FKRP-dependent glycosylation of fibronectin regulates muscle pathology in muscular dystrophy

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The muscular dystrophies encompass a broad range of pathologies with varied clinical outcomes. In the case of patients carrying defects in fukutin-related protein (FKRP), these diverse pathologies arise from mutations within the same gene. This is surprising as FKRP is a glycosyltransferase, whose only identified function is to transfer ribitol-5-phosphate to α-dystroglycan (α-DG). Although this modification is critical for extracellular matrix attachment, α-DG’s glycosylation status relates poorly to disease severity, suggesting the existence of unidentified FKRP targets. Here we reveal that FKRP directs sialylation of fibronectin, a process essential for collagen recruitment to the muscle basement membrane. Thus, our results reveal that FKRP simultaneously regulates the two major muscle-ECM linkages essential for fibre survival, and establishes a new disease axis for the muscular dystrophies.
The dystroglycanopathies are amongst the most enigmatic of all muscular dystrophies (MDs)\textsuperscript{1–3}, largely due to heterogeneity of distinct clinical presentations that remain unexplained at a mechanistic level\textsuperscript{4–7}. Within this class of disorders, mutations in FKRP result in a particularly varied pathological spectrum, with the presence of disease modifiers and novel modes of FKRP action, both being put forward as possible explanations for this phenotypic variation\textsuperscript{1,8}. Furthermore, the glycosylation status of dystroglycan, the only known target of FKRP activity, corresponds poorly to phenotype severity\textsuperscript{9}, an observation that suggests the possibility of dystroglycan-independent modes of FKRP function.

**Results**

**fkrp mutants exhibit a loss of muscle integrity and function.** In order to better understand the basis for this diversity of phenotypic severity, we developed zebrafish loss-of-function models of FKRP deficiency using distinct genome editing strategies (Extended Data Fig. 1). The *fkrp* mutant fish generated by these different processes possessed similar phenotypes and were shown to generate nonsense-mediated decay of *fkrp* transcripts and a consequential loss of protein by western blot (Extended Data Fig. 1d, e). Furthermore, *fkrp* mutant larvae possessed a significant loss of (IIH6) reactivity, a marker of glycosylated dystroglycan, FKRP’s canonical target. This loss of immunoreactivity could not be attributed to an overall loss of dystroglycan protein, or loss of dystroglycan’s core-binding partner, Laminin, whose deposition was actually significantly increased at the muscle basement membrane in homozygous mutant *fkrp* larvae (Extended Data Fig. 2). We, therefore, concluded that the mutations we have generated resulted in the loss of FKRP function. Consequently, we reasoned that by directly comparing the phenotype of our newly generated *fkrp*\textsuperscript{−/−} alleles (Extended Data Figs. 1–3) to that which presents in *dag1* (dag1\textsuperscript{−/−}) mutants, the canonical target of FKRP glycosylation\textsuperscript{10}, novel modes of FKRP function could be identified.

To perform these analyses, the phenotypes of *fkrp*\textsuperscript{−/−} and *dag1*\textsuperscript{−/−} mutants were compared for muscle structure and integrity defects at 3 and 5 days post fertilisation (dpf), a period during which progressive muscular dystrophy and muscle basement membrane (MBM) attachment defects were evident in our previously generated zebrafish models of muscular dystrophy\textsuperscript{11–13}. Both models exhibited the expected fibre detachment pathology over this period, the prevalence of which increased with age and *fkrp* mutants also demonstrated Evan’s blue dye (EBD) uptake into muscle fibres, which provides evidence for sarcolemmal defects in these mutants (Fig. 1a–c and Extended Data Figs. 2d, f–g). Mutants also exhibited a phenotypically proportional loss of maximal active force generation (Fig. 1e)\textsuperscript{14}. Furthermore, both *dag1* and *fkrp* mutants possess highly similar retinal basement membrane defects that reflect dystrophin
glycoprotein complex (DGC) component deposition deficits that accompany a lack of a-dg-mediated attachment (Extended Data Fig. 3). The similar fibre detachment and retinal basement membrane phenotypes of these mutants are consistent with the failure of FKRP-dependent a-dg glycosylation, resulting in a loss of cellular attachment in these different contexts. We could provide no evidence for the previously reported neuronal or vascular defects in fkrp morpholino-injected fish by staining for neuronal synapses, fluorescent-dextran-based angiography or EBD vascular injection, which revealed only muscle-related uptake within damaged fibres (Extended Data Fig. 2f-g)\textsuperscript{13,15}. 

**Muscle basement membrane defects in fkrp mutants.** Intriguingly, however, fkrp\textsuperscript{−/−} mutant larvae also possessed a specific phenotype, not evident in dag1\textsuperscript{−/−} mutants. fkrp homozygous mutants exhibited muscle fibres that crossed or bypassed attachment to the MBM of the vertical myosepta, a phenotype indicative of MBM failure (Fig. 1a, b, d and Extended Data Fig. 1d). As the identical fibre-crossing phenotype had been observed previously in fibronectin loss-of-function larvae\textsuperscript{16}, we postulated that FKRP may play a role in regulating fibronectin function. Fibronectin presented as an attractive candidate for a FKRP target protein as it is highly decorated with N- and O-linked glycans and plays a central role in forming and maintaining the MBM\textsuperscript{16–18}. Furthermore, one of the principal roles of fibronectin is to recruit collagen to stabilise BM formation. This biological function is a fundamental axis of tissue integrity in many organ systems, critical both to tissue homeostasis and to diseases such as cancer, where failure in BM cell adhesion plays a key role in the transition to metastasis\textsuperscript{19}. To determine if disruption of collagen recruitment and consequent MBM formation could explain the phenotypes we observed, fkrp\textsuperscript{−/−}, dag1\textsuperscript{−/−} and wild-type control fish were immunohistochemically stained for fibronectin at 1 dpf and for collagen-1a at 5 dpf, which are the respective time points at which fibronectin and collagen accumulation can be visualised and quantitated. Fibronectin localised correctly to the MBM in mutant and control fish (Fig. 2a, e) but unexpectedly exhibited a significantly higher (P < 0.00001) deposition in the fkrp\textsuperscript{−/−} fish than controls (Fig. 2c). Collagen-1 protein deposition, in contrast, was dramatically and significantly reduced (P < 0.00001) in fkrp\textsuperscript{−/−} mutants compared to control and dag1\textsuperscript{−/−} larvae (Fig. 2b, f) at the MBM. Next, transmission electron microscopy (TEM) was used to ascertain collagen deposition between the MBM of the zebrafish vertical myosepta in fkrp\textsuperscript{−/−} (n = 9), dag1\textsuperscript{−/−} (n = 7) and wild-type controls (n = 8) at 7 dpf. At this stage of development, electron-dense collagen fibrils align between adjacent MBMs at the vertical myosepta, providing structural integrity\textsuperscript{20}. As expected, dag1\textsuperscript{−/−} mutants exhibited normal localisation of fibrillar collagen at the MBM, however, in fkrp\textsuperscript{−/−} mutants, no fibrillar collagen could be detected, confirming the immunohistochemistry-based findings (Fig. 2c, d). To determine if collagen loss in fkrp\textsuperscript{−/−} fish resulted from a lack of gene expression, transcript levels of collagen-1a, the main collagen expressed at the MBM\textsuperscript{20}, was investigated. However, neither collagen-1a nor fibronectin gene expression levels were reduced in fkrp\textsuperscript{−/−} mutants. In fact, mRNA levels for collagen-1a, were found to be significantly increased (P < 0.005) in fkrp\textsuperscript{−/−} mutants (Extended Data Fig. 4), indicating that a lack of gene expression is not the cause of the loss of collagen-1a at the MBM of fkrp\textsuperscript{−/−} mutants (Extended Data Fig. 4a). Next, we sought to determine the physiological consequence for the loss of collagen deposition evident at the MBM of fkrp\textsuperscript{−/−} mutants. Since collagen is the major ECM constituent that directs passive resistance to strain at the MBM\textsuperscript{21}, we developed an assay to measure muscle passive force. Our results would predict that fkrp\textsuperscript{−/−} mutants would exhibit severe reductions in passive force transmission within the larval myotome. In line with this premise, subjecting larval zebrafish to a newly developed passive force assay\textsuperscript{22} revealed that fkrp\textsuperscript{−/−} mutants exhibited a severe and highly significant reduction in their ability to transmit passive force (Fig. 2g), a reduction not evident in either dag1\textsuperscript{−/−} mutants nor wild-type sibling controls (Fig. 2g).

**Altered fibronectin glycosylation in FKRP patient myoblasts.** In order to determine the disease relevance of our observations in the fkrp mutant zebrafish model, the role of human fibronectin impairment in muscle cell pathology was investigated in patient-derived FKRP-deficient primary myoblast cells (Table 1 and Extended Data Fig. 4b). To undertake these analyses, two distinct FKRP-deficient patient myoblast cell lines from either end of the FKRP clinical spectrum were differentiated to form myotubes: those derived from a patient with LGMD2I, a disease with a milder clinical presentation, and cells derived from a patient with a severe CMD. These cells were compared to identically established control lines derived from healthy muscle biopsies (Fig. 3a–d). As FKRP is known to catalyse the transfer of ribitol-5-phosphate to O-mannosyl glycan chains on α-DG, we investigated if any glycosylation changes between fibronectin isolated from control and FKRP mutant cells could be identified. Fibronectin from human patient cell lysate was immunoprecipitated and glycans were released and analysed by porous graphitised carbon–liquid chromatography–mass spectroscopy PGC–LC–MS/MS (Fig. 3a)\textsuperscript{23}. An analysis of O-glycans did not reveal any trace of altered O-mannosyl glycans on fibronectin nor changes of mucin-type O-glycans between wild-type and control cells (Extended Data Fig. 5). Unexpectedly, however, we detected a highly significant and specific reduction of N-glycan sialylation on fibronectin derived from LGMD2I and CMD patient cells when compared to control samples. Specifically, two peaks, m/z at 965.9 and 1111.4, corresponding to biantennary, sialylated N-glycans with compositions of NeuAc\textsubscript{A}Hex\textsubscript{A}HexNAc\textsubscript{A} and NeuA\textsubscript{C}Hex\textsubscript{A}HexNAc\textsubscript{A}, respectively, were significantly reduced on fibronectin in patient cells when compared to the healthy controls (Fig. 3a, b). In contrast, no general loss of sialylation was detected in the N-glycans derived from total cell proteins of healthy control and patient samples (Extended Data Fig. 6), indicating that this decrease in sialylation is specific for fibronectin glycosylation rather than representing a global deficit in protein sialylation in patient cells.

Our analysis thus determined that fibronectin N-glycans exhibited a similarly dramatic decrease in sialylation in cells derived from patients that spanned the FKRP clinical spectrum. As a cluster of these biantennary glycans resides within the collagen-binding domain of fibronectin\textsuperscript{17}, we next determined if a defect in collagen–fibronectin binding could be detected in patient samples, and if this defect scaled with clinical severity. To undertake these analyses, fibronectin was immunoprecipitated and subject to quantitative proteomic analysis to determine the relative abundance of co-precipitated collagen\textsuperscript{24}. This analysis revealed that the amount of collagen that co-immunoprecipitated with fibronectin was significantly less in both patient cell lines when compared to healthy controls, suggesting a loss of interaction between fibronectin and collagen, the level of which tracked with clinical severity of the patient cells from which protein was derived (Fig. 3c). To directly assay and quantify fibronectin–collagen binding in vitro, we undertook surface plasma resonance analyses. Using this method, the binding of native or desialylated fibronectin to immobilised collagen was assessed (Extended Data Fig. 7). This study revealed that the binding affinity of desialylated fibronectin to collagen was...
The fibronectin–collagen axis is disrupted at the muscle basement membrane in fkrp mutants. a–b’ Fibronectin and collagen immunochemistry. Whole-mount staining and confocal imaging of zebrafish myotomes centred on myotome 10 (1 dpf) and myotome 12 (5 dpf). Images are z projections of the mediolateral extent of the myotome stained for F-actin (red) to mark muscle fibres and DAPI (blue) for nuclei. a Fibronectin (Fn) staining (green) at the vertical myosepta at 1 dpf, images representative of n = 9, three larvae from three separate clutches on different weeks, most severe and weakest phenotypes excluded (scale bar = 30 μm). b’ dag1+/−, a” fkrp−/−. b Collagen-1 (Col1a, green) staining of the muscle basement membrane in wild-type sibling fish. b’ dag1+/− and b” fkrp−/−, white arrow: absent collagen, scale bar = 40 μm. a–b” Lateral views anterior to the left. c Transmission electron micrographs (TEM) of longitudinal sections of 7 dpf zebrafish myotome centred on the muscle basement membrane (red arrowheads). Red arrows: absent collagen fibrils, red star: fibre detachment from vertical myosepta, scale bar = 0.5 μm. Wild-type siblings, c’, dag1+/−, c” fkrp−/−, micrographs representative of (n = 9), three larvae from three separate clutches on different weeks, most severe and weakest phenotypes excluded. d Schematic of fibre-crossing and detachment model at the Myotendinous Junction (MTJ) and disease progression, d’ canonical DGC axis in dag1+/− and, d” combined detachment crossing in fkrp−/−. e, f Measurement of max intensity from fibronectin (e) and collagen (f) staining analysed by two-way ANOVA. *** (P = <0.0001), plotted points outside 95% confidence interval, box represents 5–95%, median centre line, whiskers = SEM, Tukey’s multiple comparison analysis, three independent experiments. g Passive force measurement at 6 dpf, calculated by plotting the maximum active force at given loads, as passive tension (mN) against external stretch (Lo%), with representative linear regression analysis of the plotted points of fkrp+/+ sibling (sib); black line (n = 8), fkrp−/−, red line (n = 6) and dag1+/− blue line (n = 5) was used to test the significance of the differences (***(P = <0.0001), error bars = SEM.

Table 1 Clinical histological phenotype of patient myoblast samples.

| LGMD2I                                      | CMD                                      |
|---------------------------------------------|------------------------------------------|
| Well organised muscle tissue with normotrophic fibres and subsarcolemmal nuclei. Presence of some hypotrophic fibres. No degenerative aspects are observed. No pathological accumulation of PAS positive material. Predominance of type I fibre. Relative hypotrophy of type II fibres. Conclusion: Modest, non-specific, myogenic changes. | Severe alteration of the tissue organisation due to abundant peri and endomysial connective tissue, fibre anisometry with normo-, hypop-, atrophic and various hypertrophic fibres some of which with splittings, central nuclei and rare degenerate fibres. Conclusion: Morphological features indicative of a severe dystrophic degeneration, compatible with the diagnosis of congenital muscular dystrophy |
significantly reduced compared to the fully sialylated native fibronectin (Fig. 3d), suggesting that the decrease in fibronectin sialylation may be causative of the collagen deposition deficits evident in fkrp<sup>−/−</sup> mutants. To further test this hypothesis, fully sialylated or desialylated fibronectin was injected into homozygous fkrp mutants, with both forms of fibronectin being tagged with Alexa488 dye to track localisation upon injection. In wild-type and mutant larvae, both native and desialylated fibronectin localised correctly to MBM boundaries (Fig. 3e). Next, we examined collagen expression at the myoseptal boundaries...
after fibronectin injection (Fig. 3f). This analysis revealed that collagen was expressed at the myoseptal boundaries of wild-type fish injected with sialylated or desialylated fibronectin. Furthermore, fkrp−/− fish injected with fully sialylated fibronectin possessed significantly more collagen at the MBM compared to uninjected mutants (Fig. 3f). By contrast, fkrp−/− mutant larvae injected with desialylated fibronectin failed to rescue the lack of collagen at the MBM evident in these mutants (Fig. 3f, g). The results confirmed that only sialylated fibronectin can rescue the collagen deposition deficits evident in fkrp−/− mutants. To test if these fibronectin rescue experiments could physiologically improve fkrp−/− mutant muscle function, passive force was measured. Injection of fkrp−/− larvae with natively glycosylated, but not desialylated, fibronectin rescued the ability of these mutants to transmit passive force when compared to sham-injected (PBS) fkrp−/− controls (Fig. 3h). This finding suggests that the fibronectin sialylation pathway could provide a potential novel target for therapy development for FKRP-deficient patients. Collectively, these results surprisingly reveal that the FKRP-dependent sialylation of fibronectin is necessary for fibronectin–collagen binding and maintenance of MBM integrity. Our data also have the potential to explain an unusual aspect of the FKRP clinical phenotype, namely the lack or late presentation in patients of joint contractures, stiffness of the joints that results from fibronectin–collagen accumulation, a pathology associated with the vast majority of other muscular dystrophies21,23.

**Fibronectin Golgi localisation and sialylation requires Fkrp.** Next, we sought to determine the mechanistic basis by which FKRP regulates fibronectin sialylation. We examined three distinct possibilities. Firstly, we examined if FKRP could catalyse fibronectin sialylation directly. Although FKRP contains no protein domains that suggest that it could itself catalyse fibronectin sialylation, we specifically examined this possibility by desialylating fibronectin and providing it as a substrate for recombinant FKRP protein and CMP-sialic acid donor substrate (Extended Data Fig. 9). Analyses of glycan addition by PGC immunoprecipitation studies from the identical human patient and control human myotubes (Extended Data Fig. 8). This observation suggests that muscle cells may actively survey fibronectin sialylation, and regulate sialyltransferase expression by undertaking comparative RNA Seq analyses in patient and control human myotubes (Extended Data Fig. 8). However, this analysis failed to detect any downregulation in the expression of any sialylation pathway gene. Intriguingly, ST6Gal1, one of the enzymes potentially responsible for the terminal α2,6-sialylation process that is defective in FKRP mutants, was found to be strongly upregulated in FKRP-deficient myotubes (Extended Data Fig. 8)26. This observation suggests that muscle cells may actively survey fibronectin sialylation, and regulate sialyltransferase levels accordingly. The final hypothesis we tested was that FKRP may act as an obligate binding partner for a yet-to-be-identified sialytransferase or a sialytransferase-binding protein. Such a model is supported by the observation that FKRP has previously been shown to exist in a large multimeric complex comprising several high-molecular- weight components within the Golgi27. Immunoprecipitation and mass spectrometry SWATH analyses detected no association between FKRP and known sialyltransferase enzymes (Table 2). However, it did reveal that non-muscle myosin10 bound to FKRP, an association considered significant, given that previous studies have revealed that myosin10 can be tethered through its carboxyterminus to the aminoterminus of glycosyltransferases28,29. Furthermore, reverse immunoprecipitation and mass spectrometry SWATH analyses assaying fibronectin from human control cells, revealed that myosin10 was also found associated with fibronectin (Table 3), a result confirmed by independent co-immunoprecipitation analyses using an anti-myosin10 antibody, which readily detects fibronectin in control and patient samples (Extended Data Fig. 9a). Furthermore, co-immunoprecipitation studies from the identical human patient

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**Table 2 Top ten proteins detected by mass spectrometry from FKRP pull down.**

| No. | Unused | Total | Accession | Name Description |
|-----|--------|-------|-----------|------------------|
| 1   | 160.45 | 160.45| sp|Q8VD5 | MYH9 MOUSE | Myosin-9 OS = Mus musculus GN = Myh9 PE = 1 SV = 4 |
| 2   | 60.11  | 60.11 | sp|Q9IH9 | MY18A MOUSE | Unconventional myosin-XVIIa OS = Mus musculus GN = Myo18a PE = 1 SV = 2 |
| 3   | 21.6   | 46.44 | sp|Q61879 | MYH10 MOUSE | Myosin-10 OS = Mus musculus GN = Myh10 PE = 1 SV = 2 |
| 4   | 28.46  | 28.46 | sp|Q01853 | TER4A MOUSE | Transitional endoplasmic reticulum ATPase OS = Mus musculus GN = Vcp PE = 1 SV = 4 |
| 5   | 21.2   | 21.26 | sp|Q8C1G8 | ANMS MOUSE | Protein arginine N-methyltransferase 5 OS = Mus musculus GN = Prmt5 PE = 1 SV = 3 |
| 6   | 11.52  | 11.55 | sp|Q60605 | MYL6 MOUSE | Myosin light polypeptide 6 OS = Mus musculus GN = Myl6 PE = 1 SV = 3 |
| 7   | 22.71  | 22.71 | sp|P05213 | TBA1B MOUSE | Tubulin alpha-1B chain OS = Mus musculus GN = Tub1b PE = 1 SV = 2 |
| 8   | 2.11   | 20.92 | sp|P68369 | TBA1A MOUSE | ATP synthase subunit beta, mitochondrial OS = Mus musculus GN = Atp5b PE = 1 SV = 2 |
| 9   | 16.35  | 16.46 | sp|P56480 | ATPB MOUSE | Histone H2B type 1-P OS = Mus musculus GN = Hist1h2bp PE = 1 SV = 3 |

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**Table 3 Top ten proteins detected on mass spectroscopy from fibronectin pull down.**

| No. | Unused | Total | Accession | Name Description |
|-----|--------|-------|-----------|------------------|
| 1   | 230.11 | 230.11| sp|P35579 | MYH9_HUMAN | Myosin-9 OS = Homo sapiens GN = Myh9 PE = 1 SV = 4 |
| 2   | 49.63  | 49.63 | sp|P02751 | FNC_HUMAN | Fibronectin OS = Homo sapiens GN = Fn1 PE = 1 SV = 4 |
| 3   | 32.51  | 32.51 | sp|P04264 | K2C1_HUMAN | Keratin, type II cytoskeletal 1 OS = Homo sapiens GN = Krt1 PE = 1 SV = 6 |
| 4   | 17.05  | 34.27 | sp|P35580 | MYH10_HUMAN | Myosin-10 OS = Homo sapiens GN = Myh10 PE = 1 SV = 3 |
| 5   | 16.15  | 16.33 | sp|P13645 | K1C10_HUMAN | Keratin, type I cytoskeletal 10 OS = Homo sapiens GN = Krt10 PE = 1 SV = 6 |
| 6   | 9.7    | 9.7   | sp|P35527 | KIC9_HUMAN | Keratin, type I cytoskeletal 9 OS = Homo sapiens GN = Krt9 PE = 1 SV = 3 |
| 7   | 6.2    | 6.2   | sp|P81605 | DCD_HUMAN | Dermcidin OS = Homo sapiens GN = Dcd PE = 1 SV = 2 |
| 8   | 4      | 4.01  | sp|P04259 | K2C6B_HUMAN | Keratin, type II cytoskeletal 6B OS = Homo sapiens GN = Krt6b PE = 1 SV = 5 |
| 9   | 3.52   | 3.54  | sp|P60709 | ACTB_HUMAN | Actin, cytoplasmic 1 OS = Homo sapiens GN = Actb PE = 1 SV = 1 |
| 10  | 2      | 2     | sp|P21333 | FLNA_HUMAN | Filamin-A OS = Homo sapiens GN = Flna PE = 1 SV = 4 |

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myotube membrane-enriched samples using a FKRP antibody readily detect Myosin-10 in control samples, but do not detect Myosin-10 in pulldowns conducted on patient cell lines (Extended Data Fig. 9b). Collectively, these results suggest a model whereby loss of FKRP function disrupts the localisation and tethering of fibronectin to Myosin-10 within the Golgi, a process critical for fibronectin maturation and sialylation, which in turn is required for collagen–fibronectin binding. In line with a possible role for myosin10 in fibronectin maturation, previous studies on myosin10 mutant mice have detected collagen deposition deficits in alveolar interstitial ECM30–32. Interestingly, the interstitial alveolar ECM in the lung is required for maintaining its elastic properties in a manner analogous to the MBM.

Since non-muscle myosins have also been shown to stabilise high-molecular-weight protein complexes, such as actin scaffolds in the trans-Golgi while they are being sialylated, we postulated that a similar mechanism may regulate localisation of fibronectin and its consequent sialylation in the Golgi33,34. Furthermore, Golgi dysfunction in dystroglycanopathy was hypothesised as a contributing factor for the heterogeneity of these disorders in Fukutin-deficient mouse cardiac cells35. To examine this question, confocal and stimulated depletion emission (STED) microscopy were used to determine if fibronectin was mislocalised in the trans-Golgi of patient cells (Fig. 4a and Extended Data Fig. 10a–d). Using this approach, fibronectin was found to be co-localised with myosin10 in the Golgi of control cells, a
distribution that was significantly altered in patient-derived cell lines (Fig. 4c), the severity of which correlated with the clinical phenotype of the patients from which cells were derived. Specifically, myosin10 was found to be less fragmented within patient cells compared to controls, when its localisation in the Golgi was rendered in 3D, suggesting that a loss of myosin10-associated functions occurs specifically within patient cells (Supplementary Video 1 and Extended Data Fig. 10c, d). Furthermore, Gaussian fit analyses of the STED-derived data revealed that fibronectin and myosin10 were mislocalised specifically within the trans-Golgi, which is the site within the Golgi where sialylation occurs (Fig. 4b, d). In addition, analysis of 3D-rendered Airyscan super-resolution data also confirmed that the relative localisation of fibronectin and Myosin-10 was altered within the trans-Golgi of patient cells when compared to controls (Fig. 4e–h, Extended Data Fig. 10c, d, and Supplementary Video 2). We conclude that FKRP is required to correctly localise fibronectin within the trans-Golgi, a process that is essential for its correct sialylation (Fig. 4e).

**Discussion**

Overall, the results we describe here, taken together with previously published results, reveal that FKRP functions to regulate the two most prominent ECM cell-binding adhesion complexes in skeletal muscle. Firstly, FKRP catalyses the addition of the two most prominent ECM cell-binding adhesion complexes on FKRP structure in patient cells, scale bar 2 µm. Schematic of FKRP action. The schematic illustrates that in healthy control cells, FKRP is required to correctly localise fibronectin within the trans-Golgi, which is the site within the Golgi where sialylation occurs (Fig. 4b, d). In addition, analysis of 3D-rendered Airyscan super-resolution data also confirmed that the relative localisation of fibronectin and Myosin-10 was altered within the trans-Golgi of patient cells when compared to controls (Fig. 4e–h, Extended Data Fig. 10c, d, and Supplementary Video 2). We conclude that FKRP is required to correctly localise fibronectin within the trans-Golgi, a process that is essential for its correct sialylation (Fig. 4e).

**Methods**

**Zebrafish strains, maintenance and husbandry.** The strains used in the experiments were Tubingen (Tu) (referred to in the text as wild-type, dag^{hu2072} and fkrp^{pc36(-1:5bp)} (referred to in the text as fkrp^{−/−}, and is the allele used for the majority of experiments), fkrp^{pc37(exon3)}, Tg (acta1:GFP-PennCherry:CAAX). Adult zebrafish were kept as previously described and bred under animal ethics breeding license number ERM/1448, issued by Monash Animal Services Ethics Committee. Embryos were maintained in E3 medium (5 mM NaCl, 0.2 mM KCl, 0.4 mM CaCl2, 0.9 mM MgCl2 and 2% methylene blue) in deO2H2O and incubated at 28.5 °C. Embryos/larvae were staged as per described criteria and were culled using 0.04% tricaine diluted in distilled water. All experimental procedures were reviewed and approved by Monash Animal Services Ethics Committee prior to commencement under project ID: 5652.

**Generating fkrp mutant lines.** fkrp^{pc36(-1:9bp)} mRNAs encoding a pair of Zinc-finger nucleases targeted to the fkrp locus (CompoZ™ Knockout Zinc Finger Nuclease, Sigma-Aldrich, lot number 0326124MN) were injected into single-cell zebrafish embryos at a final concentration of 50 ng/µl. To identify individual fish that were carrying fkrp mutations, injected fish were raised to sexual maturity and crossed to wild-type, DNA was extracted and genotyped using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, Catalogue no. XNATR-1KT) and screened by High-Resolution Melt analysis (HRM) on a Roche LightCycler® 480 II using LightCycler® 480 High-Resolution Melting Master mix (Catalogue No. 40909631001), with the following primers: fkrp melt-F: TCACTTTGGGCTCAATTCG and fkrp melt-R: TCTTCTTCAGCAGCTTGT. Mutations were sequence-confirmed by BigDye sequencing at the Garvan Molecular Genetics Facility (Darlinghurst, NSW). Zebrafish were genotyped by sequencing using the following genotyping primers: fkrp_Fwd: CGAGTCCA ACGTGAGGACAG and fkrp_Rev: AGCTAAACAAGGCCGAGTA. fkrp^{pc37(exon3)}, Alt-R CRISPR-Cas9 CRNAs were targeted to the start and end of exon 3 of the fkrp locus in zebrafish: fkrp1: TTTGGCTCTTCAGGAGGAGC, fkrp2: GGAACATGAAATTTCGAG (IDT) and injected into single-cell zebrafish embryos at a final concentration of 36 ng/µl together with Alt-R S.p. Cas9 Nuclease (IDT) at 0.5 µg/µl. In 20 mM HEPS, 150 mM KCl, pH 7.5. To identify individual fish that were passing on fkrp mutations, injected fish were raised to sexual maturity and crossed to wild-type, DNA was extracted and genotyped using the following primers: Forward: fkrp_cispr_frw: TTTGCCAGCTCGTGTAA and fkrp_cispr_rev: GCCAGTCTGCTGACGTT.

**qPCR.** RNA was extracted from larvae or cells to generate RNA for cDNA production as previously described. In total, 20 ng of cDNA samples were run in triplicate with LightCycler® 480 SYBR Green 1 Master reaction mix (Roche) using the primers listed in Supplementary Table 1. Reactions were run on the Roche LightCycler® 480 Real-Time PCR Machine. CT values for each sample set were normalised against the housekeeper genes, before comparing the data sets.
RNA sequencing and analysis. In total, 600,000 cells from human myoblast cell lines CMD, LGMD2I and control, were harvested and RNA-extracted using RNAspin columns (IQGEN, Cat 74204). RNA concentrations were measured using Quibit RNA HS Assay Kit (Life Technologies, Cat Q32855) on a Denovix Fluorometer. Samples were processed with 200 ng of the total RNA. PolyA RNA seq libraries were generated using Illumina TruSeq Stranded mRNA Sample prep, protocol 15031047 RevE Oct 2013. Libraries were prepared according to the Illumina standard RNA protocol. Each library was single-end with a 50 nt read length. Sequencing was performed on the Illumina HiSeq. 3000. RNA library preparation and sequencing were carried out by the Monash University Medical Genomics Facility. Sequencing reads were filtered and trimmed with Trimmomatic (v 0.36, Phred trim threshold 0 and 10% of nucleotide bases below 15, minimum read length of 27 nt) and mapped to the human genome (GENCODE’s GRC38 primary assembly, annotation v24) with STAR (v 2.5.2b)43. Read-to-gene assignment was done with (anti-β- DG mouse IgG 1:50, Novocastra), IIH6 (anti-α-DG mouse IgM, Merck-Millipore 05-593 1:50), pan-laminin (anti-rabbit IgG polyclonal 1:100, Sigma), fibronectin (rabbit 1:200, Sigma) and Collagen-1 (rabbit, Abcam, 1:100) in PBST overnight at room temperature. Sections were incubated in secondary antibodies: (goat anti-mouse IgM Alexa Fluor 594, Invitrogen; 1:500, goat anti-mouse IgG Abberior580 1:250 and goat anti-rabbit IgG Abberior635 1:250 were used. Cells on coverslips were mounted in ProLong® Gold antifade (Invitrogen) and imaged.

Western blot and co-immunoprecipitation. Larval heads were removed and used for genotyping, bodies were snap-frozen, homogenised in immunoprecipitation buffer (PBST)20. In all, 30 μg of protein was loaded on a 4–12% Bis-Tris Protein Gel MES Buffer (ThermoFisher Scientific). Primary antibodies were prepared in TBS blocking buffer (LI-COR) fluor polarization 1:500 fluor 48, 1:500, Beta-Tubulin-Loading Control (Abcam: ab664468, Golgi 50 kda, IgG rabbit (Abcam: ab6640) 1:10,000, incubated in secondary antibody IRDye 800CW goat anti-rabbit (LI-COR) 1:2000 and scanned on a infrared scanner (Odyssey). Immunoprecipitation utilised 10ug of antibody, fibronectin or FKRP, and a protein-G dynabead kit (Thermofisher).

Immunohistochemistry. For retina-based experiments, humanically killed 5-dpf embryos were incubated in 4% PFA at room temperature for 3 h. The fish were incubated in 30% sucrose in PBS at 4 °C overnight. Fish were aligned in OCT-embedding medium and snap-frozen. Using a cryostat, samples were trimmed to single slice was imaged. Co-localisation analysis was performed on the STED and confocal images using the Imaris (Bitplane) Coloc module. Golgi co-localisation to fibronectin (n = 5) from confocal images was investigated. Co-localisation analysis on the STED images were examined for the co-localisation of myh10 and fibronectin within the Golgi (n = 9).

Transmission electron microscopy. Larvae were fixed in Karnovsky fixative (4% glutaraldehyde, 2.5% glutaraldehyde, 0.1 M Na-cacodylate, 10 mM MgCl2, and distilled water)50. This was followed by post-fixation in osmium tetroxide plus potassium ferricyanide (1% OsO4, 1.5% K3Fe(III)CN6 and 0.065 M Na-cacodylate buffer). Larvae were dehydrated through ascending concentrations of acetone (50%, 70%, 90% and 100%). Embedding consisted of a solution of ascending concentrations of Epon-Araldite resin: propylene oxide, and the resin was polymerised at 60 °C for 48 h. Sections were cut and then stained with 2% uranyl acetate followed by 1% lead citrate. Images were taken on a Hitachi H7500 with a Gatan Multiscan 791 digital camera.

Physiology and functional studies. In total, 6 dpf larvae were anaesthetised in tricaine and placed in MOPS-buffered physiological solution. Small aluminium foil sheets were folded into clips on both sides of the trunk musculature of each fish. Holes were then punched in these aluminium clips, enabling the fish to be mounted horizontally onto an in vitro muscle physiology tester apparatus (model 1500 A, force transducer 403 A, Aurora Scientific, Ontario, Canada). One hook was attached to a force transducer and the other hook was anchored to a motor that allowed graduated fine-length adjustments. The optimum muscle length (Lo) was determined for maximum active force generation by applying single-twist stimulations while lengthening step-wise, until the force detected no longer increased with stretch. The fish was stretched in increments of 5% of Lo and held at that length for 300 ms to measure the passive force response. The initial peak of the force-time graph was used for analysis. This was repeated up to 25% of Lo stretch for each zebrafish larva25. All measurements were carried out at room temperature.

Culture of human myoblasts. Three human myoblast lines were obtained from the European Biobank in Milan (Human Ethics ID: 5652): a healthy control and two muscular dystrophy lines: a male 15-month-old CMD patient with FKRP mutations: c.693 G > C, p.Ile48Thr and a male 4.5 years old with LGMD2I with FKRP mutations: c.826 C > A, p.Leu276Ile. Use of patient cell lines at The Australian Regenerative Medicine Institute, Monash University, was conducted with ethical oversight and review via the Monash Human Research Ethics Committee project number CF14/3369-2014001793. Cells were cultured and differentiated to myotubes and allowed to mature for 5 days as per standard operating procedures outlined by the Euro Biobank: http://www.eurobiobank.org/

Quantitative proteome analysis. Larvae or cell lysates were homogenised in immunoprecipitation buffer (Pierce) and incubated with 10 μg of human fibronectin antibody (Sigma) bound to IgG beads as per the manufacturer’s instructions. After the prescribed PBS and PBST wash steps, the pulled-down fraction was subjected to on-bead trypsin digestion. Briefly, beads were reconstituted to 100 μL in 100 mM triethylammonium bicarbonate containing 1% sodium deoxycholate, reduced, alkylated and trypsin- digested. Digests were then separated from beads and purified by self-packed C18 tips. Samples were injected onto a C18 reversed-phase (RP) trap chip (ChromXP, 120 Å, 3 μm) using a nanolC 400 with chipLPC

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system (SCIEX) and separated on a 15-cm × 200-µm analytical RP chip column within 1 h, and acetonitrile gradients (2–35%). Eluting peptides were analysed using a TripleTOF 6600 (SCIEX) mass spectrometer in positive ion mode with a spray voltage of 2.5 kV. For data-dependent acquisition (DDA), a 0.25±MS scan was followed by 20 × 0.1-s MS/MS scans of the most intense ions in the MS scan. For SWATH acquisition, 50 fixed 10-m/z windows (800–1300 m/z) were used. A 0.1±MS scan was followed by 50 × 0.06-s SWATH MS/MS acquisitions at the range between 100 and 2000 m/z. DDA data were processed and searched through a Mascot searching engine. The result was used to generate a spectral library for SWATH quantitative analysis. SWATH data were analysed using Skyline. Integrated peak area of the top five most abundant transitions were summed as the quantitation value of each protein-specific peptide. The values of collagen-specific peptide (FYYTVLEDCGK) were normalised to the quantitative value of each protein-specific peptide of the glycopeptide (FTYTVLEDGCTK) to obtain the relative abundance values.

Glycan release and MS analysis. Fibronectin was pulled down as described above and fractions separated in SDS-PAGE and elecblotted to the PVDF membrane.

Collagen-fibronectin SPR. Biacore analyses were carried on a T200 (GE Healthcare) using the dedicated control and evaluation software. Human fibro¬
nectin (Millipore EMD) and human collagen-1α (Sigma) were immobilised onto individual flow cells onto the surface of a CM5 Chip (GE Healthcare) by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) coupling at 50 µg/ml in NaOAc buffer, pH 4.0 (collagen) or pH 4.2 (fibronectin). Unreacted sites were capped with 1 M ethanolamine. Control surfaces were prepared in parallel by activation (ECD/NCHS) and deactivation (ethanolamine) of the flow cell. Serial (doubling) dilutions of analyte from 100 to 1.57 mM were prepared in Running Buffer (10 mM HEPES, pH 7.4, 150 mM NaCl and 0.005% (w/v) Tween 20) and injected over the control and cognate ligand surface for 2 min at 30 µl/min. The dissociation was monitored for 10 min and double regeneration carried out with a 30-s pulse of 0.1 mM glycine-HCl, pH 2.0, followed by a 30-s pulse of 25 mM NaOH/0.5 M NaCl. For each experiment, seven concentrations, one in duplicate, and a zero (buffer only), were performed. Sensorgrams were double-

Sialytransferase assays. Coding sequence of full-length human FKRP with C-
terminal FLAG tag was synthesised (IDTDNA) and ligated into pGen2.1 vector to generate pGen2.1-hFKRP. In all, 320 µg of pGen2.1-hFKRP was used to transfect 160 ml HEK293T cells at a density of 2.5 × 10⁵ cells/ml. Ninety-six hours post transfection, cells were pelleted and solubilised in 20 ml 25 mM HEPES, pH = 7.5, 150 mM KCl, 1× Protease cocktail and 1% Triton X-100. Lysate was then clarified by centrifugation at 5000×g for 10 min. The supernatant was loaded on the anti-FLAG M2 agarose gel (Sigma). After washing by 5× volume column of PBS containing 300 mM NaCl, bound hFKRP was eluted by 0.1 M glycine-HCl and the pH was immediately brought back to 7.5 by 0.5 M NaOH, followed by 3 volumes of 1% (w/v) Hepes, pH 7.5, Elution, and concentrated and buffer-exchanged to 25 mM HEPES, pH 7.5, and 50 mM KCl. MBS-sialyltransferase assays were conducted in a final volume of 100 µl containing 25 mM MES, pH 6.4, 20 mM MgCl₂, 2 mM CaCl₂, 5 µg of desialylated fibronectin using neuraminidase (NEB) as per the manufacturer’s instructions, 1 mM CMP-Neu5Ac, 5 µg of desialylated fibronectin and the enzyme to be tested. For negative controls, water was used instead of CMP-Neu5Ac. Reactions were conducted at 37 °C for 6 h. Reactions were brought to 1% sodium deoxycholate and 50 mM triethyraminomycin bicarbonate, reduced by 2.5 mM DTT and alkylated by 5 mM IAA. In total, 0.5 µg of sequencing-grade trypsin was added and incubated at 37 °C for 16 h. Tryptic digests were brought to 1% formic acid to precipitate sodium deoxycholate and spun at 13,000 × g for 10 min. Supernatants were transferred to new tubes and dried in a Speedvac (Thermo Fisher Scientific). Digested peptides were resuspended in 2% ACN containing 0.1% formic acid and injected for LC–MS analysis. A SWATH method was used for data acquisition and analysis. Sialylation transfer activity was monitored and quantified via sialyla-

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Additional information
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