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Mitochondrial dysfunction in skeletal muscle of fukutin-deficient mice is resistant to exercise- and 5-aminoimidazole-4-carboxamide ribonucleotide-induced rescue

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Abstract

Disruptions in the dystrophin–glycoprotein complex (DGC) are clearly the primary basis underlying various forms of muscular dystrophies and dystroglycanopathies, but the cellular consequences of DGC disruption are still being investigated. Mitochondrial abnormalities are becoming an apparent consequence and contributor to dystrophy disease pathology. Herein, we demonstrate that muscle-specific deletion of the fukutin gene (*Myf5/fktn*-KO mice (*Fktn* KO)), a model of secondary dystroglycanopathy, results in ~30% lower muscle strength ($P < 0.001$) and 16% lower mitochondrial respiratory function ($P = 0.002$) compared to healthy littermate controls (LM). We also observed ~80% lower expression of the gene for peroxisome proliferator-activated receptor- γ coactivator 1 α (*PGC-1 α*) ($P = 0.004$), a primary transcription factor for mitochondrial biogenesis, in *Fktn* KO mice that likely contributes to the mitochondrial defects. *PGC-1 α* is post-translationally regulated via phosphorylation by AMP-activated protein kinase (AMPK). Treatment with the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) failed to rescue mitochondrial deficits in *Fktn* KO mice ($P = 0.458$) but did have beneficial (~30% greater) effects on recovery of muscle contractility following injury in both LM and *Fktn* KO mice compared to saline treatment ($P = 0.006$). The beneficial effects of AMPK stimulation via AICAR on muscle contractile function may be partially explained by AMPK's other role of regulating

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AUTHOR CONTRIBUTIONS

The experiments were performed at the University of Georgia in the laboratories of J.A.C or A.M.B. Experiment were designed by W.M.S., A.S.N., A.M.B. and J.A.C.; data acquisition and analysis/interpretation was done by all authors.; W.M.S. drafted the manuscript and A.S.N., A.M.B. and J.A.C. critically revised it. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

COMPETING INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data will be made freely available upon request.

skeletal muscle autophagy, a cellular process critical for clearance of damaged and/or dysfunctional organelles. Two primary conclusions can be drawn from this data: (1) fukutin deletion produces intrinsic muscular metabolic defects that likely contribute to dystroglycanopathy disease pathology, and (2) AICAR treatment accelerates recovery of muscle contractile function following injury suggesting AMPK signalling as a possible target for therapeutic strategies.

Keywords

dystroglycan; fukutin; mitochondrial oxygen consumption; mitochondrial respiration; muscle regeneration; muscle torque; muscular dystrophy; oxidative plasticity

1 | INTRODUCTION

Muscular dystrophy is characterized by progressive muscle weakness and atrophy due to the loss of structural integrity of the dystrophin–glycoprotein complex (DGC) within muscle cells. The DGC provides a critical link between the extracellular matrix of skeletal muscle cells and the intracellular cytoskeleton (Ervasti, 2007; Ervasti & Campbell, 1991). Without proper function of this protein complex, muscle fibres are susceptible to injury, leading to a progressive muscle dysfunction (Nigro & Piluso, 2015). One important functional component of the DGC is the extracellular protein α -dystroglycan (α DG) that acts as a receptor for extracellular matrix proteins such as laminin (Ibraghimov-Beskrovnaya et al., 1992). Disruptions in the function of α DG give rise to dystroglycanopathies characterized by early onset of muscle weakness and progressive loss of muscle contractile function (Taniguchi-Ikeda, Morioka, Iijima, & Toda, 2016). The fukutin protein, encoded by the *FKTN* gene, is one of a cascade of proteins responsible for glycosylation of α DG, a post-translational modification necessary for α DG binding to laminin and other extracellular matrix receptors (Sheikh, Halmo, & Wells, 2017). Mutations in the *FKTN* gene have been directly linked to α DG dysfunction and subsequent muscle dystroglycanopathy (Kobayashi et al., 1998). A fukutin knockout mouse (Fktn KO) model accurately recapitulates many of the characteristics observed in human dystroglycanopathies as the muscles are characterized by chronic weakness, a perpetual cycle of injury and repair, and abnormal muscle regeneration (Beedle et al., 2012; Foltz et al., 2016b). That said, several pathological features of dystroglycanopathies remain undefined, and herein we sought to interrogate mitochondrial respiratory function based on our previous work which eluded to possible mitochondrial alterations in Fktn KO muscle (Foltz et al., 2016a).

Skeletal muscle mitochondria are a critical network of organelles within the muscle cell responsible for energy production, redox control, ion homeostasis and signalling. The health of the mitochondrial network can influence the health and function of the muscle cell. Maintaining a healthy mitochondrial network is a balance between the addition of new mitochondria to the network, i.e. mitochondrial biogenesis, and the removal of damaged or dysfunctional mitochondria from the network, i.e. mitochondrial autophagy. Previously, we identified a mild reduction in succinate dehydrogenase activity, a marker of mitochondrial content, in Fktn KO mice compared to wild-type controls suggesting that mitochondrial maintenance (biogenesis and/or autophagy) is detrimentally impacted by Fktn KO-induced

disruption of the DGC (Foltz et al., 2016a). Our findings are in line with other studies highlighting compromised mitochondrial respiratory function and content in mouse models of DGC, suggesting that DGC disruptions are linked to the metabolic health of the muscle.

If mitochondrial maintenance is altered in Fktn KO mice, then targeting mitochondrial biogenesis or autophagy may be a viable therapy to explore for treatment of secondary dystroglycanopathies. One potential target is the cellular energy-sensing AMP-activated protein kinase (AMPK), which is critical for post-translational modifications to peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α ; a metabolic gene transcription factor) and ULK1 (a kinase that initiates autophagosome assembly) (Kim, Kundu, Viollet, & Guan, 2011; Narkar et al., 2008). As demonstrated by Pauly et al. (2012), pharmacological treatment of dystrophin-deficient *mdx* mice with the AMPK agonist 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) enhanced autophagy and ameliorated several of the pathological characteristics common to *mdx* mice including muscle weakness and some metabolic defects. These results highlight the therapeutic potential of AMPK stimulation via AICAR to potentially correct metabolic and contractile abnormalities in Fktn KO mice.

The purpose of this study was to determine the extent to which Fktn KO disrupted mitochondrial respiratory function in skeletal muscle and then test if enhancing mitochondrial quality in Fktn KO mice affects muscle contractile function. We hypothesized that Fktn KO mice will have lower mitochondrial respiratory function than control mice, and that enhancing the mitochondrial network will be beneficial for muscle contractile function. To test this hypothesis, we utilized highly sensitive and reproducible electrophysiological techniques to measure both muscle contractility and mitochondrial quality in Fktn KO mice.

2 | METHODS

2.1 | Ethical approval

All animal protocols were approved by the University of Georgia Animal Care and Use Committee (A2017 08-004) under the national guidelines set by the Association for Assessment and Accreditation of Laboratory Animal Care. All experiments were carried out according to the guidelines described by Grundy (2015).

2.2 | Animals and study design

Male and female *Myf5/Fktn* knockout (Fktn KO) mice (previously described; Beedle et al., 2012) and littermate controls (LM) were generously donated by Dr Aaron Beedle (formerly University of Georgia) at 8–12 weeks of age and were maintained at 20–23°C on a 12:12 h light–dark cycle with food and water *ad libitum*. Both male and female LM and Fktn KO mice were used for all studies with no special consideration for sex, as in our previous reports (Beedle et al., 2012; Foltz et al., 2016 a,b) involving these mice have not revealed clear disease-related phenotypical differences. LM and Fktn KO mice (63% male, 37% female) were randomized into either saline (LM $n = 13$, Fktn KO $n = 9$) or AICAR (LM $n = 13$, Fktn KO $n = 7$) treatment groups. An additional 10 LM and 10 Fktn KO mice were killed at the beginning of the study for the initial characterization of mitochondrial respiratory function. Mice received daily intraperitoneal injections of saline (200 μ l) or

AICAR ($500 \text{ mg (kg body mass)}^{-1}$) for a total of 4 weeks (Narkar et al., 2008; Pauly et al., 2012). After 2 weeks of treatment, *in vivo* peak isometric torque was assessed on the dorsiflexor muscles of the left hindlimb just before the same limb of all mice was injured with a $40 \mu\text{l}$ intramuscular injection of cardiotoxin into the anterior and posterior compartments of the lower hindlimb (Naja kaouthia, 0.071 mg ml^{-1} ; cat. no. C9759, Sigma-Aldrich, St Louis, MO, USA; Nichenko et al., 2016); the contralateral limb served as the uninjured control. Following the muscle injury, all mice continued to receive treatment for 2 weeks at which point they were killed with graduated CO_2 in a pre-empted container followed by cervical dislocation. Immediately prior to killing, *in vivo* peak isometric torque was assessed on the dorsiflexor muscles of the left (injured) mouse hindlimb. At killing, injured and uninjured gastrocnemius and tibialis anterior muscles were extracted and weighed. A fraction of the gastrocnemius was immediately processed for measurements of mitochondrial respiratory function, while the remaining portion was homogenized and prepared for assessment of mitochondrial enzyme activities and western blotting. The tibialis anterior muscle was processed for histology and microscopy and stored at -80°C .

2.3 | *In vivo* muscle contractile function

In vivo peak isometric torque of the ankle dorsiflexors was assessed as previously described (Baltgalvis, Call, Nikas, & Lowe, 2009). Briefly, anaesthesia was induced using an induction chamber and 5% isoflurane in oxygen. Anaesthesia was maintained using 1.5% isoflurane at an oxygen flow rate of 0.4 l min^{-1} . The left hindlimb was depilated and aseptically prepared and the foot placed in a foot-plate attached to a servomotor (Model 300C-LR; Aurora Scientific, Aurora, Ontario, Canada). Platinum–iridium needles (Model E2–12; Grass Technologies, West Warwick, RI, USA) were placed on either side of the left common peroneal nerve to elicit contraction of the dorsi-flexors muscles. Peak isometric torque was defined as the greatest torque measured by a 300B-LR servomotor (Aurora Scientific) during a 200 ms stimulation using 1 ms square-wave pulses at 300 Hz and increasing voltage from 3.0 to 9.0 V (models S48 and SIU5; Grass Technologies). All torque values were normalized by mouse body mass.

2.4 | Acute stimulation protocol

To assess the integrity of mitochondrial biogenesis signalling in Fktn KO muscle, eight additional untreated mice (4 LM; 4 Fktn KO, 100% female) underwent a 30 min *in vivo* electrical stimulation protocol to simulate an acute bout of exercise as previously described (Southern et al., 2019). Briefly, mice were anesthetized with isoflurane (1.5% to 2.0%) and platinum–iridium needle electrodes were placed around the sciatic nerve. The electrical stimulation protocol consisted of 10 sets of 1800 contractions (parameters: pulse frequency = 100, pulse width = 0.1, pulses per train = 1, train frequency = 10 Hz) conducted over 30 min. At the end of the protocol, gastrocnemius muscles were quickly harvested, flash-frozen in liquid nitrogen, and stored at -80°C for qRT-PCR analysis.

2.5 | Mitochondrial assays

Mitochondrial respiratory function was assessed as previously described (Southern et al., 2019). Briefly, portions of the medial and lateral gastrocnemius muscles from uninjured limbs were dissected on a chilled aluminium block in 4°C buffer X ($7.23 \text{ mM K}_2\text{EGTA}$,

2.77 mM Ca-K₂EGTA, 20 mM imidazole, 20 mM taurine, 5.7 mM ATP, 14.3 mM PCr, 6.56 mM MgCl₂·6H₂O, 50 mM K-MES) into thin muscle fibre bundles (<1 mg). Muscle fibres were permeabilized in buffer X and saponin (50 µg ml⁻¹) for 30 min at 4°C. Muscle fibre bundles were rinsed for 15 min in buffer Z (105 mM K-MES, 30 mM KCl, 10 mM KH₂PO₄, 5 mM MgCl₂, 0.5 mg ml⁻¹ BSA, 1 mM EGTA) at 4°C. All measurements were performed using a Clark-type electrode (Oxygraph Plus System, Hansatech Instruments, King's Lynn, UK) at 25°C. Muscle fibre bundles (~2.5 mg for all samples) were added to the chamber and mitochondrial leak respiration (addition of 10 mM glutamate, 5 mM malate) and mitochondrial state 3 respiration (addition of 2.5 mM ADP and 10 mM succinate) were measured. Cytochrome *c* (10 µM) was also added to measure the integrity of the outer mitochondrial membrane. Mitochondrial respiration was terminated by the addition of cyanide (2 mM).

Mitochondrial content was assessed using citrate synthase and succinate dehydrogenase enzyme activities as previously described (Nichenko et al., 2016; Southern et al., 2017). Briefly, a fraction of the gastrocnemius muscles were homogenized in a glass tissue grinder in 33 mM phosphate buffer (pH 7.0) at a muscle:buffer ratio of 1:20. Citrate synthase activity was assessed by combining 5 µl of homogenate, 173.74 µl of 100 mM Tris buffer (pH 8.0), 17.51 µl of 5,5-dithiobis-(2-nitrobenzoic acid (DTNB), 8.75 µl of acetyl CoA, and 20 µl of oxaloacetate in a well of a 96-well plate and measuring absorbance at 405 nm every 10 s for 3 min. Succinate dehydrogenase activity was assessed by combining 4 µl of homogenate, 10 µl of 0.5 M sodium succinate, 10 µl of 0.01 M sodium cyanide and 280 µl of cytochrome *c* solution (33% 0.1 mM cytochrome *c*, 13% 0.004 M AlCl₃/CaCl₂) in a well of a 96-well plate and measuring absorbance at 550 nm every 20 s for 5 min. Enzyme activities were normalized to mg of tissue in the sample homogenate.

2.6 | Western blotting

For protein content analysis, protein concentration of muscle lysates was determined by measuring absorbance at 280 nm via a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and 30 µg of total protein from injured and uninjured gastrocnemius muscles was separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane as previously described (Nichenko et al., 2016). The following antibodies (Cell Signaling Technology, Danvers, MA, USA) were used: AMPK (RRID: AB_330331, 1:1000), pAMPK (RRID: AB_330330, 1:1000), Beclin-1 (RRID:AB_1903911, 1:1000), LC3B (RRID:AB_915950, 1:1000), SQSTM1/p62 (RRID: AB_1841064, 1:1000), BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3) (RRID:AB_2259284, 1:1000) and dynamin-related protein 1 (Drp1) (D6C7, 1:1000). Mouse or rabbit horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, CA, USA) were detected using enhanced chemiluminescence on a ChemiDoc imaging system (Bio-Rad). Immunoblots were normalized to total protein in lane via stain-free gel imaging (Collins, An, Peller, & Bowser, 2015; Vigelsø et al., 2015; Zeitler, Gerrer, Haas, & Jiménez-Soto, 2016) and quantified using Bio-Rad Image Lab software.

2.7 | Immunofluorescence microscopy

A subset of five mice were randomly selected from each experimental group for immunofluorescence analysis. Seven-micrometre tissue cryosections were mounted on microscope slides and processed for immunofluorescence analysis of centrally nucleated fibres (CNF) and embryonic myosin heavy chain (eMHC) similar to previous work (Foltz et al., 2016b). Sections were wet with Dulbecco's phosphate-buffered saline (no Ca^{2+} or Mg^{2+} , Corning; Corning, NY, USA); incubated for 30 min in blocking solution (5% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in Dulbecco's phosphate-buffered saline); incubated overnight at 4°C with primary antibody; washed three times; incubated for 30 min with secondary antibody, stains or dyes; washed three times; and mounted with PermaFluor (Thermo Fisher Scientific). Reagents used for visualization were: F1.652 against embryonic myosin heavy chain (1:40, Developmental Studies Hybridoma Bank, Iowa City, IA, USA); goat anti-mouse IgG1-Alexa546 (1:500, Thermo Fisher Scientific); membrane marker wheat germ agglutinin (WGA)-coupled Alexa488 (Thermo Fisher Scientific, 1:100); muscle marker actin-Alexa647 (Thermo Fisher Scientific, 1:40); and nuclear stain 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, 1:10,000). Four filter epifluorescence images were obtained for whole TA sections using the VS-120 slide scanner (Olympus, Tokyo, Japan) with $\times 10$ objective and imported into ImagePro Premier 3D v9.1 (Media Cybernetics, Rockville, MD, USA) as three colour images for analysis (centrally nucleated and eMHC counts: eMHC/WGA-A488/DAPI; fibre size: WGA-A488/actin-A647/DAPI). Centrally nucleated and eMHC-positive fibres were analysed by manual count out of total fibres for entire tibialis anterior sections. The smart segmentation function in ImagePro Premier was used to partially automate detection of fibre perimeters. All sections were manually verified for fibre detection and corrected as necessary. Minimum diameter (μm) was collected for all fibre annotations on a section. A bin size of 3.5 μm for fibre sizes was selected by taking the average h calculated for each sample using the formula $h = 3.5\sigma/n^{1/3}$ (where h is the optimized bin size, σ is standard deviation, n is number of observations) and rounding up to the nearest 0.5. Fibre sizes for each sample were binned and reported as mean $\pm$ standard error for each group. All image analyses were performed blinded.

2.8 | Gene expression

cDNA was generated from isolated gastrocnemius muscle RNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). iQ SYBR Green Supermix (Bio-Rad) and sequence-specific primers were used to assess mRNA levels for the following genes: *PGC-1 α* (forward: 5'-AGC CGT GAC CAC TGA CAA CGA G-3'; reverse: 5'-GCT GCA TGG TTC TGA GTG CTA AG-3') (Lin et al., 2004). Gene expression analysis was carried out by qRT-PCR on a Bio-Rad CFX instrument as previously described (Southern et al., 2019). Data were normalized to *18S* (forward: 5'-TTG ATT AAG TCC CTG CCC TTT GT-3'; reverse: 5'-CGA TCC GAG GGC CTA ACT A-3') (Jenkins, Witkowski, Spangenburg, & Hagberg, 2009) and relative gene expression was calculated using the $2^{-\text{CT}}$ method.

2.9 | Statistics

Data are presented in the Results as means $\pm$ SD. A multi-factor repeated measures analysis of variance (ANOVA) was used to analyse genotype (LM, Fktn KO), treatment (saline, AICAR), and limb (injured, uninjured) conditions. The between-subject factors were genotype and treatment while the repeated measure factor was limb. Two-way ANOVA was used to analyse the remaining data. All data were required to pass normality (Shapiro–Wilk) and equal variance (Brown–Forsythe *F* test) tests before proceeding with the ANOVA. Differences among groups are only reported where significant interactions were observed and subsequently tested with Tukey's *post hoc* test using JMP statistical software (SAS Institute, Cary, NC, USA). Group main effects are reported where significant interactions were not observed. An α level of 0.05 was used for all analyses.

3 | RESULTS

3.1 | Characterization of contractile and metabolic function of Fktn KO mice

In agreement with previous reports, Fktn KO mice had ~21% lower body mass compared to healthy LM control mice ($P < 0.001$; Figure 1a; Beedle et al., 2012). *In vivo* assessment of peak-isometric torque revealed that Fktn KO mice were ~30% weaker than LM controls even after accounting for the differences in body mass ($P < 0.001$; Figure 1b). Gastrocnemius muscle mass normalized to body mass was not different between LM and Fktn KO mice (LM: 5.43 ± 0.52 mg g⁻¹ vs. KO: 5.11 ± 0.89 mg g⁻¹). Tibialis anterior muscle mass normalized to body mass was slightly greater in the Fktn KO mice compared to LM (LM: 1.73 ± 0.14 mg g⁻¹ vs. KO: 2.03 ± 0.28 mg g⁻¹). State 3 mitochondrial respiration from permeabilized muscle fibres was also ~16% less in Fktn KO mice compared to LM mice suggesting that fukutin deletion may interfere with metabolic processes in skeletal muscle ($P = 0.002$; Figure 1c).

3.2 | Effects of AICAR treatment on mitochondrial content and function in Fktn KO mice

In an effort to rescue the loss of mitochondrial respiratory function, we treated LM and Fktn KO mice with AICAR, a drug previously shown to improve mitochondrial quality in dystrophic muscle via the AMPK–PGC-1 α molecular pathway. Interestingly, after 4 weeks of treatment, AICAR did not improve mitochondrial content or function in either LM or Fktn KO mice (Figure 2a–c), but rather resulted in a slight (~7%) reduction in mitochondrial content irrespective of genotype ($P = 0.010$, Figure 2b,c).

3.3 | Blunted PGC-1 α gene expression in Fktn KO mice

Dysfunctional mitochondrial biogenesis could be a potential mechanism explaining the lower mitochondrial respiratory function and content observed in Fktn KO mice. In order to test whether Fktn KO mice had disrupted mitochondrial biogenesis signalling, we administered an acute exercise stimulus to a hindlimb of LM and Fktn KO mice and measured markers of mitochondrial biogenesis, namely pAMPK content and PGC-1 α gene expression. Exercised limbs had ~80% greater pAMPK/AMPK protein expression compared to control limbs irrespective of genotype ($P = 0.040$; Figure 3a,b), demonstrating that exercise-induced AMPK signalling was intact in Fktn KO mice. Interestingly, the same did

not hold true for post-exercise *PGC-1 α* gene expression as LM mice had a ~3-fold increase in *PGC-1 α* expression while the Fktn KO mice did not have a significant change in expression levels (interaction: $P = 0.004$; Figure 3c). Together these data suggest that Fktn KO mice have blunted signalling for mitochondrial biogenesis through impaired activation of PGC-1 α signalling.

3.4 | Effects of AICAR treatment on recovery of muscle contractile function following muscle injury

We have previously shown that disruptions in mitochondrial maintenance can have an impact on recovery of muscle contractile function following muscle injury (Nichenko et al., 2016). Thus, in light of the mitochondrial dysfunction in Fktn KO mice, we investigated (1) whether Fktn KO mice would have delayed recovery of muscle contractile function following injury and (2) whether AICAR treatment would enhance the recovery process. Prior to injury, Fktn KO mice had ~22% lower peak-isometric torque compared to LM controls, independent of saline or AICAR treatment ($P < 0.001$; Figure 4a). Two weeks after the injury, the untreated LM and Fktn KO mice had recovered to ~60% of pre-injury peak-isometric torque indicating that Fktn KO mice, despite having mitochondrial abnormalities, did not have delayed recovery of muscle contractility. Interestingly, AICAR-treated mice had ~30% greater peak-isometric torque 14 days after injury compared to untreated mice independent of genotype ($P = 0.006$), which suggests that AICAR treatment reduced damage and/or enhanced the recovery of muscle contractile function for both LM and Fktn KO mice (Figure 4b).

Immunohistological analysis of muscle fibres expressing eMHC and CNFs was performed to interrogate aspects of the muscle regeneration process in LM control and Fktn KO mice. Specifically, eMHC was interpreted as indicating acute/ongoing fibre regeneration and CNF was interpreted as evidence of past/historical fibre regeneration. There was a significant interaction between genotype and limb (uninjured vs. injured) for CNFs indicating greater historical regeneration in injured LM muscle and both uninjured and injured Fktn KO muscle compared to uninjured LM muscle ($P < 0.001$; Figure 5a). There was a significant interaction between genotype and treatment (saline vs. AICAR) for eMHC indicating less ongoing regeneration in AICAR-treated Fktn KO muscles ($P = 0.014$; Figure 5b). These data support the notion that muscle damage (CNF) was not affected by AICAR; rather AICAR enhances muscle regeneration after injury (Figure 5a,b). While all groups had similar muscle fibre numbers (Figure 5c), analysis of muscle fibre size revealed that Fktn KO mice and injured muscles (irrespective of genotype) had smaller fibre diameter with no effect of AICAR treatment detected (chi-square distribution leftward shift; uninjured vs. injury, $P = 0.02$; LM vs. Fktn KO, $P = 0.007$; Figure 5d,e). Together with the analyses of mitochondrial respiration and AMPK and PGC1- α signalling (Figures 2 and 3), these data suggest that AICAR treatment aids in recovery of muscle contractile function, but this action is independent of changes in mitochondrial respiratory function.

3.5 | Effects of AICAR treatment on autophagy following muscle injury

Given that our previously published work demonstrated that autophagy plays an important role in regeneration of muscle following injury (Nichenko et al., 2016), we investigated

various markers of autophagy as a potential mechanism for AICAR aiding in recovery of muscle contractile function. Autophagy is a cellular recycling process that identifies, removes and degrades damaged or dysfunctional cellular components via autophagosomes. Expression of Beclin1 (or Atg6), an important marker of cellular autophagy which contributes to the formation of autophagosomes, was greater in Fktn KO mice compared to LM mice independent of injury or treatment (injury $\times$ genotype $\times$ treatment interaction $P=0.039$; Figure 6a). Interestingly, AICAR treatment resulted in greater Beclin1 expression in the 2-week post-injury limb of LM mice, but not Fktn KO mice (Figure 6a).

The final step of autophagy is the degradation of the autophagosome, and the level of activity of this process can be indirectly characterized by measuring protein content levels of the conjugated form of microtubule-associated proteins 1A/1B light chain 3A (LC3-II), as well as the relative degradation of polyubiquitin-binding protein p62/SQSTM1 (p62). LC3-II content was elevated by $\sim 52\%$ in Fktn KO mice compared to LM mice (Figure 6b; $P<0.002$) and by $\sim 32\%$ in the injured limb compared to uninjured limb (Figure 6b; $P<0.001$), but these effects were independent of AICAR treatment. Interestingly, degradation of p62 was $\sim 54\%$ greater in AICAR-treated mice compared to untreated mice (Figure 6b; $P=0.031$).

BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (Bnip3) and dynamin-related protein 1 (Drp1) are proteins involved in mitochondrial specific autophagy, known as mitophagy. Bnip3, which is specifically responsible for tagging mitochondria for degradation, was $\sim 60\text{--}100\%$ greater in the AICAR-treated LM injured, Fktn KO uninjured and Fktn KO injured mice compared to corresponding untreated mice (Figure 6c; limb $\times$ genotype $\times$ treatment interaction: $P=0.016$). Drp1, which facilitates fission of damaged mitochondria from the network, was also elevated ($\sim 70\%$) in response AICAR treatment, but only in LM-injured AICAR-treated mice (Figure 6c; injury $\times$ genotype $\times$ treatment interaction: $P=0.037$).

4 | DISCUSSION

Disruptions in the function of α DG within the DGC give rise to a collection of muscle myopathies termed secondary dystroglycanopathies that are characterized by early onset of muscle weakness and progressive loss of muscle contractile function. Importantly, in order to develop targeted treatments for these myopathies, we must first understand how disruptions in the DGC may manifest in muscle pathophysiology. In this study, we reveal that fukutin deletion leads to reductions in mitochondrial respiratory function and content compared to LM control mice, which is likely the result of impaired mitochondrial biogenesis. These results point to the possibility of a link between muscle metabolic function and proper function of α DG meaning that the resulting metabolic disruptions stemming from loss of fukutin could be an important component of the disease pathology. Secondly, we demonstrate that the AMPK agonist AICAR potentially accelerates muscle recovery from injury, making the AMPK signalling pathway an intriguing option for future research involving treatments for dystroglycanopathies.

In recent years, it has become apparent that mitochondrial abnormalities are a common phenotype presenting in mice with various forms of DGC disruptions (Millay et al., 2008; Pambianco et al., 2016; Pauly et al., 2012; Vila et al., 2017). However, research involving mitochondria in the context of dystroglycanopathies has yet to elucidate whether the mitochondrial abnormalities are intrinsic manifestations or secondary consequences of disease pathology. Herein, we present data that suggest that mitochondrial defects are intrinsic to the disease pathology, as Fktn KO mice have impaired *PGC-1 α* transcription following acute exercise. This data suggests that poor mitochondrial quality in Fktn KO mice is likely to stem from modifications to the *PGC-1 α* gene that is derived from altered DGC signalling. This idea is supported by the recent work performed by Pambianco et al. (2016) who demonstrated that DGC disruption via deletion of α -sarcoglycan leads to reduced acetylation and a condensed chromatin structure of the *PGC-1 α* promoter. This nuclear modification arising from a dysfunctional DGC resulted in diminished mitochondrial function due to reduced mitochondrial proteins that are under the regulatory control of *PGC-1 α* . *PGC-1 α* is likely not the only metabolic gene that affected by disruptions in the DGC, as gene expression profiling performed on human tissue revealed that 26 nuclear encoded mitochondrial genes were down-regulated in dystrophin- and α -SG-deficient patients (Chen, Zhao, Borup, & Hoffman, 2000). The downregulation of these metabolic genes could potentially be the result of overactive Ca^{2+} signalling in the muscle cells creating a negative feedback loop that drives down the metabolic gene expression. Together these data suggest that mitochondrial defects are linked to altered DGC function, but more research is necessary to explore whether these defects are directly linked to or simply accompany the DGC disruptions. Nevertheless, these data do provide a potentially interesting avenue for future research regarding treatments strategies for secondary dystroglycanopathies.

Activation of the AMPK signalling pathway is one of the primary mechanisms underlying mitochondrial biogenesis, and AICAR, being an AMPK agonist, is a drug that has been shown to stimulate the mitochondrial biogenesis process. In this study, we attempted to exploit the benefits of AICAR on mitochondrial biogenesis to rescue the mitochondrial defects observed in Fktn KO mice. However, AICAR had no beneficial effects on mitochondrial content or function, which could potentially be due to faulty *PGC-1 α* transcription given that AICAR stimulates mitochondrial biogenesis through AMPK-induced activation of *PGC-1 α* . Minimal effects of AICAR on mitochondria have also been reported by Pauly et al. (2012) who treated dystrophin-deficient *mdx* mice with AICAR for 4 weeks and found no changes in mitochondrial content or respiratory function. Irrespective of the reason for AICAR having minimal effects on muscle mitochondria, these data inform future research to employ other drugs to target alternative pathways in order to correct mitochondrial defects in Fktn KO mice.

Our group has previously published a report with histological evidence suggesting that Fktn KO mice have abnormal muscle regeneration following severe muscle injury (Foltz et al., 2016b). Thus, to determine whether the histological alterations in Fktn KO mice translated to functional deficits in recovery and to see if AICAR could help correct any abnormal recovery, we measured muscle contractile function in LM and Fktn KO mice with and without AICAR treatment following an injury. Interestingly, despite our previously

published data, Fktn KO mice did not have any signs of delayed regeneration at 2 weeks following the injury. This is not to say that a delay in functional recovery would never happen in Fktn KO mice, as one injury may not be sufficient to observe defects in functional recovery. Perhaps, repeated cardiotoxin injuries would have revealed a regeneration deficit that only becomes apparent after chronic repeated injuries. Our results demonstrate that Fktn KO mice have the ability to undergo normal functional regeneration following a severe injury, but it remains to be seen whether further injuries or ageing would have an effect on this finding.

While AICAR treatment did not have any beneficial effects on mitochondria in Fktn KO mice, it did have a beneficial effect on recovery of muscle contractile function following muscle injury in both LM and Fktn KO mice. Initially, we hypothesized that AICAR treatment would be beneficial for Fktn KO mice after injury because it would enhance mitochondrial maintenance, but given the lack of effect of AICAR on mitochondria, it remains to be seen how AICAR would lead to improved recovery after injury. AICAR is known to stimulate autophagy, and we have previously reported that enhancing autophagy prior to an injury can lead to accelerated recovery of muscle contractile function (Nichenko et al., 2016). In this study, we found that AICAR treatment did indeed augment autophagy in both LM and Fktn KO mice, which is a possible explanation for AICAR's effect on recovery of muscle contractile function. Alternatively, AICAR has also been shown to induce satellite cell proliferation (Fu, Zhu, Dodson, & Du, 2015), which may be a mechanism for enhancing recovery following injury (Jahnke et al., 2012). In conclusion, AICAR accelerates functional recovery of muscle after injury, but further research is needed to identify AICAR's mechanism of action and thus optimize treatment efficacy.

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New Findings

What is the central question of this study?

Does fukutin deficiency in skeletal muscle cause mitochondrial dysfunction, and if so, can AMP-activated protein kinase (AMPK) stimulation via 5-aminoimidazole-4-carboxamide ribonucleotide attenuate this through regulation of mitochondrial biogenesis and autophagy?

What is the main finding and its importance?

Mitochondrial dysfunction is associated with fukutin deficiency and AMPK stimulation may benefit muscle contractility to a greater extent than mitochondrial function.

FIGURE 1.

Fktn KO mice have lower body mass, muscle strength, and mitochondrial respiratory function compared to LM control mice. (a) Body mass of LM ($n = 26$) and Fktn KO ($n = 16$) mice; $*P < 0.001$. (b) Peak isometric torque of dorsiflexor muscles normalized to body mass of LM ($n = 26$) and Fktn KO ($n = 16$) mice; $*P < 0.001$. (c) Mitochondrial respiratory function normalized by grams wet weight of permeabilized muscle fibres from LM and Fktn KO mice ($n = 20$ permeabilized fibre bundles from $n = 10$ mice for each group); $*P = 0.002$. Data are presented as means $\pm$ SD

FIGURE 2.

Mitochondrial deficits in Fktn KO mice are still present after 4 weeks of AICAR treatment.

(a) Mitochondrial respiratory function normalized by grams wet weight of permeabilized muscle fibres (main effect of genotype, $P = 0.004$), (b) citrate synthase activity (CS) (two-way ANOVA, $P = 0.01$ for main effect of treatment), and (c) succinate dehydrogenase (SDH) activity (two-way ANOVA, $P = 0.04$ for main effect of treatment and $P < 0.001$ for main effect of genotype) from LM and Fktn KO mice after 4 weeks of saline or AICAR treatment (Saline: LM $n = 13$, Fktn KO $n = 9$; AICAR: LM $n = 13$, Fktn KO $n = 7$). Data are presented as means $\pm$ SD

FIGURE 3.

Altered marker of mitochondrial biogenesis signalling in Fktn KO mice. (a) Representative immunoblot and (b) semi-quantitative analysis of pAMPK and AMPK relative protein expression 3 h after completion of stimulation protocol for stimulated (Stim) and non-stimulated (No Stim) gastrocnemius muscles of LM ($n = 4$) and Fktn KO ($n = 4$) mice. Blots are normalized to total protein as a loading control. (c) *PGC-1α* gene expression 3 h after completion of stimulation protocol for stimulated and non-stimulated limbs of LM and Fktn KO mice; two-way repeated measures ANOVA, interaction $P < 0.01$, *Significantly different from all other experimental groups. Data are presented as means $\pm$ SD

FIGURE 4.

AICAR treatment aids in recovery of muscle strength following muscle injury. (a) Pre-cardiotoxin injury muscle strength from LM and Fktn KO mice treated with saline (LM $n = 13$, Fktn KO $n = 9$) or AICAR (LM $n = 13$, Fktn KO $n = 7$; two-way ANOVA, main effect of genotype $P < 0.001$). (b) Muscle strength 14 days after cardiotoxin injury for LM and Fktn KO mice treated with saline (LM $n = 12$, Fktn KO $n = 9$) or AICAR (LM $n = 10$, Fktn KO $n = 6$; main effect of treatment $P = 0.006$). Data are presented as means $\pm$ SD

FIGURE 5.

The effects of AICAR treatment on metrics of muscle fibre regeneration for uninjured and injured muscles from LM and Fktn KO mice. (a) Percentage of total fibre with centrally located nuclei (two-way repeated measures ANOVA, genotype $\times$ limb interaction $P < 0.001$) from LM and Fktn KO mice treated with saline (LM $n = 5$, Fktn KO $n = 5$) or AICAR (LM $n = 5$, Fktn KO $n = 5$). (b) Percentage of total fibres with positive embryonic myosin heavy chain (eMHC) staining (treatment $\times$ genotype interaction, $P = 0.014$). (c) Total number of fibres. (d,e) Fibre size distribution for injured and uninjured muscles from LM (uninjured: continuous line, injured: dashed line) and Fktn KO (uninjured: filled bar, injured: hatched bar) mice treated with (d) saline or (e) AICAR (chi-square distribution analysis; uninjured vs. injury, $P = 0.02$; LM vs. Fktn KO, $P = 0.007$). Data are presented as means $\pm$ SD. (f)

Representative injured and uninjured tibialis anterior images from LM and Fktn KO mice treated with saline (top row) or AICAR (bottom row). Wheat germ agglutinin (WGA) (green), embryonic myosin heavy chain (red), and nuclear (blue; DAPI) stains are shown for all groups. Scale bar: 100 μm for all images

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FIGURE 6.

Effects of AICAR treatment on markers of autophagy from injured and uninjured muscles of LM and Fktn KO mice following 4 weeks of either saline or AICAR treatment.

Representative immunoblots and semi-quantitative analyses of relative protein expression normalized to total protein content for (a) Beclin (two-way repeated measures ANOVA, interaction $P = 0.039$), (b) LC3 II (main effect of genotype $P < 0.002$; injury $P < 0.001$), p62 (main effect of treatment $P = 0.031$), (c) Bnip3 (interaction $P = 0.016$) and Drp1 (interaction $P = 0.037$). *Significantly different from denoted experimental groups. Data are presented as means $\pm$ SD