NAD+ improves neuromuscular development in a zebrafish model of FKRP-associated dystroglycanopathy

Erin C. Bailey 1,4, Sarah S. Alrowaished 1, Elisabeth A. Kilroy 4, Emma S. Crooks 1, Daisy M. Drinkert 3, Chaya M. Karunasiri 1,5, Joseph J. Belanger 1,6, Andre Khalil 2,4, Joshua B. Kelley 3,4 and Clarissa A. Henry 1,4*

Abstract

Background: Secondary dystroglycanopathies are muscular dystrophies that result from mutations in genes that participate in Dystroglycan glycosylation. Glycosylation of Dystroglycan is essential for muscle fibers to adhere to the muscle extracellular matrix (myomatrix). Although the myomatrix is disrupted in a number of secondary dystroglycanopathies, it is unknown whether improving the myomatrix is beneficial for these conditions. We previously determined that either NAD+ supplementation or overexpression of Paxillin are sufficient to improve muscle structure and the myomatrix in a zebrafish model of primary dystroglycanopathy. Here, we investigate how these modulations affect neuromuscular phenotypes in zebrafish fukutin-related protein (fkrp) morphants modeling FKRP-associated secondary dystroglycanopathy.

Results: We found that NAD+ supplementation prior to muscle development improved muscle structure, myotendinous junction structure, and muscle function in fkrp morphants. However, Paxillin overexpression did not improve any of these parameters in fkrp morphants. As movement also requires neuromuscular junction formation, we examined early neuromuscular junction development in fkrp morphants. The length of neuromuscular junctions was disrupted in fkrp morphants. NAD+ supplementation prior to neuromuscular junction development improved length. We investigated NMJ formation in dystroglycan (dag1) morphants and found that although NMJ morphology is disrupted in dag1 morphants, NAD+ is not sufficient to improve NMJ morphology in dag1 morphants. Ubiquitous overexpression of Fkrp rescued the fkrp morphant phenotype but muscle-specific overexpression only improved myotendinous junction structure.

Conclusions: These data indicate that Fkrp plays an early and essential role in muscle, myotendinous junction, and neuromuscular junction development. These data also indicate that, at least in the zebrafish model, FKRP-associated dystroglycanopathy does not exactly phenocopy DG-deficiency. Paxillin overexpression improves muscle structure in dag1 morphants but not fkrp morphants. In contrast, NAD+ supplementation improves NMJ morphology in fkrp morphants but not dag1 morphants. Finally, these data show that muscle-specific expression of Fkrp is insufficient to rescue muscle development and homeostasis.

Keywords: Zebrafish, Dystroglycanopathy, FKRP, NAD+, Neuromuscular junction

* Correspondence: Clarissa.Henry@maine.edu
1 School of Biology and Ecology, University of Maine, Orono, ME 04469, USA
4 Graduate School of Biomedical Sciences and Engineering, University of Maine, 217 Hitchner Hall, Orono, ME 04469, USA

Full list of author information is available at the end of the article

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Background
Muscle is a highly conserved tissue that is part of the neuromusculoskeletal system and is essential for strength, locomotion, and health. The neuromuscular junction (NMJ) initiates muscle contraction, whereas the myotendinous junction (MTJ) is the major site of force transmission from muscle to the skeletal system. Muscle fibers adhere to their surrounding extracellular matrix (ECM), the MTJ, and the NMJ; and these adhesion complexes are critical for muscle development and homeostasis (reviewed in [26]). The muscle extracellular matrix and adhesion complexes at MTJs and NMJs are specialized and allow the muscle to adhere to and interact with neurons and tendons during development and homeostasis. Thus, regulation of cell-ECM adhesion is essential for development and homeostasis of the neuromusculoskeletal system.

The dystrophin-glycoprotein complex (DGC) is an essential component of muscle-ECM adhesion. The DGC is a multi-protein complex that indirectly links the actin cytoskeleton of muscle fibers to the laminin-rich basement membrane in the ECM [18]. The DGC is thought to provide mechanical stabilization during muscle contraction [18, 60] in addition to its role as a signaling complex [12]. Different variants of the DGC are present at the MTJ versus the NMJ. Whereas Dystrophin is the predominant protein that connects the actin cytoskeleton to the transmembrane protein Dystroglycan (DG) at the MTJ [18], Utrophin mainly establishes this connection at the NMJ [16]. Despite these differences, DG is present and highly glycosylated at both the MTJ and the NMJ. DG glycosylation is required for DG binding to basement membrane proteins such as laminin, agrin, and perlecan [8, 20, 49]. Dystroglycanopathies result from mutations in genes responsible for glycosylation of alpha-DG (DG contains two subunits: a transmembrane beta subunit and an extracellular alpha subunit) [23, 33, 51]. Thus far, 16 gene products that participate in DG glycosylation have been identified [52]. In addition to muscle degeneration, dystroglycanopathies are frequently associated with central nervous system defects such as intellectual disability and brain malformation [52]. The role of DG glycosylation in the peripheral nervous system is less understood although abnormal NMJs have been reported after birth in a couple of dystroglycanopathy mouse models [13, 32, 43, 54, 61]. Because NMJ defects have been observed after embryonic development, the most prevalent hypothesis is that postsynaptic DG does not play a role in NMJ formation, but is important for NMJ stabilization [52]. For example, patients with mutations in GMPPPB (required for GDP-mannose formation [36]) show decreased action potentials with repeated nerve stimulation [37]. Given the plethora of glycosylated proteins at the NMJ, it is possible that a subset of dystroglycanopathy genes may be required outside of muscle tissue for NMJ development.

FKRP-associated dystroglycanopathy results from mutations in FKRP, which encodes an enzyme critical for DG glycosylation. FKRP works in concert with Fukutin to transfer CDP-ribitol synthesized by Isoprenoid Synthase Domain-Containing (ISPD) protein to alpha-DG [21, 38] to form a tandem ribitol-5-phosphate that links xylose with N-acetylgalactosamine [69]. This process is necessary for DG glycosylation, which is important for DG binding to basement membrane proteins [8, 20, 49]. Patients with FKRP-associated dystroglycanopathy present with phenotypic variability; however, muscle weakness and elevated serum creatine kinase are consistently present [37]. Depending on the molecular basis of the FKRP mutation, individuals with this condition may develop limb-girdle muscular dystrophy 2I (LGMD2I) or congenital muscular dystrophy with or without eye and brain involvement [67, 75]. There is some genotype-phenotype correlation in patients with C-terminal FKRP mutations tending to be more severe [4, 6, 67, 75]. However, there is remarkable phenotypic variation among individuals with the same mutation [67]. For example, a study of 25 patients homozygous for c826C/A found significant variation in muscle pathology, and levels of glycosylated DG [67]. Neither the histopathological alterations nor levels of DG glycosylation correlated with age of onset or walking function. Interestingly, whereas one set of siblings presented with similar clinical and histopathological features, a second set of siblings had dramatic variation in the age of onset (12 vs 27 years old) [67]. These data suggest that there are modifying factors—environmental and/or genetic—that affect progression of FKRP-associated dystroglycanopathy. These data also suggest that it is imperative to study features other than muscle pathology and/or levels of DG glycosylation. Mouse [1] and zebrafish models of FKRP deficiency have been used to study mechanisms associated with FKRP-associated dystroglycanopathy. Zebrafish models of FKRP-associated dystroglycanopathy include morpholino (MO) models [39, 72] and a more recently generated fkrp mutant [63]. These studies indicate that the fkrp morphants phenocopy the fkrp mutant, that fkrp deficiency results in impaired muscle development and wasting, and that laminin deposition at the MTJ is disrupted in Fkrp-deficient zebrafish.

Laminin is a major component of the basement membrane that surrounds muscle. Laminin is a heterotrimeric protein with an alpha, beta, and gamma chain. Adhesion of muscle fibers to laminin is...
necessary for muscle development and homeostasis [2, 28, 29, 64, 65, 80]. The principle laminin isoform in mature vertebrate skeletal muscle is laminin-211 (alpha2 beta1 gamma1). Mutations in the human laminin alpha2 gene result in MDC1A, a common congenital muscular dystrophy [31]. A different laminin isoform, laminin-111, is the major isoform expressed during muscle development, and laminin-111 is required for muscle development in mouse and zebrafish [2, 65]. Laminin-111 can partially compensate for laminin-211. Overexpression of laminin alpha1 slows the progression of dystrophy in laminin alpha2 mutant mice [19, 57]. Injection of laminin-111 protein directly into muscle of mdx mice modeling Duchenne muscular dystrophy (DMD) improves muscle structure and function [56]. These data indicate that understanding mechanisms that mediate laminin-111 expression and polymerization during muscle development could provide information for future therapeutic development. We identified a novel pathway required for laminin-111 organization at the zebrafish MTJ during muscle development. Nicotinamide Riboside Kinase 2b (previously called muscle Integrin binding protein) [44, 45] is necessary for normal laminin-111 organization [24]. Yeast and human Nrk2s function in an alternative salvage pathway that generates Nicotinamide Adenine Dinucleotide (NAD+) [7, 70]. Exogenous NAD+ rescues MTJ morphogenesis in Nrk2b-deficient zebrafish, indicating that zebrafish Nrk2b also functions to generate NAD+ [24]. Given that NAD+ biosynthesis is necessary for normal laminin-111 organization during muscle development, we asked whether exogenous NAD+ would be sufficient to improve muscle structure and function in zebrafish modeling muscular dystrophies. NAD+ supplementation increases laminin organization and reduces muscle degeneration in zebrafish deficient for either of the laminin-211 receptors (DG or Integrin alpha7 (Itga7)) [25]. As vitamin B3 is a precursor for NAD+, we asked whether vitamin supplementation would increase NAD+ and improve muscle structure and function in DG-deficient zebrafish. Supplementation with EmergenC packets that contain B vitamins (chosen because they are water soluble) improves muscle structure and motility in DG-deficient zebrafish.

The two major transmembrane receptors that anchor muscle cells to laminin in their ECM are DG (described above) [18, 20] and Integrin alpha7 (Itga7) [11, 66]. Itga7 is also required for muscle homeostasis: mutations in Itga7 lead to congenital muscular dystrophy with Itga7 deficiency [30]. These two cell adhesion complexes display some degree of functional redundancy in both zebrafish and mouse models. Pharmacologically increasing levels of Itga7 compensates for the loss of Dystrophin in a mouse model of DMD [62, 76]. Given that NAD+ is sufficient to improve muscle structure in zebrafish deficient for either laminin receptor complex (DG or Itga7), we hypothesized that NAD+ increases laminin organization by increasing clustering of the remaining receptor [25]. Our model is that in the absence of Itga7, NAD+ increases DG clustering, thus improving muscle-ECM adhesion. Similarly, we hypothesize that in the absence of DG, NAD+ increases Itga7 clustering, thus improving muscle-ECM adhesion. This model leads to the question of whether, if DG is present but hypoglycosylated, NAD+ would be sufficient to improve muscle structure in a zebrafish secondary dystroglycanopathy model. Our previous data showed beneficial effects of NAD+ when administered prior to muscle development [25]. Whether NAD+ is beneficial after initial muscle development in this context has not yet been determined.

Paxillin is an Integrin-associated adaptor protein that concentrates at the MTJ during muscle development [14]. Paxillin is an essential signaling nexus that regulates cell adhesion, morphology, and migration [15]. Paxillin participates in the Nrk2b-laminin pathway. Nrk2b is cell-autonomously required for subcellular concentration of Paxillin at the MTJ, and Paxillin overexpression rescues muscle development Nrk2b-deficient zebrafish [24]. DG is required for normal Paxillin concentration at the MTJ: DG-deficient zebrafish show reduced concentration of Paxillin at the MTJ. Addition of NAD+ improves Paxillin concentration at the MTJ and Paxillin overexpression reduces muscle degeneration in DG-deficient zebrafish [25]. In contrast, Paxillin overexpression does not rescue zebrafish deficient for Itga7 [25]. These data suggest that Paxillin functions downstream of NAD+ to improve muscle resilience in DG-deficient zebrafish. However, whether Paxillin concentration at the MTJ is disrupted in zebrafish models of secondary dystroglycanopathy and whether Paxillin overexpression ameliorates muscle degeneration in zebrafish models of secondary dystroglycanopathy have not been investigated.

We used the fkrp morphant model of FKRP-associated dystroglycanopathy [39, 72] to address unanswered questions regarding NAD+ regulation of the ECM in a secondary dystroglycanopathy. In addition, given the data indicating that there is not a strict correlation between DG glycosylation levels and phenotype, we investigated neuromuscular junction development in fkrp morphants. Supplementing fkrp morphants with NAD+ at gastrulation improves
laminin polymerization at the MTJ, muscle structure, and muscle function. Despite the fact that Paxillin localization is disrupted in fkrp morphants, Paxillin overexpression failed to rescue any of these phenotypes. Early NMJ development was disrupted in fkrp morphants and improved with NAD+. To our knowledge, this is the first report of initial NMJ formation being disrupted in an animal model of secondary dystroglycanopathies. As initial NMJ development has not been investigated in an animal model of DG deficiency, we analyzed early NMJ development in dag1 morphants. NMJ development is disrupted in dag1 morphants, although to a lesser extent than fkrp morphants. In contrast to fkrp morphants, NAD+ supplementation did not improve NMJ development in dag1 morphants. Finally, we show that muscle-specific overexpression of fkrp improved MTJ morphology but was not sufficient to improve muscle structure or function. Taken together, these data indicate that, at least in the zebrafish, FKRPs-associated dystroglycanopathy does not phenocopy DG deficiency. Furthermore, these data show that Fkrp is required for normal NMJ development and is required in tissues other than muscle.

Methods

Zebrafish husbandry and transgenic lines
All embryos were obtained from natural spawnings of adult zebrafish maintained on a 14-h light/10-h dark cycle at 28.5°C. Embryos were reared in 1X embryo rearing medium (ERM) with methylene blue and staged according to [40]. The following strains were utilized/generated: AB, Tg (fltl:EGFP) [42], Tg (ef1α: xbp1Δ-GFP) [46] (a kind gift from Dr. Shao Jun Du), Tg (hsp70l:pxn-EGFP) [10], Tg (hsp70l:fkrp-EGFP), and Tg(-503unc:fkrp-EGFP). The Tg (hsp70l:fkrp-EGFP) and Tg(-503unc:fkrp-EGFP) lines were generated via the gateway cloning system. A sequence surrounding the fkrp gene was amplified with NCBI-verified primers and was gated and inserted into the pDONR221 vector (Invitrogen). The heat shock gateway 222 5′ vector, GFP gateway 366 3′ vector, and the gateway 394 destination vector [41] were used along with the donor vector and LR clonase II for the Tg (hsp70l:fkrp-EGFP) line. Components remained the same for the Tg(-503unc:fkrp-EGFP) line with the exception of the -503unc zebrafish muscle-specific promoter [5], which was used in place of the heat shock gateway 222 5′ vector. Plasmids were injected at the single cell stage as previously described [24].

MO injections
Antisense MOs were obtained from Gene Tools, LLC, and hydrated at 65°C for 10 min with sterile water to generate 1-mM stocks. For fkrp morphant experiments, the previously published fkrp MO2 (5′-CTTGTGGTTTATA TGGCAGAAAGAGT-3′) [39] was utilized and injected into the yolk of 1–2-cell stage embryos so that embryos received approximately 3.2 ng of MO. For dag1 morphant experiments, the previously published dag1 MO (dag1 MO1) (5′-CATGCCCTGCTTTATTTTCCCCTGCG-3′ [53] and an additional slightly overlapping dag1 MO (dag1 MO2) (5′-CCCTCCTCGCTACAAAAAGAGGA CGT-3′) were co-injected into the yolk of 1–2 cell stage embryos so that embryos received approximately 12 ng of each dag1 MO1 and dag1 MO2. Embryos utilized in experiments alongside NAD+ and EmergenC-treated embryos were separated into 60-mm Petri dishes with 25 embryos per dish in 10 mL 1X ERM.

NAD+ and EmergenC supplementation

Fkrp morphants receiving EmergenC treatment (Alacer Corp) were separated into 60-mm Petri dishes with 25 embryos per dish in 10 mL 1X ERM-EmergenC solution. The NAD+ solution was prepared as previously described [24] except in sterile water. The EmergenC solution was diluted in 1X ERM so that the level of niacin in the solution was equal to 7.61 μM. Supplementation began at 6 h post fertilization (hpf) or 24 hpf depending on the experiment, and the solution was changed every 24 h until embryo fixation.

Expression of constructs with the heat shock promoter

To constitutively overexpress Fkrp, uninjected and injected Tg (hsp70l:fkrp-EGFP) embryos were heat shocked at 38°C for 1.25–2 h at the 15 somite stage. To overexpress Paxillin, Tg (hsp70l:pnn-EGFP) were heat shocked at 38°C for 1.5 h at 12 hpf, then for 1.5–2 h daily until fixation. Heat-shocked fish were immediately transferred to 28°C.

Mobility assays

Touch response analysis was performed at 3 dpf. Larvae were placed in 1X ERM and stimulated at the posterior end with fishing wire. The number of touches to evoke an escape response was recorded. For larvae that did not respond after 50 touches, 50 was recorded as their touches to response.

Phalloidin staining, bungarotoxin staining, and immunohistochemistry

All embryos were fixed in 4% Paraformaldehyde (PFA) for 2–4 h at room temperature, depending on the stage. After fixation, embryos received five rinses in PBS-0.1% Tween 20. Unless co-stained with alpha-bungarotoxin, embryos stained with phalloidin were permeabilized for 1.5 h in PBS-2% Triton-X-100, followed by incubation in 1/20 phalloidin-546 (Invitrogen) in PBS-2% Triton-X-100 or PBS-0.1% Tween 20 overnight at 4°C. Embryos stained with...
alpha-bungarotoxin (Molecular Probes) and SV2 were permeabilized in 1 mg/ml collagenase in 1X PBS for 75 min, then were stained with 1/500 alpha-bungarotoxin-647 for 2–4 h at room temperature. For co-staining, 1/20 phalloidin-546 was added to the alpha-bungarotoxin solution in antibody block. All embryos were then subjected to five rinses for 5 min each in PBS-0.1% Tween 20, followed by an overnight incubation in antibody block (5% BSA, 1% DMSO, 1% Triton-X-100, 0.2% saponin in 1X PBS) for embryos receiving subsequent immunohistochemistry treatments. Primary antibodies were added at a concentration of 1/50 (anti-laminin (Thermo Scientific), anti-SV2 (Developmental Studies Hybridoma Bank), anti-Paxillin (BD Biosciences), anti-beta-DG (Novocastra-Leica)) in antibody block and incubated for 2–8 h at room temperature followed by overnight at 4°C. Embryos treated with anti-SV2 were incubated for 3 days at 4°C following the initial overnight incubation. All embryos were then treated with antibody block for 8 h at room temperature or overnight at 4°C, followed by an overnight incubation 4°C plus 2–6 h at room temperature in secondary antibody (Invitrogen) diluted 1/150–1/200 in block. Embryos were rinsed out of secondary antibody with PBS-0.1% Tween 20 prior to deyolking, mounting, and imaging.

**Imaging**

Fixed and stained embryos were deyolked, then mounted and imaged in 80% glycerol. Fluorescent images of fixed, stained fish were captured at ×20 with an Olympus Fluoview IX-81 inverted microscope with FV1000 confocal system or at ×25 with a Leica DMi8 confocal microscope. Images for Tg (ef1α:xbp18-GFP) were acquired at ×10 on a Zeiss Axio Imager Z1 microscope with a Zeiss ApoTome or at ×5 on a Zeiss microscope with Vivatome attachment. Exposure time was kept consistent between all embryo groups within an experiment.

**Statistical analysis**

All data were log transformed in GraphPad PRISM 8 before performing statistical comparisons. All subsequent analyses were performed in GraphPad PRISM 8. Statistical comparisons of data were performed using a two-tailed student t test for comparisons of two groups, and an ordinary one-way ANOVA with Tukey’s ad hoc analysis for assays with three or more groups. Categorical data were scored in using Fisher’s exact test for two categories and the chi square test for three or more categories.

**Image analysis**

Muscle fiber degeneration was quantified by counting the number of muscle segments with degeneration per embryo and calculating the percent of myotomes with muscle degeneration. MTJ angles were analyzed in FIJI by using the angle tool to measure the angles formed by the MTJ. For laminin and beta-DG staining analyses, images were blinded using a Perl script and embryos were scored according to their relative staining intensity (Additional file 1: Figure S1). Intersegmental vessel (ISV) lengths were measured in FIJI using the segmented line tool.

For anisotropy analysis, a polygon was drawn in eight myotomes per image analyzed using the polygonal lasso tool in Adobe Photoshop. The xy coordinates of each polygon were recorded. Masks were generated from the coordinates, and 2DWTMM analysis was performed on each of these masks as previously described [24, 25].

For analysis of xbp1 transgenic embryos, fluorescence intensity was quantified using FIJI. A polygon was drawn around the dorsal muscle above the notochord for all embryos in the trunk region. The average pixel intensity (mean gray value) was calculated. The mean average pixel intensity for all controls was calculated. These values were used to determine the percent average pixel intensity for all embryos. Images for all xbp1 transgenic embryos were blinded using a Perl script prior to analysis.

For NMJ analyses, confocal images were processed into maximum intensity projections of fast-twitch (distributed) and slow-twitch (myoseptal) innervation using FIJI and were subsequently blinded using a Perl script. Masks for the fish, horizontal myoseptum, and myoseptal innervation were generated using FIJI. The masks were imported into MATLAB (Mathworks) and used to segment acetylcholine receptor (AChR) and Synaptic vesicle protein 2 (SV2) fluorescence channels. The fluorescence images were enhanced using adaptive histogram equalization (MATLAB “adapthisteq” function) and then denoised with a 1-pixel radius gaussian filter (MATLAB “imgaussfilt” function). Single masks of the fluorescence channels were then generated using a threshold of 30 on each channel, which were then combined with an “or” statement. Thresholds up to 58 and down to 10 yielded the same trend in the data, indicating that the measurements chosen were not overly sensitive to this parameter. The masks were skeletonized, and subsequently cleaned and despurred using the MATLAB “bwlabel” command. Branchpoints were also identified using the MATLAB “bwlabel” command. The fish, horizontal myoseptum, and myoseptal innervation masks were used to define the muscle segments, and the indicated measurements were performed for each muscle segment across all embryos analyzed.

**Results**

**NAD+ supplementation prior to muscle development improves muscle and MTJ structure**

Injection of previously published fkrp MOs recapitulated the previously described phenotype of fkrp morphants.
Fig. 1 (See legend on next page.)
prove motility in dag1 ECM organization, reduce muscle degeneration, and im-
function was disrupted by quantifying the number of touches required to induce an escape response. We asked if NAD+/EmergenC is sufficient to improve laminin organization by potentiating clustering of the other major laminin receptors in muscles, Integrins alpha6/beta1 and alpha7/beta1. We found that laminin (Fig. 1A, B, J) is reduced in fkrp morphants compared to controls and that beta-DG is concentrated at the MTJ, although sometimes slightly reduced (Fig. 1D, E, K). We next asked whether muscle function was disrupted by quantifying the number of touches required to induce an escape response (Fig. 1O). These data indicate that NAD+ or EmergenC supplementation during gastrulation is necessary for normal muscle development and function.

We previously showed that either NAD+ or EmergenC supplementation at gastrulation is sufficient to improve ECM organization, reduce muscle degeneration, and improve motility in dag1 morphants [25]. We hypothesized that NAD+/EmergenC increased laminin organization by potentiating clustering of the other major laminin receptors in muscles, Integrins alpha6/beta1 and alpha7/beta1. We asked if NAD+/EmergenC is sufficient to improve laminin organization in the context of impaired DG glycosylation because hypoglycosylated DG could hinder increased laminin organization. NAD+ or EmergenC supplementation at gastrulation increased the concentration of laminin at the MTJ (Fig. 1C, J), decreased MTJ angles (Fig. 1M), decreased muscle degeneration (Fig. 1N), improved muscle fiber organization (Fig. 1L), and significantly reduced the touches required to evoke an escape response (Fig. 1O). These data indicate that NAD+ or EmergenC supplementation during gastrulation is sufficient to improve muscle development and function in Fkrp-deficient zebrafish.

NAD+/EmergenC are not sufficient to improve abnormal vascularization, midbrain-hindbrain development, or the increased unfolded protein response in fkrp morphants

Fkrp is required for normal zebrafish vascularization: fkrp morphants and the recently described fkrp mutant exhibit shorter intersegmental vessel (ISV) lengths compared to wild-type embryos [63, 78]. Fli1:EGFP transgenics injected with fkrp MOs exhibited shorter ISV lengths (including truncated vessels) than uninjected controls (Fig. 2E, F, H). NAD+ supplementation had no significant effect on ISV length in fkrp morphants (Fig. 2G, H). We also analyzed ISV length when normalized to the width of the embryos because muscle development is disrupted in fkrp morphants. The same result was observed with normalized data (not shown). These data suggest that: (1) NAD+ supplementation is not sufficient to improve vascularization in fkrp morphants, and (2) the vascularization defects observed in fkrp morphants are likely a consequence of fkrp knockdow...
Fig. 2 NAD+ and EmergenC supplementation at gastrulation do not significantly improve the UPR or vascularization in fkrp morphants. (A–C1) Anterior left, dorsal top, side-mounted 3 dpf embryos expressing Tg(ef1α:xbp1δ-GFP). Fluorescence intensity was kept constant within an experiment (see methods). Numbered panels are merged with the brightfield channel. (A, A1) Control embryo. Note the low relative expression of Xbp1 compared to morphants. (B, B1) fkrp morphant. (C, C1) fkrp morphant treated with EmergenC at gastrulation. Note that fluorescence intensity is similar to that of untreated morphant. (D) Quantification of Xbp1 fluorescence intensity as a percent of the wild-type value for all groups imaged. Fluorescence intensity is significantly increased in morphants. There is no significant difference in fluorescence intensity between untreated m orphants (n = 15 embryos) and morphants receiving EmergenC (n = 21 embryos). (E–G) Anterior left, dorsal top, side-mounted 2 dpf embryos expressing Tg(fli1:EGFP) focused on the ISVs. (E) Control embryo. (F) fkrp morphant embryo. Note that some ISVs are truncated (white arrowhead). (G) fkrp morphant embryo treated with NAD+ at gastrulation. Truncated ISVs are still present in NAD+ treated morphants (white arrowhead). (H) Quantification of ISV length. ISV length is reduced in fkrp morphants (n = 201 vessels) compared with uninjected controls (n = 84 vessels). NAD+ supplementation (n = 166 vessels) does not rescue ISV length in fkrp morphants. Scalebars are 50 μm. *p < 0.05, **p < 0.01, ***p < 0.001, ns non-significant.
Fig. 3 (See legend on next page.)

26 hpf AB Controls

A

A2

A3

phalloidin paxillin

26 hpf fkrp MOs

B

B2

B3

AB fkrp MOs

72 hpf

C

C1

C2

Tg(hsp70:pxn-EGFP)

Controls, 72 hpf

D

D1

D2

Phalloidin

E

E1

E2

Tg(hsp70:pxn-EGFP) MOs, 72 hpf

F

MTJ angles

G

Fiber Degeneration

H

Escape response

% myotomes with degeneration

MTJ angles (°)

Touches to escape response

AB PxnGFP fkrp PxnGFP Ctrl Ctrl MO fkrp MO

*** ns

*** ns

Fig. 3 (See legend on next page.)
and not aberrant muscle development and/or homeostasis.

Zebrafish deficient for Fkrp exhibit abnormal midbrain-hindbrain boundary formation that has been likened to cobbledstone lissencephaly [72]. We also observed abnormal midbrain-hindbrain boundary formation in fkrp morphants at 26 hpf (n = 8) compared with uninjected controls. NAD+ was not sufficient to rescue midbrain-hindbrain boundary formation (n = 8, data not shown). Zebrafish models of FKRP-associated dystroglycanopathy are also associated with endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) [47]. One marker of the UPR is increased activation of the xbp1 transcript, which is upregulated in fkrp morphants [47] and the recently described fkrp mutant [63]. To determine whether EmergenC could improve ER stress in fkrp morphants, we injected fkrp MOs into the Tg (e1ta: xbp1Δ-GFP) line that allows visualization of xbp1 activation [46]. Injection of fkrp MOs increased the UPR (Fig. 2B) compared with controls (Fig. 2A). However, untreated and EmergenC-treated fkrp morphants did not significantly differ in relative fluorescence intensity (Fig. 2C, D). Taken together, these results suggest that the benefits of NAD+ supplementation may be limited to muscle in Fkrp-deficient zebrafish.

Paxillin overexpression does not significantly reduce muscle degeneration

Paxillin is an Integrin-associated protein that is required for cell adhesion to the ECM [74]. Paxillin concentrates at MTJs in wild-type muscle [114], Fig. 3. Paxillin plays a role in the Nrk2b-NAD+-laminin cell adhesion pathway. Paxillin concentration at the MTJ is disrupted in nrk2b morphants and rescued with NAD+ [24]. Paxillin overexpression rescues nrk2b morphants [24]. Similarly, concentration of Paxillin at the MTJ is disrupted in dag1 morphants and is improved with NAD+. Paxillin overexpression improves muscle structure in dag1 morphants [25]. It is not known if either Paxillin concentration at the MTJ is disrupted in animal models of secondary dystroglycanopathy or if Paxillin overexpression improves muscle structure in these models. Paxillin concentration at the MTJ was disrupted in 26 hpf fkrp morphants (Fig. 3B) compared with controls (Fig. 3A). Paxillin does concentrate at the center of the MTJ adjacent to muscle pioneers that are slow-twitch fibers (Fig. 3B at the crux of the v-shaped MTJ, this was observed in 14/16 fkrp morphants). However, Paxillin concentration at the MTJ adjacent to fast-twitch muscle is disrupted (Fig. 3B2, arrow). These data suggest that Fkrp is required for normal development of the MTJ adjacent to fast-twitch muscle. Overexpression of Paxillin was sufficient for Paxillin to concentrate at the fast-muscle MTJ in fkrp morphants (Fig. 3E1 arrowhead). However, in contrast to what was previously observed in dag1 morphants [25], Paxillin overexpression did not reduce muscle degeneration or improve the escape response in fkrp morphants (Fig. 3G-H). Paxillin overexpression actually increased MTJ angles (Fig. 3F). These data indicate that, in contrast to dag1 morphants, Paxillin overexpression is not sufficient to improve muscle structure in fkrp morphants.

NAD+ supplementation after initial muscle development improves MTJs but not muscle structure

NAD+/EmergenC supplementation prior to muscle development is sufficient to improve muscle structure and function in dag1 [25] and fkrp morphant embryos (Fig. 1). It is not known whether NAD+ or EmergenC can improve muscle structure and function after initial muscle development. We supplemented fkrp morphants with NAD+ or EmergenC at 24 hpf (after initial muscle development). Both NAD+ and EmergenC supplementation significantly improved MTJ angles (Fig. 4F). In contrast, neither NAD+...
Fig. 4 (See legend on next page.)
focused on analysis of innervation of fast-twitch structure at 72 hpf was analyzed by staining for SV2 interrupted in secondary dystroglycanopathies. NMJ for NMJ maturation/stabilization/regeneration [27]. It is generally thought that DG is necessary for normal NMJ formation during muscle birth in mouse [32, 35, 43]. DG glycosylation is also required for NMJ development but is required of branching. As mentioned above, DG contributes to NMJ maturation and stabilization [27]. Given the above data showing that initial NMJ development is disrupted in fkrp morphants, we asked whether DG is also required for NMJ development. Skeleton length was slightly, but significantly, reduced in dag1 morphants (Fig. 6B) compared to controls (Fig. 6A). However, NAD+ supplementation at gastrulation did not significantly increase skeleton length in dag1 morphants despite rescuing other aspects of the phenotype (Fig. 6C, D, F). This result contrasts with what we observed in fkrp morphants supplemented with NAD+. Thus, we compared the relative severity of NMJ skeleton length defects in fkrp morphants versus dag1 morphants. On average, dag1 morphant skeleton length was 83.9% of that of un.injected controls. In contrast, fkrp morphant skeleton length on average was 51.4% of that of un injected controls (Fig. 6G). Thus, NMJs are more severely disrupted in fkrp morphants compared to dag1 morphants.

Somewhat surprisingly, we found that NAD+ supplementation at 24 hpf actually significantly worsened the escape response in fkrp morphants (Fig. 4, p < 0.05) despite the fact that we did not observe a significant deterioration of muscle structure. Thus, we asked how NAD+ supplementation at 24 hpf affected NMJ structure (Fig. 7). The effects of NAD+ on NMJ structure correlated with the motility defects: NAD+ at 24 hpf resulted in significantly shorter skeleton lengths (Fig. 7E). There was no significant difference between 24 hpf-treated and untreated morphants in terms of branching frequency (Fig. 7F).

Muscle-specific expression of fkrp is not sufficient to rescue fkrp morphants
The above data indicate that Fkrp is necessary for early NMJ development and that disruption in NMJ morphology with 24 hpf NAD+ treatment correlates with worse motility. These results raise the possibility that Fkrp function is required in non-muscle tissues. We tested this hypothesis by expressing Fkrp in a muscle-specific manner. To confirm that constitutive Fkrp

NMJ development is disrupted and partially improved with NAD+ supplementation in fkrp morphants
The DGC is necessary for NMJ maturation after birth in mouse [32, 35, 43]. DG glycosylation is also necessary for normal NMJ formation during muscle regeneration [22]. It is generally thought that DG is not required for NMJ development but is required for NMJ maturation/stabilization/regeneration [27]. We asked whether initial NMJ development is disrupted in secondary dystroglycanopathies. NMJ structure at 72 hpf was analyzed by staining for SV2 and postsynaptic AChRs (alpha-bungarotoxin). We focused on analysis of innervation of fast-twitch fibers (distributed innervation, see skeletons Fig. 5). There is an extensive network of distributed NMJs in 72 hpf control embryos (Fig. 5A). This network is qualitatively disrupted in fkrp morphants where there appeared to be shorter chains of NMJs (Fig. 5B). Supplementation of NAD+ or EmergenC improved NMJ length, although NMJs were not fully restored (Fig. 5C, D). In order to quantify innervation, we developed a semi-automated technique in MATLAB to skeletonize NMJs (see methods). This analysis showed that although branching frequency was normal, skeleton length was reduced in fkrp morphants (Fig. 5E, F). NAD+ or EmergenC supplementation at 6 hpf slightly, but significantly, improved skeleton length in fkrp morphants (Fig. 5E). These results suggest that glycosylated DG is necessary for innervation, but not branching.
Fig. 5 (See legend on next page.)
overexpression could rescue fkrp morphants, we injected fkrp MOs into a Tg(hsp70::fkrp-EGFP) line we generated (Fig. 8A). Previous data suggest that overexpression of fkrp can be deleterious [77]. Although we observed a slight increase in MTJ angles (Fig. 8C), we did not observe any significantly adverse effects of Fkrp-EGFP on fiber degeneration or the escape response in control embryos (expression was induced at the 15 somite stage) (Fig. 8A, D, E). Global overexpression of Fkrp-EGFP in fkrp morphants improved fiber resiliency, MTJ angles, and the escape response compared to control morphants (Fig. 8B–E). These data indicate that (1) overexpression of Fkrp is not toxic under these conditions and (2) EGFP does not deleteriously affect Fkrp function. We next asked whether muscle-specific expression was sufficient to rescue the phenotype. We generated a transgenic line expressing Fkrp-EGFP under control of the -503unc promoter [5].

Muscle-specific overexpression of Fkrp did not affect muscle morphology in control embryos (Fig. 8F–P2). Muscle-specific overexpression of Fkrp in fkrp morphants decreased MTJ angles compared to EGFP negative control morphants (Fig. 8I). However, no other metrics of muscle structure/function were improved. Muscle-specific overexpression neither ameliorated fiber detachment (Fig. 8G, H, J) nor reduced touches required to induce an escape response (Fig. 8K). These data indicate that expression of Fkrp in muscle is not sufficient to rescue muscle morphology or function.

Discussion

Dystroglycanopathies are a relatively understudied subset of muscular dystrophies. One aspect of dystroglycanopathies that is not well understood is how the myomatrix is impacted. Whether strategies that improve the myomatrix and muscle structure in a primary dystroglycanopathy (DG deficiency) are efficacious in secondary dystroglycanopathies are also not known. We previously showed that either NAD+ supplementation or overexpression of an Integrin-associated adaptor protein (Paxillin) are sufficient to improve muscle structure and function in DG-deficient zebrafish. Here, we show that muscle phenotypes in a zebrafish model of FKRP-associated dystroglycanopathy are improved with NAD+ supplementation but not Paxillin overexpression. Although the mechanisms are not known, these data clearly indicate that primary and secondary dystroglycanopathies show some differences. Our data also suggest that there are differences even within secondary dystroglycanopathies. Whereas muscle-specific expression of Large is sufficient to rescue muscle structure and function in Large/myd mutant mice [27], we found that muscle-specific expression of fkrp is not sufficient to improve muscle structure/function in fkrp morphant zebrafish. Finally, our data show that NMJ formation is disrupted earlier in development than has previously been observed in a primary and secondary dystroglycanopathy model. Taken together, these data indicate that it is necessary to study the secondary dystroglycanopathies individually within the context of the group as a whole.

Cell-matrix adhesion and secondary dystroglycanopathy

Adhesion of muscle fibers to the MTJ is necessary for muscle development and homeostasis. We previously identified a cell adhesion pathway that contributes to laminin organization at the MTJ: Nrk2b-mediated NAD+ production potentiates laminin organization at the MTJ during zebrafish muscle development [24]. We then demonstrated that exogenous NAD+ is sufficient to improve laminin organization, muscle structure, and muscle function in zebrafish deficient for either DG or Itga7 [25]. DG and Itga7 are both transmembrane receptors for laminin. We hypothesized that NAD+ improved laminin organization, at least in part, by increasing clustering of the remaining receptor (DG in Itga7-deficient embryos, and Itga7 in DG-deficient embryos). Here, we asked whether laminin organization would be increased if DG is present but hypoglycosylated. The rationale was that it is possible that the presence of hypoglycosylated DG that cannot bind laminin could inhibit the improvement of laminin organization with NAD+. The ribitol 5-phosphate transferase FKRP is necessary for glycosylation of DG [21, 38]. We asked if NAD+ was sufficient to improve cell-matrix adhesion in fkrp morphant zebrafish embryos. NAD+ supplementation prior to muscle development was sufficient to improve laminin polymerization, muscle structure, and the escape response.
Fig. 6 (See legend on next page.)
Organization of laminin at the MTJ is disrupted in zebrafish deficient either for DG [25] or Fkrp [39, 47, 72] (Fig. 1). Laminin is required for concentration of the Integrin adaptor protein Paxillin to the MTJ [24]. Paxillin is an intracellular protein that localizes to cell-ECM adhesion complexes [15] and modulates ECM composition at the developing MTJ [34]. Previous data regarding the beneficial potential of Paxillin in the context of aberrant muscle development/homeostasis are contradictory. Paxillin overexpression worsens muscle damage in ethanol-treated zebrafish [10]. However, Paxillin overexpression is sufficient to improve MTJ morphology in Nrk2b-deficient zebrafish [24]. Paxillin overexpression also improves laminin organization and reduces muscle degeneration in dag1 morphant zebrafish [25]. We hypothesized that Paxillin overexpression would improve muscle structure and function in fkrp morphants where DG is present but not properly glycosylated. Interestingly, we found that Paxillin expression trended towards reducing degeneration and improving the escape response, but the effects were not significant (Fig. 3). This result suggests the hypothesis that, in contrast to the situation where DG is absent, the presence of hypoglycosylated DG in the membrane prevents Paxillin-mediated stabilization of muscle fibers.

Benefits of NAD+ and EmergenC supplementation may be restricted to the neuromusculoskeletal system

Both fkrp morphants and mutants have an increased UPR. Xph1, a marker of the UPR, is upregulated in both fkrp morphants and fkrp mutants [47, 63]. Expression of bip, a marker of the UPR, is upregulated in fkrp morphants at 28 hpf, especially in the neural floor plate and the hatching gland [47]. However, gbyt1lb (large2) morphants with hypoglycosylated DG, dag1 morphants, and sly/lam1c mutants do not exhibit bip upregulation [47], suggesting that activation of the UPR may result directly from loss of Fkrp. We asked if EmergenC supplementation was sufficient to reduce the UPR in fkrp morphants. We found that 3 dpf Tg (ef1axbip18-GFP) embryos injected with fkrp MOs have increased xbp1-GFP fluorescence compared to control embryos. EmergenC supplementation at gastrulation was not sufficient to reduce xbp1-GFP fluorescence. Taken together, these data suggest that activation of the UPR occurs independently of muscle-cell matrix adhesion and DG glycosylation and is likely a direct consequence of Fkrp knockdown.

Fkrp morphants and fkrp mutants also exhibit aberrant vascular development, including reduced ISV lengths [63, 78]. One study suggests that impaired vascularization directly results from loss of Fkrp and that muscle phenotypes and vascular phenotypes are independent of one another [78]. Here, we demonstrate that NAD+ supplementation at gastrulation is not sufficient to rescue truncated ISVs in fkrp morphants. Given that NAD+ improves muscle structure and function in fkrp morphants, our data suggest that vascular phenotypes observed in fkrp morphants are not dependent on muscle phenotypes. This supports the previous hypothesis that abnormal vascular development in zebrafish models of FKRP-associated dystroglycanopathy is a direct result of loss of Fkrp.

Timing of NAD+ intervention is critical

Gene therapy [55, 71, 73, 77], estrogen receptor modulators [79], and exogenous ribitol supplementation [9] improve muscle structure in FKRP-associated dystroglycanopathy models. The efficacy of adenoviral gene therapy and ribitol supplementation decreases if administered later in the mouse's lifespan [9, 77], suggesting that early intervention is most beneficial. Our data regarding the timing of NAD+ and EmergenC supplementation in fkrp morphants are consistent with the data mentioned above. We found that NAD+ (or EmergenC) supplementation prior to muscle development increased laminin organization, reduced muscle degeneration, improved muscle function, and improved NMJ structure (Fig. 1). In contrast, NAD+ (or EmergenC) supplementation after initial muscle development only improved MTJs and not muscle structure/function (Fig. 4).
Fig. 7 (See legend on next page.)
Our data do not resolve why MTJ morphology is improved with late NAD+ supplementation or muscle-specific overexpression of Fkrp. Improved MTJ morphology does not always correlate with fiber degeneration: *tiga7* morphants exhibit muscle fiber detachment but have normal MTJ morphology. NAD+ supplementation improves MTJ angles, but not fiber degeneration in *dag1/tiga7* double morphants [25]. Movement is not necessary for MTJ chevron formation in zebrafish embryos [59]. We show that motility is not improved in *fkrp* morphants receiving NAD+ or EmergenC after muscle development, further suggesting that improved MTJ morphology does not always correlate with mobility.

While this manuscript was in preparation, it was shown that administration of pentetic acid at 48 hpf improved muscle and pericardial phenotypes in a *fkrp* mutant zebrafish model of LGMD2I [63]. Pentetic acid is a chelating agent that binds Ca2+ and Mg2+. The mechanism by which this improves muscle phenotypes is currently unknown. However, abnormal Ca2+ levels have been implicated in DMD and inducing an influx of Ca2+ is sufficient to induce dystrophy [50]. Together, these data suggest that different therapeutic avenues have different time windows of efficacy in multiple animal models.

**Dag1 and fkrp are required for proper NMJ development**

NMJ development requires orchestrated interactions between muscle cells and motor neurons. The DGC is a major component of NMJs. Thus, it is not surprising that NMJ morphology is disrupted in multiple dystroglycanopathies. Fukutin-deficient chimeric mice have abnormal AChR clustering and NMJs at postnatal day 15 [61]. Newborn pups homozygous for *Large/myd* mutations have altered NMJs as well [32]. These data clearly indicate that NMJs are abnormal after birth. What is not clear is at which point in embryonic development NMJ disruption occurs. Do NMJs develop normally and then degenerate or is initial NMJ development abnormal? Here, we provide evidence that early NMJ development is slightly disrupted in DG-deficient zebrafish, with NMJ chains that innervate fast-twitch muscle being approximately 84% of control chain length. To our knowledge, this is the earliest developmental stage at which NMJ disruption has been observed in a primary or secondary dystroglycanopathy. Interestingly, *fkrp* morphants exhibit more dramatic disruption of NMJ morphology, with NMJ chains about half of control chain length. We do not know the mechanisms underlying the more severe disruption in NMJ development in *fkrp* versus *dag1* morphants. One possibility is that hypoglycosylated *dag1* acts as a “dominant negative” and thus disrupts NMJ development more than when *dag1* is not present. Additionally, there are many glycosylated proteins at the NMJ, such as Lrp4, Musk, and Agrin. Thus, the more severe disruption in *fkrp* morphants could also reflect a requirement of Fkrp for glycosylation of other NMJ resident proteins.

**Muscle-specific overexpression of Fkrp is not sufficient to rescue fkrp morphants**

Overexpression of LARGE is sufficient to synthesize glycan-enriched alpha-DG and is sufficient to improve laminin-binding activity in multiple dystroglycanopathy patient cell lines [3]. Muscle-specific expression of *Large* is sufficient to rescue muscle structure, neurotransmission, and NMJs in *Large/myd* mutant mice [27]. NMJ defects in *Large/myd* mutant mice were observed after birth [32]. The above data suggest that, in the context of Large-associated dystroglycanopathy, NMJ defects are secondary to muscle disruption. Given that we observed very early developmental disruption of NMJs, we could not necessarily conclude that NMJ disruption in Fkrp-deficient embryos is secondary to the muscle phenotype. Thus, we tested whether muscle-specific expression of Fkrp would be sufficient to rescue neuromuscular function in *fkrp* morphant embryos. We first showed that global expression of Fkrp (under control of the heat shock promoter) is sufficient to rescue *fkrp* morphant embryos. This result indicates that the Fkrp-EGFP fusion protein is functional.

Next, we expressed Fkrp in muscle under control of the muscle-specific -503unc promoter [5]. Muscle-specific expression of Fkrp was sufficient to slightly, but significantly, reduce MTJ angles. However, muscle-specific expression of Fkrp was not sufficient to reduce muscle degeneration or improve the escape response (Fig. 8). We do not know
Fig. 8 (See legend on next page.)
earlier intervention has improved outcomes in future and a reduced variation in muscle degeneration. Fkfrp is efficacious. There was a slight improvement in MTJ structure after initial muscle development was not particularly effective. 

**Fig. 8** Muscle-specific overexpression of Fkrp improves MTJ angles, but not motility or fiber resiliency. (A–B2) Anterior left, dorsal top, side-mounted embryos at 72 hpf stained for f-actin (phalloidin, gray) and expressing Fkrp-EGFP (green). (A–A2) Control Tg (hsp70:fkrp-EGFP) embryo expressing Fkrp in the fibers. (B–B2) Fkrp morphant Tg (hsp70:fkrp-EGFP) embryo expressing Fkrp in fibers. (C) MTJ angles of Tg (hsp70:fkrp-EGFP) control (n = 264 MTJs) and fkrp morphant (n = 340 MTJs) embryos and A8 controls and morphants (n = 141, 194 MTJs) at 72 hpf. Constitutive expression of Fkrp improves MTJ angles in morphants. (D) Constitutive expression of Fkrp in morphants (n = 72 embryos) significantly reduces the number of myotymes with fiber degeneration compared with control morphants (n = 35 embryos). (E) The escape response is significantly lower in fkrp morphants constitutively overexpressing Fkrp (n = 82 embryos) compared to control morphants (n = 44 embryos). (F–H2) Anterior left, dorsal top, side-mounted embryos at 72 hpf stained for f-actin (phalloidin, gray) and expressing Fkrp-EGFP under control of the muscle-specific -503unc promoter (green). (F) Control TgI(-503unc-fkrp-EGFP) embryo expressing Fkrp specifically in muscle fibers. (G) Fkrp morphant embryo on TgI(-503unc-fkrp-EGFP) background lacking Fkrp expression in muscle fibers. (H) Fkrp morphant TgI(-503unc-fkrp-EGFP) embryo expressing Fkrp specifically in muscle fibers does not show improved muscle organization. (I) Muscle specific expression of FKR in fkrp morphants (n = 158 MTJs) significantly improves MTJ angles compared to control morphants (n = 153 MTJs). (J) Muscle-specific overexpression of Fkrp (n = 45 embryos) does not significantly lower the percent of myotymes with muscle degeneration in control morphants (n = 47 embryos). (K) There is not a significant difference in the number of touches required to induce an escape response in fkrp morphants that overexpress Fkrp (n = 73 embryos) in muscle versus those that do not (n = 79 embryos). Scalebars are 50 μm. *p < 0.05, **p < 0.01, ns non-significant.
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