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Title:
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Date:
2021-08-01

Citation:
Hardee, J. P., Caldow, M. K., Chan, A. S. M., Plenderleith, S. K., Trieu, J., Koopman, R. & Lynch, G. S. (2021). Dystrophin deficiency disrupts muscle clock expression and mitochondrial quality control in mdx mice. American Journal of Physiology Cell Physiology, 321 (2), pp.C288-C296. https://doi.org/10.1152/ajpcell.00188.2021.

Persistent Link:
https://hdl.handle.net/11343/311455

Dystrophin deficiency disrupts muscle clock expression and mitochondrial quality control in *mdx* mice

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Declarations of interest: none

Word Count: 4534 (all sections except abstract)

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ABSTRACT

Impaired oxidative capacity and mitochondrial function contribute to the dystrophic pathology in muscles of Duchenne muscular dystrophy (DMD) patients and in relevant mouse models of the disease. Emerging evidence suggests an association between disrupted core clock expression and mitochondrial quality control, but this has not been established in muscles lacking dystrophin. We examined the diurnal regulation of muscle core clock and mitochondrial quality control expression in dystrophin-deficient C57BL/10ScSn-*Dmd*^{mdx} (*mdx*) mice, an established model of DMD. Male C57BL/10 (BL/10; n=18) and *mdx* mice (n=18) were examined every 4 hours beginning at the dark cycle. Throughout the entire light-dark cycle, extensor digitorum longus (EDL) muscles from *mdx* mice had decreased core clock mRNA expression (*Arntl, Cry1, Cry2, Nr1d2*; $p<0.05$) and disrupted mitochondrial quality control mRNA expression related to biogenesis (decreased; *Ppargc1a, Esrra*; $p<0.05$), fission (increased; *Dnm1l*; $p<0.01$), fusion (decreased; *Opa1, Mfn1*; $p<0.05$) and autophagy/mitophagy (decreased: *Bnip3*; $p<0.05$; increased: *Becn1*; $p<0.05$). Cosinor analysis revealed a decrease in the rhythmicity parameters mesor and amplitude for *Arntl*, *Cry1*, *Cry2*, *Per2*, *and Nr1d1* ($p<0.001$) in *mdx* mice. Diurnal oscillations in *Esrra*, *Sirt1*, *Map1lc3b* and *Sqstm1* were absent in *mdx* mice, along with decreased mesor and amplitude of *Ppargc1a* mRNA expression ($p<0.01$). The expression of proteins involved in mitochondrial biogenesis (decreased: PPARGC1A, $p<0.05$) and autophagy/mitophagy (increased: MAP1LC3BII, SQSTM1, BNIP3; $p<0.05$) were also dysregulated in tibialis anterior muscles of *mdx* mice. These findings suggest that dystrophin deficiency in *mdx* mice impairs the regulation of the core clock and mitochondrial quality control, with relevance to DMD and related disorders.

Keywords: Muscular dystrophy, diurnal variation, dystrophin, mitochondria

INTRODUCTION

The circadian core clock is a molecular oscillator that regulates physiological processes relevant to skeletal muscle homeostasis. Diurnal regulation of mRNA expression is regulated by a set of core clock proteins, the transcriptional activators circadian locomotor output cycles kaput (*Clock*) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (*Arntl*; also known as *Bmal*), and function through coordinated negative transcription-translation feedback loops generated by the transcriptional repressors period 1/2/3 (*Per1/2/3*) and cryptochrome 1/2 (*Cry1/2*) (1). The core clock regulates daily gene expression patterns involved in metabolism, signal transduction and transcription (2, 3), with the transcription of specific metabolic pathways tightly linked to the regulation of cellular metabolism (1). There is crosstalk between the expression and activity of core clock components and mitochondrial homeostasis (4). Disruptions to core clock components impair mitochondrial quality control and respiratory function, whereas alterations in cellular metabolism and metabolic intermediates influencing clock regulation (4, 5). Evidence suggests that disruptions to the core clock in muscle can impair cellular processes regulating contractile and metabolic function (6-8), which has important implications in muscle-related disorders such as cancer and muscular dystrophies.

In Duchenne muscular dystrophy (DMD) and murine models of disease, muscles exhibit impaired mitochondrial content and function (9-11), which has been implicated in the pathophysiology. Mitochondrial homeostasis is dynamically regulated through processes related to biogenesis, dynamics (fission/fusion events), and mitophagy (12). Given that disruption of these cellular processes has been implicated in DMD, an improved understanding of the underlying mechanisms regulating mitochondrial quality control is required to develop effective treatments. Despite independent evidence for altered core clock and mitochondrial

quality control mechanisms in dystrophic pathology (13, 14), how these cellular processes fluctuate throughout the diurnal cycle in muscles lacking dystrophin has yet to be examined. We therefore examined the expression of the core clock and mitochondrial quality control throughout the light-dark cycle (assessed every 4 hours) in *mdx* and age-matched control BL/10 mice. Our findings suggest dystrophin deficiency in muscles of *mdx* mice impairs the regulation of the core clock and mitochondrial quality control, with relevance to DMD and related muscle disorders.

MATERIALS AND METHODS

Animals and Experimental Design

Male, C57BL/10 (BL/10) and dystrophin deficient C57BL/10ScSn-*Dmd*^{mdx} (*mdx*) mice were obtained from the Animal Resources Centre (Canning Vale, WA, Australia) at 4-5 weeks of age. All mice were housed under a 12-hour light/dark cycle and provided access to drinking water and standard chow *ad libitum*. Following 2-weeks of acclimation to the light-dark cycle, mice were killed in an alternating fashion every 4 h beginning at the onset of the dark cycle (Zeitgeber time (ZT) 12). Mice were anesthetized by sodium pentobarbitone (120 mg/kg), tissues were rapidly excised, weighed, and frozen in liquid nitrogen, and then mice were euthanized by cardiac excision while under deep anesthesia. All experiments were approved by the Animal Ethics Committee of The University of Melbourne (AEC 1613961).

RNA extraction and qPCR

Total RNA was extracted from extensor digitorum longus (EDL) muscle and qPCR was performed in triplicate as described previously (15). mRNA expression was normalized to *Hprt1*, which was not altered by dystrophin deficiency or time of day. Primers used in this study have been published previously (19).

Immunoblotting

Protein was isolated from the tibialis anterior (TA) muscles and western blot performed as described previously (15, 16). Protein content was determined via Bio-Rad DC protein assay as per manufacturer's instructions (Bio-Rad). Samples were separated by SDS-PAGE using Criterion TGX Stain-Free Precast Gels (Bio-Rad) and transferred to 0.45 µm PVDF via

wet transfer at 4°C (100V for 60 min). Membranes were incubated with primary and secondary antibodies before being treated with enhanced chemiluminescence (Super Signal West Femto; Thermo Scientific) and visualized using ChemiDoc imaging system (Bio-Rad). Antibodies used in this study have been published previously (17-19). Blots were quantified using ImageJ software (NIH) and normalized to total protein.

To determine differences in protein expression, samples were analyzed as described previously (18-20). An equal amount of protein from each genotype (n=3 samples/timepoint) was pooled to generate diurnal expression patterns for both groups on a single gel, as shown in Figures 1C and 4A. To determine the effects of dystrophin deficiency throughout the entire light-dark cycle, all six timepoints were grouped and differences between BL10 and *mdx* mice were assessed by Student's t-test. In contrast to previous studies (18-20), we did not perform statistical analysis across genotypes within light or dark cycles (e.g., n=3 genotype), but representative curves are presented for qualitative assessment and to guide future studies examining protein expression.

Statistical Analysis

Data are presented as mean ± standard error of the mean unless noted in the Figure Legends. In Figures 1 and 4, an unpaired Student's t-test was performed to determine differences in body mass (n=18 genotype), muscle mass (n=18 genotype), mRNA expression independent of time (n=18 genotype), and protein expression independent of time (n=6 genotype) between BL/10 and *mdx* mice. In Figure 2, a one-way analysis of variance (ANOVA) was performed to determine if there were differences in mRNA expression over time. If this condition was met, then the rhythmicity of gene expression was evaluated using a cosine wave

fitted by the least squares method using a predefined period of 24 h. If a significant rhythm was detected across both genotypes (e.g., amplitude statistically greater than zero), the cosine curve parameters mesor, amplitude and acrophase, were compared using an unpaired Student's t-test. The parameters were defined as: Mesor, the mid-value of the cosine curve representing a rhythm-adjusted mean; Amplitude, the one-half distance between peak and trough; Acrophase, the time of the peak of fitted curve, representing the average time of high values in the data. In Figure 3, a two-way ANOVA (genotype × time) with post hoc comparison by the Fisher's LSD test was used to determine differences between BL/10 and *mdx* mice. When an 'interaction' or 'main effect of time' was observed, rhythmicity of gene expression was evaluated using a cosine wave fitted by the least squares method as described for Figure 2. GraphPad Prism 8 software (GraphPad, San Diego, CA) and Microsoft Excel (Microsoft, Redmond, WA) were used for data processing and analyses.

RESULTS

Dystrophin deficiency in mdx mice disrupts core clock and mitochondrial quality control mRNA expression.

We examined muscles from dystrophin deficient *mdx* and age-matched BL/10 mice every 4 hours beginning at the start of the dark cycle (Fig. 1A). As expected, tibialis anterior (TA) and extensor digitorum longus (EDL) muscles of *mdx* mice were heavier independent of body mass (Fig. 1B; TA: $p<0.0001$; EDL: $p<0.001$), exhibited a loss of dystrophin protein isoform 427 (DP427) protein expression (Fig. 1C; $p<0.0001$), and a reciprocal upregulation of utrophin (UTRN) protein expression (Fig. 1C; $p<0.0001$). We then assessed whether the relative mRNA expression of the core clock and mitochondrial quality control were dysregulated throughout the entire light-dark cycle in muscles of *mdx* mice. We found that *Arntl*, *Cry1*, *Cry2*, and *Nr1d2* mRNA expression were decreased in muscles of *mdx* mice (Figure 1D; $p<0.05$). The changes in core clock components coincided with disrupted regulation of mitochondrial quality control related to biogenesis (decreased; *Ppargc1a, Esrra*; $p<0.05$), fission (increased; *Dnm1l*; $p<0.01$), fusion (decreased; *Opa1, Mfn1*; $p<0.05$) and mitophagy (decreased: *Bnip3*; $p<0.05$; increased: *Becn1*; $p<0.05$). Similar findings in gene expression were also observed in skeletal muscle biopsies of patients diagnosed with DMD compared to healthy controls when examining a published RNA sequencing dataset (Fig. 1F) (21). Overall, these results demonstrate that dystrophin deficiency in muscles of *mdx* mice and human patients disrupts the absolute core clock and mitochondrial quality control mRNA expression.

Dystrophin deficiency in mdx mice disrupts diurnal oscillations in core clock mRNA expression.

The core clock genes exhibited significant differences in mRNA expression across timepoints in BL/10 and *mdx* mice when examined by one-way ANOVA (Fig. 2; all $p<0.05$ except *Cry1* and *Cry2* in *mdx* mice; $p<0.10$). Cosinor analysis revealed that diurnal oscillations in core clock mRNA expression occurred in both BL/10 and *mdx* mice (Fig. 2; $p<0.05$). The mesor of *Arntl*, *Cry1*, *Cry2*, *Per2*, *Nr1d1*, and *Nr1d2* mRNA expression were decreased in *mdx* mice (Fig. 2; $p<0.001$). The amplitude of *Arntl*, *Cry1*, *Cry2*, *Per2*, and *Nr1d1* mRNA expression were also decreased in *mdx* mice (Fig. 2J; $p<0.001$). Interestingly, the mRNA expression of several core clock genes (*Cry1*, *Per1*, *Per2*, *Nr1d1*, *Nr1d2*) exhibited trends towards alterations in acrophase (Fig. 2K; $p<0.10$), but only *Per3* mRNA expression reached statistical significance ($p<0.05$). These results demonstrate that dystrophin deficiency in muscles of *mdx* mice suppressed absolute and diurnal oscillations of core clock mRNA expression.

Dystrophin deficiency in mdx mice disrupts diurnal oscillations in mitochondrial quality control mRNA expression.

Given the disruptions to core clock mRNA expression, we assessed differences in mitochondrial quality control mRNA expression by a two-way ANOVA (genotype × time) with post hoc analysis. When an 'interaction' or 'main effect of time' was observed, rhythmicity was evaluated using a cosine wave fitted by the least squares method, as described previously. Cosinor analysis revealed diurnal oscillations of mitochondrial quality control mRNA expression occurred in 8 out of 13 genes (*Ppargc1a*, *Esrra*, *Sirt1*, *Map1lc3*, *Sqstm1*, *Bnip3*, *Becn1*, *Bcl2*; $p<0.05$) examined in BL/10 mice (Fig. 3), whereas this occurred in only 4 out of 13 genes (*Ppargc1a*, *Bnip3*, *Becn1*, *Bcl2*; $p<0.05$) in *mdx* mice (Fig. 3). We then assessed whether dystrophin deficiency altered the rhythmicity parameters mesor, amplitude and acrophase of

these four genes common to BL/10 and *mdx* mice. While the mesor of all four genes (*Ppargc1a*, *Bnip3*, *Becn1*, *Bcl2*) were decreased in *mdx* mice (Fig. 3; $p<0.001$), only the amplitude of *Ppargc1a* mRNA expression was decreased (Fig. 3N; $p<0.01$). Interestingly, the mean difference in acrophase of *Ppargc1a*, *Becn1*, and *Bcl2* was significantly different between BL/10 and *mdx* mice (Fig. 3O; $p<0.01$). These results demonstrate that diurnal oscillations in mitochondrial biogenesis and autophagy/mitophagy mRNA expression are most sensitive to the loss of dystrophin in muscles of *mdx* mice.

We next explored how dystrophin deficiency altered the regulation of mitochondrial quality control mRNA expression of genes that did not exhibit rhythmicity in *mdx* mice. Interestingly, the mRNA expression of *Esrra* and *Sirt1* was decreased at the early phase of the dark cycle in *mdx* mice (ZT 16; Fig. 3B,C; $p<0.05$). There was a main effect to increase *Dnm1l* mRNA expression in *mdx* mice, which appeared related to elevations at the end of the dark cycle and into the early light cycle (ZT 24, 04; Fig. 3D). In contrast, there was a main effect to decrease *Opa1* and *Mfn1* mRNA expression in *mdx* mice, which appeared related to their suppression at most timepoints throughout the light-dark cycle (Fig. 3F,G; $p<0.05$). The expression of *Map1lc3b* was increased mid-way through both the dark and light cycle in *mdx* mice (ZT 16, 04; Fig. 3I; $p<0.01$). While the expression of *Sqstm1* was decreased at the start of the dark cycle in *mdx* mice (Fig. 3J; $p<0.01$), the expression was increased midway throughout the dark (ZT 20; $p<0.05$) and light cycles (ZT 08; $p<0.05$). Interestingly, the mRNA expression of *Bnip3* was decreased across multiple timepoints of the light-dark cycle (Fig. K; $p<0.05$). Collectively, these results demonstrate that dystrophin deficiency in *mdx* mice suppresses both absolute and diurnal oscillations of muscle mitochondrial quality control mRNA expression.

Dystrophin deficiency in mdx mice disrupts diurnal oscillations in mitochondrial biogenesis and mitophagy protein expression.

While the field of circadian biology and physiology has been guided mostly by the analysis of mRNA expression (22), we investigated whether the expression of proteins involved in mitochondrial biogenesis and autophagy/mitophagy were altered throughout the light-dark cycle. We found throughout the entire light-dark cycle an uncoupling of the regulators of mitochondrial biogenesis PPARGC1A (decreased; $p<0.001$), ESRRA (no change), and SIRT1 (increased; $p<0.0001$) (Fig. 4A). The loss of mitochondrial proteins COX4I1 and VDAC ($p<0.05$) coincided with increased expression of autophagy/mitophagy markers MAP1LC3BI, MAP1LC3BII, SQSTM1, and BNIP3 (Fig. 4A; $p<0.05$). Qualitatively, the expression of PPARGC1A was decreased throughout most timepoints of the light-dark cycle in *mdx* mice (Fig. 4B), while the expression of SIRT1 was higher throughout the entire light-dark cycle (Fig. 4C). The expression of COX4I1 and VDAC were decreased at the early timepoints of the dark cycle in *mdx* mice (Fig. 4D, E). The expression of MAP1LC3B, SQSTM1, and BNIP3 were increased throughout most timepoints of the light-dark cycle in *mdx* mice (Fig. 4F-H), with the greatest differences observed during the light cycle. Overall, these initial observations highlight that that the loss of dystrophin in muscles of *mdx* mice disrupts diurnal oscillations in mitochondrial biogenesis and autophagy/mitophagy protein expression.

DISCUSSION

Examination of core clock and mitochondrial quality control throughout the light-dark cycle in *mdx* and age-matched BL/10 mice revealed that dystrophin deficiency in muscles of *mdx* mice: i) suppressed core clock mRNA expression; ii) disrupted mitochondrial quality control mRNA expression related to biogenesis, fission, fusion and mitophagy; iii) suppressed diurnal oscillations in core clock and mitochondrial quality control mRNA expression; and iv) impaired the expression of proteins involved in mitochondrial biogenesis and autophagy/mitophagy. These findings suggest that dystrophin deficiency in muscles of *mdx* mice impairs regulation of the core clock and mitochondrial quality control, with relevance to the pathophysiology of DMD and related disorders.

The core clock has been implicated in the regulation of contractile and metabolic function (6-8), which is biologically and clinically relevant to multiple muscle-related disorders. When examining muscles across the entire light-dark cycle, we found dystrophin deficiency in *mdx* mice suppressed the absolute expression of several core clock mRNAs, which is consistent with the only previous investigation in mouse models of DMD (13). We also highlight that this is also observed in muscles of DMD patients (21). Importantly, we revealed for the first time that while diurnal oscillations in core clock mRNA expression were present in *mdx* mice, the rhythmicity parameters mesor and amplitude were decreased across multiple core clock genes (*Arntl*, *Cry1*, *Cry2*, *Per2*, *Nr1d1*). Interestingly, we also observed a significant change in the acrophase of *Per3* mRNA expression in *mdx* mice, with several other core clock genes showing strong trends towards differences when peak amplitude occured (*Cry1*, *Per1*, *Per2*, *Nr1d1*, *Nr1d2*). Determining the intrinsic relationship between the dystrophin-glycoprotein complex, mechanical signaling, and core clock regulation, will require further interrogation.

Previous studies demonstrated that *Arntl* knockout in *mdx* mice aggravated the dystrophic pathology, impaired regenerative capacity, and reduced survival (13), whereas treatment with the REV-ERB antagonist SR8278 improved muscle function associated with enhanced mitochondrial biogenesis and oxidative capacity (14). Whether current therapeutic interventions which aim to improve the pathophysiology in DMD also restore basal and diurnal oscillations in core clock mRNA expression has yet to be examined. Nonetheless, the findings highlight core clock expression is altered throughout the light-dark cycle in muscles of *mdx* mice, and that modulation of the core clock may be a therapeutic target to address aspects of the dystrophic pathology.

Mitochondrial function is critical for the maintenance of muscle structure and function, and disruptions to mitochondrial quality control underlie muscle-related disorders, including DMD (23). Disruptions to core clock components can impair mitochondrial homeostasis and respiratory function, while alterations in cellular metabolism and metabolic intermediates can influence core clock regulation (4, 5). We found that dystrophin deficiency in *mdx* mice suppressed the absolute and diurnal oscillations of key regulators involved in mitochondrial quality control. Importantly, we show for the first time that several of these genes exhibited diurnal oscillations in healthy skeletal muscle, but this rhythmicity was altered or completely abrogated in muscles lacking dystrophin. For example, diurnal oscillations of *Esrra* and *Sirt1* mRNA expression, key regulators of mitochondrial biogenesis, were not observed in *mdx* mice. Further, while muscles of *mdx* mice retained some rhythmicity of genes involved in mitochondrial biogenesis (*Ppargc1a*) and autophagy/mitophagy (*Bnip3*, *Becn1*, *Bcl2*), the parameters mesor, amplitude and acrophase, were significantly altered. The changes in mRNA expression related to mitochondrial biogenesis and autophagy/mitophagy were also coupled to

alterations in protein expression throughout the light-dark cycle. These findings suggest an inability to induce mitochondrial biogenesis in the active dark cycle and an overactivation of autophagy/mitophagy during the inactive light cycle. This is consistent with our recent findings of impaired transcriptional remodeling to chronic low-frequency electrical stimulation in dystrophic skeletal muscles (16). Further, the increase in autophagy/mitophagy proteins may indicate an accumulation of dysfunctional mitochondria, a hallmark of the dystrophic pathology (9-11). While previous studies targeting mitochondrial biogenesis, autophagy and mitophagy in *mdx* mice improved the dystrophic pathology (23-25), whether these adaptations alter the regulation of the core clock has not been addressed. Future studies are warranted to determine the interaction between the core clock and mitochondrial quality control in the pathophysiology of muscular dystrophy, and implications on the rhythmicity of gene/protein expression should be considered.

Dystrophin deficiency in *mdx* mice altered absolute and diurnal oscillations of the core clock and mitochondrial quality control throughout the light-dark cycle. Further, changes in mRNA and protein expression involving mitochondrial biogenesis and autophagy/mitophagy were tightly coupled in muscles of dystrophic mice. Importantly, we identified unique time-of-day differences in the expression of core clock and mitochondrial quality control, which should be considered in future studies. It is feasible that therapeutic strategies could be optimized by an improved understanding of diurnal oscillations in cellular physiology. Collectively, these results suggest dystrophin deficiency in *mdx* mice impairs muscle regulation of the core clock and mitochondrial quality control (Fig. 4J), with relevance to DMD and related disorders.

ACKNOWLEDGMENTS

The authors thank Dr. Christopher A. Wolff and Dr. Jennifer L. Steiner for thoughtful discussions regarding procedures for statistical analyses of circadian rhythms. J.P.H and M.K.C. were each supported by a McKenzie Research Fellowship from The University of Melbourne. This work was supported by research grants to GSL from the National Health and Medical Research Council (GNT1124474).

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FIGURE LEGENDS

Figure 1. Experimental approach and validation of dystrophin deficiency. (A) Experimental approach. Mice were killed every 4 h beginning at the start of the dark cycle (n=3 timepoint/genotype). (B) Body mass, tibialis anterior (TA) muscle mass, and extensor digitorum longus (EDL) muscle mass (n=18 genotype). (C) Dystrophin (DP427) and utrophin (UTRN) protein expression in TA muscles throughout the entire light-dark cycle (n=6 genotype). An equal amount of protein from each genotype was pooled (n=3 genotype/timepoint) to generate diurnal expression patterns on the same gel, with the original western blots shown as representative image (n=6 genotype). (D) Core clock and (E) mitochondrial quality control mRNA expression in EDL muscles throughout the entire light-dark cycle (n=3 timepoint/group). (F) Heatmap showing the expression of core clock and mitochondrial quality control genes manually curated from a published dataset in healthy human controls (n=7) and Duchenne muscular dystrophy (DMD) patients (n=6) (21). Data are means ± standard error. Unpaired Student's t-test (B-E); †p <0.10, *p < 0.05, **p < 0.01, ***p < 0.001. C57BL/10 (BL/10), C57BL/10ScSn-*Dmd*^{mdx} (*mdx*), Total protein (TP).

Figure 2. Dystrophin deficiency in *mdx* mice disrupts core clock mRNA expression. (A-I) Core clock mRNA expression in extensor digitorum longus (EDL) muscles at each timepoint throughout the entire light-dark cycle (n=3 timepoint/genotype). The curved lines represent the cosine wave fitted by the least squares method. The triangles on the right side of the graph represent the rhythmicity parameter mesor. (J) Amplitude of core clock mRNA expression in EDL muscles throughout the entire light-dark cycle (n=3 genotype). (K) Acrophase of core clock mRNA expression in EDL muscles throughout the entire light-dark cycle (n=3 genotype).

Data are means ± standard error, except (J) which is means ± 95% confidence interval. A one-way analysis of variance (ANOVA) was performed to determine if there were differences in gene expression. If this condition was met, then the rhythmicity of gene expression was evaluated using a cosine wave fitted by the least squares method. If a significant rhythm was detected across both genotypes (e.g., amplitude statistically greater than zero), the cosine curve parameters mesor, amplitude and acrophase, were compared by unpaired Student's t-test. †p <0.10, *p < 0.05, **p < 0.01, ***p < 0.001. C57BL/10 (BL/10), C57BL/10ScSn-*Dmd*^{mdx} (*mdx*), confidence interval (CI).

Figure 3. Dystrophin deficiency in *mdx* mice disrupts mitochondrial quality control mRNA expression. (A-M) Mitochondrial quality control mRNA expression in extensor digitorum longus (EDL) muscles at each timepoint throughout the entire light-dark cycle (n=3 timepoint/genotype). The straight lines are connecting data points for the two-way ANOVA. The dotted curved lines represent the cosine wave fitted by the least squares method. The triangles on the right side of the graph represent the rhythmicity parameter, mesor. (N) Amplitude of core clock mRNA expression in EDL muscles throughout the entire light-dark cycle (n=3 genotype). (O) Acrophase of core clock mRNA expression in EDL muscles throughout the entire light-dark cycle (n=3 genotype). Data are means ± standard error, except (N) which is means ± 95% confidence interval. A two-way ANOVA (genotype × time) with post hoc comparison by the Fisher's LSD test was used to determine differences between BL/10 and *mdx* mice. When an 'interaction' or 'main effect of time' was observed, rhythmicity of gene expression was evaluated using a cosine wave fitted by the least squares method. If a significant rhythm was detected across both genotypes (e.g., amplitude statistically greater

than zero), the cosine curve parameters mesor, amplitude and acrophase, were compared by unpaired Student's t-test. †p <0.10, *p < 0.05, **p < 0.01, ***p < 0.001. C57BL/10 (BL/10), C57BL/10ScSn-*Dmd*^{mdx} (*mdx*), confidence interval (CI).

Figure 4. Dystrophin deficiency in *mdx* mice disrupts mitochondrial biogenesis and autophagy/mitophagy protein expression. (A) Mitochondrial quality control protein expression in tibialis anterior (TA) muscles throughout the entire light-dark cycle. An equal amount of protein from each genotype was pooled (n=3 genotype/timepoint) to generate diurnal expression patterns on the same gel, with the original western blots shown as representative image (n=6 genotype). (B-I) Mitochondrial quality control protein expression in TA muscles at each timepoint throughout the entire light-dark cycle, as described in (A) (n=6 genotype). (J) Dystrophin deficiency in muscles of *mdx* mice impairs the regulation of the core clock and mitochondrial quality control. Created with BioRender.com. Data are means ± standard error. Unpaired Student's t-test (A); †p <0.10, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. C57BL/10 (BL/10), C57BL/10ScSn-*Dmd*^{mdx} (*mdx*), Total protein (TP).