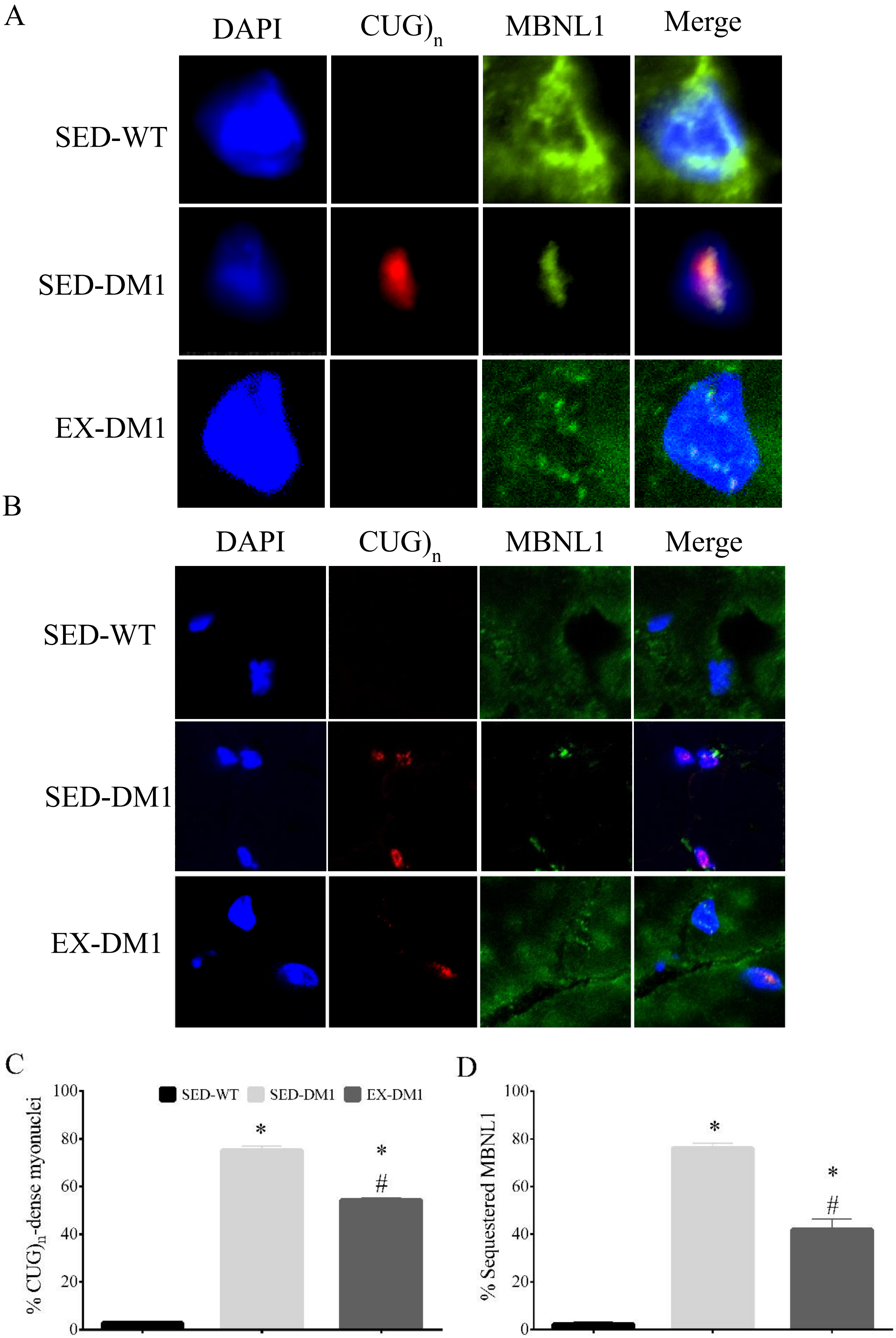
A
DAPI
CUG)n
MBNL1
Merge
SED-WT
SED-DM1
EX-DM1
B
DAPI
CUG)n
MBNL1
Merge
SED-WT
SED-DM1
EX-DM1
C
% CUG)n-dense myonuclei
100
80
60
40
20
0
SED-WT
SED-DM1
EX-DM1
*
*
#
D
% Sequestered MBNL1
100
80
60
40
20
0
*
*
#

Figure 9. Exercise-induced reduction in CUG)n-dense myonuclei and liberation of MBNL1 sequestration. Representative high (A) and lower magnification (B) images of combination fluorescence in situ hybridization probing for CUG repeats [CUG)$_n$] with IF targeting MBNL1 in EDL muscle sections from SED-WT, SED-DM1 and EX-DM1 animals. DAPI (denoting myonuclei) and merged images are also shown. Graphical summary of the percentage of myonuclei with CUG)$_n$-foci (C) and myonuclei with sequestered MBNL1 (D). n = 4; *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-DM1.

Discussion

The purpose of this study was to comprehensively examine the exercise biology of DM1. Our data are the first to demonstrate that following seven weeks of daily volitional physical activity, DM1 mice exhibit significant beneficial adaptations to their skeletal muscle at the physiological, cellular, and molecular levels. Specifically, chronic exercise led to an improvement in muscle strength and endurance, as well as an attenuation of myotonia, all hallmark signs of the DM1 pathology. At the cellular level, exercise augmented mitochondrial-specific enzyme activity, increased the sarcolemmal localization of CLC-1 protein, as well as significantly reduced the prevalence of $CUG)_n$-dense myonuclear foci with sequestered MBNL1. Exercise-induced molecular adaptations were exemplified by improved alternative splicing profiles of CLC-1 and BIN1, which support the enhanced muscle physiology. Thus, these data indicate that chronic physical activity mitigates RNA toxicity and its resultant spliceopathy, fundamental disease mechanisms of DM1, which together form the cellular foundations of physiological improvements.

Ng and colleagues recently provided the first review of DM1 exercise biology (Ng *et al.*, 2018). Although this body of literature is limited in number, a consensus emerges that exercise is a safe and modestly effective, lifestyle-based intervention to improve muscle strength, function and quality of life in DM1 patients. A notable caveat however, is that the disparate training regimes employed in these studies, the small cohort sizes, as well as the varying experimental designs utilized, indicate that caution is indeed required when discussing in broad terms the impact of exercise on DM1 patients. Clearly therefore, many more well-designed and -executed exercise trials with DM1 participants are warranted. Nevertheless, in addition to observing physiological adaptations such as enhanced strength and VO_{2max}, these DM1 participant studies have revealed some valuable clues as to the cellular alterations to exercise training, for example an increase in myofiber CSA and unaltered serum creatine kinase levels, a marker for muscle damage (Ørngreen *et al.*, 2005*b*). During the finalization of this manuscript, data from Bernard Jasmin's laboratory demonstrated that 8 weeks of habitual, voluntary wheel running partially rescued a number of mRNA alternative splicing events in DM1 mouse skeletal muscle (Ravel-Chapuis *et al.*, 2018). These very interesting results suggest that exercise positively affects the DM1 spliceopathy, however the underlying mechanism(s) linking exercise to the attenuation of missplicing remains unknown. Moreover, the influence of chronic physical activity on other critical disease characteristics, such as myotonia and CLC-1 expression, has not been examined. It is critical to thoroughly investigate the physiology of exercise adaptation DM1, as well as the cellular and molecular mechanisms that drive physical activity-induced remodelling of DM1 skeletal muscle to increase our knowledge of the basic biology of the disorder and assist in the discovery of more effective lifestyle and/or pharmacological interventions to mitigate its severity.

Utilizing $\mathrm{HSA}^{\mathrm{LR}}$ animals, an established preclinical murine model of DM1 (Mankodi *et al.*, 2000), our data demonstrate that despite the presence of typical indicators of the DM1 myopathy such as muscle atrophy, weakness, and reduced functional capacity (Jones *et al.*, 2012; Timchenko, 2013), DM1 animals voluntarily ran consistently for seven weeks, averaging 5.3 ± 1.2 km/day. This distance is less when compared to healthy FVB/n mice (i.e., >10 km/day; (Lerman *et al.*, 2002; Steiner *et al.*, 2013), the background strain for the transgenic $\mathrm{HSA}^{\mathrm{LR}}$ line. Nonetheless, these results are consistent with findings from participant studies that clearly indicate that those DM1 patients that are able to engage in a structured physical activity regimen will do so (i.e., high participant adherence rates) and find it enjoyable [e.g., high satisfaction scores; (Wright *et al.*, 1996; Ørngreen *et al.*, 2005*b*; Aldehag *et al.*, 2013; Brady *et al.*, 2014)]. Importantly, our data also show that the volume of activity completed by the DM1 animals, although modest relative to healthy mice, was indeed sufficient to elicit significant physiological improvements in strength and endurance as indicated by a battery of complementary in vivo and in situ phenotyping experiments. Physical activity-induced increases in muscle strength and fatigue-resistance in the healthy condition(Hawley *et al.*, 2014) are correlated with muscle hypertrophy and mitochondrial biogenesis, respectively (Hawley *et al.*, 2014). In DM1 mice, voluntary running lead to a significant increase in skeletal muscle CS activity and OXPHOS protein levels, which exhibit stronger associations with mitochondrial volume than does PGC-1α content (Larsen *et al.*, 2012), which was unaffected here by chronic exercise. In addition, exercise augmented mean myofiber CSA, as well as elicited a CSA frequency distribution similar to that of the SED-WT cohort without exacerbating internalized myonuclei. It is reasonable to suspect, based on similar exercise-induced alterations in healthy animals (Hawley *et al.*, 2014) that these adaptations contributed to the improved muscle strength and endurance observed in the EX-DM1 group. Furthermore, as missplicing of BIN1 mRNA is highly correlated to muscle weakness in DM1 patients (Fugier *et al.*, 2011; Nakamori *et al.*, 2013), the complete correction of this alternative splicing event by chronic physical activity may also explain the increase in skeletal muscle performance.

Prior to the current study, the only metrics of myotonia in the DM1 condition in response to physical activity were self-reported severity ratings pre- versus post- hand-training intervention (Aldehag *et al.*, 2005, 2013). The authors observed that the training protocol did not improve subjective assessment of action myotonia during hand gripping tasks. Here, we provide quantitative data that demonstrate that seven weeks of voluntary running ameliorates myotonia in vivo in DM1 mice. Previous studies employing pharmacological, cell-based technologies such as antisense oligonucleotides or recombinant adeno-associated viral vectors have shown that these interventions are able to significantly reduce myotonia in DM1 animals (Wheeler *et al.*, 2012; Bisset *et al.*, 2015). These strategies are remarkable for their efficacy in pre-clinical contexts, however the results have yet to translate into the human DM1 condition. To our knowledge, we are the first to demonstrate that a physiological approach such as chronic exercise, a modality that is safe, low-cost, and accessible to patients and their teams, is also able to significantly reduce myotonia, a cardinal disease characteristic of DM1. These improvements in myotonia were associated with a rescuing of the depressed expression of mature CLC-1 mRNA and protein in the EX-DM1 cohort. Specifically, the data show that chronic physical activity significantly increased exon 7a exclusion, as well as total CLC-1 protein content and its sarcolemmal localization. Healthy CLC-1 levels and subcellular location are largely responsible for repolarizing and relaxing myocytes after contractions (Orengo *et al.*, 2008). As such, its alternative pre-mRNA splicing to include exon 7a and subsequent nonsense-mediated mRNA degradation and sparse CLC-1 protein expression, accounts for the DM1-associated myotonia (Chau & Kalsotra, 2015). Indeed, pharmacological induction of CLC-1 in skeletal muscle significantly reduces myotonia in DM1 mice (Wheeler *et al.*, 2007). Thus, the evidence strongly support the hypothesis that the exercise-evoked enhancement of skeletal muscle CLC-1 expression provides the molecular basis for the attenuation of myotonia in DM1 animals. It is interesting to note that the selective correction of alternative splicing by habitual physical activity, namely improvement of CLC-1 and BIN1 mRNAs, but not CAMKIIβ, ATP2A1/SERCA, RYR1 and DHPR transcripts, is wholly consistent with previous studies that have also observed a treatment-induced rescue of assorted misspliced mRNAs in DM1 skeletal muscle (Kanadia *et al.*, 2006; Coonrod *et al.*, 2013; Brockhoff *et al.*, 2017). The cause of this selective recovery is unclear, but it is likely related, at least in part, to the expression, localization, and/or function of RNABPs that are affected in DM1, primarily MBNL1.

A main finding of the current study is that that voluntary running markedly diminished both the prevalence of myonuclei containing CUG)$_n$-expanded RNA, as well as sequestered MBNL1 without any change in myocellular MBNL1 content. CUG)$_n$-expansion-mediated RNA toxicity is the prevailing primary downstream disease mechanism in DM1 (Chau & Kalsotra, 2015). Specifically, in DM1 patients, the microsatellite repeat expansion mutation within the 3'-UTR of the DMPK gene results in DMPK transcripts forming hairpin secondary structures that sequester MBNL1 proteins with high affinity, which causes MBNL1 loss-of-function (Chau & Kalsotra, 2015). MBNL1 is the most critical RNABP responsible for, among other functions, the proper splicing of pre-mRNAs germane to DM1, for example CLC-1 or SERCA proteins (Nakamori *et al.*, 2013). This pathological cascade is recapitulated in the skeletal muscle of HSA^{LR} animals that were employed in the current study, albeit in these mice the CUG)$_n$-induced RNAopathy arises from toxic HSA transcripts (Mankodi *et al.*, 2000; Wheeler *et al.*, 2007). Previous work has clearly demonstrated that the dissolution of toxic CUG)$_n$-containing RNAs and dispersal of MBNL1 from myonuclear foci, either by pharmacological or transgenic means, attenuates the prevalence of alternatively-spliced mRNAs and mitigates the DM1 myopathy (Thornton *et al.*, 2017). For example, Coonrod et al. (2013) demonstrated the ability of pentamidine and its analogues to reduce CUG)$_n$-RNAs, increase the concentration of free MBNL1, and selectively rescue certain MBNL1-mediated splicing events, namely the partial rescue of SERCA exon 22 inclusion and full rescue of CLC-1 exon 7a exclusion, in pre-clinical cell and murine models of DM1. While speculative, we attribute the selective nature of the normalization of alternative splicing events elicited by chronic exercise to the amount of functional, liberated MBNL1 protein needed to regulate pre-mRNA splicing, as well as to the sensitivity displayed by each misspliced pre-mRNA toward the RNABP. Further investigation into this phenomenon of the selective correction of alternative splicing in DM1 is clearly warranted in order to optimize potential therapeutic strategies.

We postulate that AMPK and PGC-1α are integral to the mechanisms responsible for the exercise-induced attenuation of toxic RNAs, MBNL1 entrapment to myonuclear foci, and spliceopathy in DM1 animals. In the healthy condition, exercise robustly stimulates the activity, expression, and myonuclear translocation of these molecules with powerful, pleiotropic downstream effect (Kjøbsted *et al.*, 2017; Dial *et al.*, 2018*a*). For example, PGC-1α determines, maintains, and remodels neuromuscular phenotype by regulating transcriptional and posttranscriptional events, such as mRNA processing, including splicing, in skeletal muscle (Monsalve *et al.*, 2000; Martínez-Redondo *et al.*, 2016). A growing body of evidence strongly supports the concept that pharmacological AMPK activation has therapeutic potential in mitigating DM1 (Savkur *et al.*, 2001; Laustriat *et al.*, 2015; Brockhoff *et al.*, 2017; Thornton *et al.*, 2017; Dial *et al.*, 2018*a*; Ravel-Chapuis *et al.*, 2018), in part via reduced CUG)$_n$-RNA toxicity, however the mechanism for this is yet to be identified. AMPK directly interacts with a key nuclear CUG)$_n$-RNA stabilizing protein, heterogeneous nuclear ribonucleoprotein H (horn H), which reduces the stability of the expanded repeat tract, facilitating its export and thus liberation of MBNL1 from myonuclear aggregates (Kim *et al.*, 2005, 2014). AMPK and PGC-1α translocate to myonuclei in response to acute exercise, which in the DM1 context might promote the interaction between the kinase and ribonuclear protein, as well as PGC-1α splicing activity. However, our data did not demonstrate a nuclear accumulation of AMPK or PGC-1α, which we submit was likely due to the timing of the analysis (i.e., 24 hours after the final exercise bout; Supplementary Figure 1). This does not preclude the possibility that chronic exercise stimulates swift, but transient AMPK and PGC-1α myonuclear localizations with each acute bout of activity, which may effectively reduce toxic RNAs and MBNL1 loss-of-function via an hnRNP H-mediated mechanism, and/or a PGC-1α-induced missplicing correction. Consistent with this, we have observed in DM1 mice that a single bout of running augments AMPK activation status, PGC-1α expression, as well as their myonuclear translocation during the immediate-to-short-term (≤ 3 hours) post-exercise recovery (Manta and Ljubicic, unpublished observations). Ongoing experiments in our laboratory pursue this line of inquiry further, as well as whether exercise: 1) evokes physical and functional interactions between AMPK and hnRNP H, and; 2) elicits a PGC-1α-mediated splicing correction of DM1-associated mRNAs.

In summary, this study reveals for the first time in the DM1 condition that chronic, volitional exercise results in favourable physiological, myocellular, and molecular adaptations. In particular, the main findings demonstrate that in DM1 mice, habitual physical activity elicited improved strength and endurance, and limited myotonia, which were associated with corrected splicing of the CLC-1 and BIN1 mRNAs, as well as reduced CUG)$_n$-RNA toxicity and the liberation of MBNL1 from myonuclear foci. Further exploration of the molecular mechanisms by which exercise causes these alterations is warranted, as is identifying the optimal exercise prescription (i.e., frequency, intensity, mode, duration) to obtain maximal benefits. Moreover, the possibility that this safe, accessible, and affordable physiological intervention provides additive or synergistic effects to current or experimental pharmacological or cell-based therapeutic strategies is certainly worthy of future consideration, with the ultimate goal of improving the lives of individuals with DM1.

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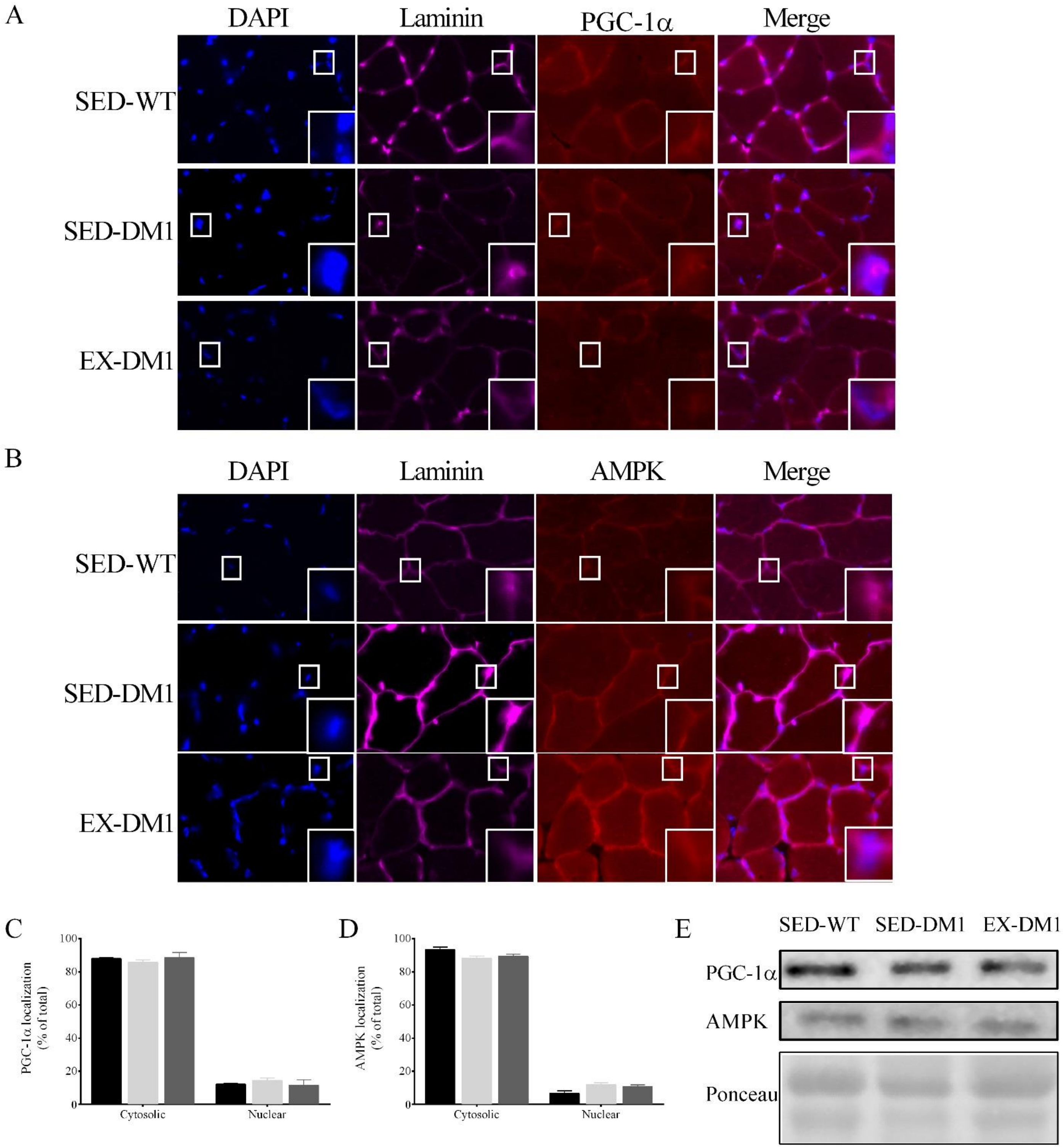
Supplementary Figure 1


A
DAPI
Laminin
PGC-1α
Merge
SED-WT
SED-DM1
EX-DM1
B
DAPI
Laminin
AMPK
Merge
SED-WT
SED-DM1
EX-DM1
C
PGC-1α localization (% of total)
0
20
40
60
80
100
Cytosolic
Nuclear
D
AMPK localization (% of total)
0
20
40
60
80
100
Cytosolic
Nuclear
E
SED-WT
SED-DM1
EX-DM1
PGC-1α
AMPK
Ponceau

Supplementary Figure 1. Subcellular localization of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and AMP-activated protein kinase (AMPK) in DM1 skeletal muscle. Representative IF images of DAPI, laminin (denoting the sarcolemma), merged overlay, along with PGC-1α (A) or AMPK (B) in EDL muscle sections from SED-WT, SED-DM1, and EX-DM1 animals. Higher magnifications are inset. Graphical summaries of PGC-1α (C) and AMPK (D) subcellular localization in cytosolic and nuclear compartments of the EDL muscles from the three experimental groups. E: Representative Western blots of whole cell PGC-1α and AMPK content, as well as a typical Ponceau, in TA muscle homogenates. n = 4 *, $p < 0.05$ vs. SED-WT; #, $p < 0.05$ vs. SED-DM1.