



Published in final edited form as:

*Trends Cell Biol.* 2021 March ; 31(3): 197–210. doi:10.1016/j.tcb.2020.11.003.

## Fukutin-Related Protein: from Pathology to Treatments

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### Abstract

Fukutin-related protein (FKRP) is a glycosyltransferase involved in the functional glycosylation of  $\alpha$ -dystroglycan (DG), a key component in the link between the cytoskeleton and the extracellular matrix (ECM). Mutations in *FKRP* lead to dystroglycanopathies with broad severity, including limb-girdle and congenital muscular dystrophy. Studies over the past five years have elucidated the function of FKRP, which has expanded the number of therapeutic opportunities for patients carrying *FKRP* mutations. These include small molecules, gene delivery and cell therapy. Here we summarize recent findings on the function of FKRP and describe available models for studying diseases and testing therapeutics. Lastly, we highlight preclinical studies that hold potential for the treatment of FKRP-associated dystroglycanopathies.

### Keywords

muscular dystrophy; dystroglycanopathy; limb-girdle; congenital muscular dystrophy;  $\alpha$ -dystroglycan; glycosylation

### Dystroglycanopathies

Muscular dystrophies (MDs) are a group of more than 50 genetic diseases characterized by progressive muscle weakness and degeneration. Among these are dystroglycanopathies, a heterogeneous subgroup characterized by abnormal **O-glycosylation (see Glossary)** of  $\alpha$ -dystroglycan (DG) [1].  $\alpha$ -DG has a fundamental role in linking the intracellular cytoskeleton with the **extracellular matrix** (ECM), but for this to occur,  $\alpha$ -DG needs to be correctly glycosylated [2, 3]. This is a complex sequential process involving more than 20 proteins, among which are included fukutin related protein (FKRP), fukutin (FKTN), protein O-mannosyl transferase (POMT1), and like-acetylglucosaminyltransferase

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(LARGE1). Mutations in each of these genes result in some form of dystroglycanopathy [4]. In this review, we focus on dystroglycanopathies caused by mutations in *FKRP*.

FKRP was first identified in 2001 based on its homology to FKTN [5]. This led to the identification of mutations in the *FKRP* coding region of patients with MDs of previously unknown genetic etiology [5–7]. Recent improvements in sequencing technology and the use of targeted panels have greatly improved diagnostics, resulting in increased detection of mutant variants and better identification of patients affected by dystroglycanopathies [8, 9]. In the last decade, multiple animal models have been developed to study FKRP, and in the last five years, several studies aiming to treat the pathology have been reported. While significant advances have been achieved, curative treatments have not yet advanced to clinical trials. Here we describe available FKRP models and summarize experimental studies that may lead to the future treatment of patients with FKRP-associated dystroglycanopathies.

## FKRP pathophysiology

FKRP is a ribitol-5-phosphate transferase that, in tandem with FKTN, adds ribitol-5-phosphate onto the M3 core O-mannosylation of  $\alpha$ -DG (Box 1) [10–12]. The presence of ribitol-5-phosphate is a pre-requisite for the subsequent addition of xylose and glucuronic acid repeats, which are necessary for proper glycosylation of  $\alpha$ -DG, and therefore, its binding to ECM ligands, in particular laminin, **agrin**, and **perlecan** in the **sarcolemma**, the neuromuscular junction and the central nervous system (CNS) (Figure 1). Additionally, in the CNS,  $\alpha$ -DG also interacts with neurexin and slit, and in the retina,  $\alpha$ -DG binds to pikachurin [13–15]. These interactions are critical for sarcolemma integrity, maintenance of the basal membrane, cell viability of muscle progenitor cells and regeneration, as well as for neuronal migration and synaptic structural maintenance [16, 17].

FKRP is localized in the Golgi apparatus and contains transmembrane, N-terminal, and C-terminal catalytic domains [18]. It has been previously proposed that FKRP forms oligomers *in vivo* [19]. A recent report indicates that FKRP is a tetramer and that its dimerization is fundamental for its activity as a ribitol-5-phosphate transferase [20]. This study also demonstrated key regions necessary for the protein's activity, among which, the zinc finger loop present in the catalytic domain (Figure 2).

## FKRP and Associated Dystroglycanopathies

Numerous mutations have been identified in the *FKRP* gene. According to the Leiden database<sup>i</sup>, **missense mutations** are the most common (63%), followed by **frameshift** (17%) and **nonsense** (13%) **mutations**. Interestingly, mutations in the catalytic domain are associated with more severe dystrophic phenotypes (Figure 2). Recessive mutations in *FKRP* are associated with a heterogeneous spectrum of dystroglycanopathies, which can range from limb-girdle muscular dystrophy (LGMDR9, previously known as LGMD2I) to several forms of congenital muscular dystrophy (MDC1C), including severe Walker-

<sup>i</sup> <http://www.dmd.nl/>

Warburg Syndrome (WWS) and muscle eye brain disease (MEB). Creatine kinase (CK), a common marker of muscle damage, has been reported to be elevated (1000–15000 U/L) in the serum of LGMDR9 and MDC1C patients, indicating muscle damage [5, 6, 21].

LGMDR9, the most common FKRP-associated dystroglycanopathy [22], manifests between the first and third decades of life. Loss of ambulation and cardiorespiratory issues are common but variable among patients [23]. The most frequently reported *FKRP* mutation for LGMDR9 is the 826C>A (L276I) [6, 24]. MDC1C is more severe, with symptoms developing in the first year of life, leading to rapid loss of ambulation, cardiorespiratory complications, and death before 10 years of age [5, 22]. At the end of the severity spectrum, WWS, a type of CMD, is accompanied by significant brain and eye abnormalities, with patients succumbing before reaching 3 years of age [7].

Muscle biopsies from these patients (both LGMDR9 and MDC1C) show a reduction in  $\alpha$ -DG glycosylation, measured by VIA4 or IIH6C4 (IIH6) antibodies, and a decrease in laminin- $\alpha$ 2 binding [25, 26]. The IIH6 antibody is specific for functional  $\alpha$ -DG glycosylation, competing for the  $\alpha$ -DG binding domain with laminin [2, 27]. While loss of IIH6 immunoreactivity has been used as a hallmark for the diagnosis of FKRP-associated dystroglycanopathies, this may vary among LGMDR9 patients [28–30], suggesting lack of clear correlation between changes in  $\alpha$ -DG glycosylation and patient phenotype.

## Models

Several models have been established to recapitulate the different phenotypes related to human *FKRP* mutations. These include FKRP mutant animal models developed in the last decade, as well as the use of patient-specific induced **pluripotent stem cells** (iPSC), which have emerged as an attractive tool to study human disease (Figure 3).

### Zebrafish models

Zebrafish (*Danio rerio*) has been widely used to model human genetic disease due to its rapid development and ease of genetic manipulation and screening [31]. Similarities between zebrafish and human skeletal muscles have triggered studies on MDs [32], and several groups have investigated FKRP function in zebrafish embryos. **Morpholino** knockdowns show muscle, ocular, vascular, and neuronal defects, recapitulating the broad spectrum of human phenotypes (Figure 3). At the cellular level, embryos show reduced IIH6 immunoreactivity and laminin binding [33–37]. It has been shown that these phenotypes can be partially rescued by co-injecting the human FKRP mRNA, demonstrating functional homology [38]. Recently, **TALENs** were used to generate two FKRP mutant zebrafish models, which recapitulate WWS and LGMDR9 phenotypes [39]. In the WWS model, a 13 base pair deletion induced a frameshift loss of function that resulted in reduced IIH6 immunoreactivity, severe neuronal, muscular and vasculature defects as well as early death. The LGMDR9 model was obtained by introducing the human FKRP mRNA sequence carrying the L276I mutation in WWS zebrafish [39].

## Mouse models

Various FKRP mutant mouse models have been generated with the goal of recapitulating the full spectrum of phenotypes observed in human patients (Figure 3). The first generated FKRP mutant mouse model was the FKRP-neo<sup>Y307N</sup> (FKRP<sup>KD</sup>), which recapitulates the MEB phenotype [40]. Of note, addition of the neomycin selection cassette (neo) in intron 2 was required to decrease FKRP expression and achieve the desired phenotype. Later on, a model for LGMDR9 (FKRP<sub>MD</sub>) was derived from this FKRP<sup>KD</sup> by rescuing FKRP expression in the CNS [41]. In the last decade, Qi Long Lu and colleagues have generated several mouse models that are broadly used by the FKRP scientific community. Among these, FKRP-neo<sup>P448L</sup> and FKRP<sup>P448L</sup> mice, which carry the P448L mutation that is associated with MDC1C in humans [42, 43]. As mentioned above, the conservation of the neomycin selection cassette aggravates disease phenotype. Whereas the FKRP-neo<sup>P448L</sup> mice show MD as well as brain and eye abnormalities, displaying a phenotype between MDC1C and embryonic lethality [42], the FKRP<sup>P448L</sup> mouse model (following excision of the neomycin cassette), shows a less severe phenotype, which was found restricted to skeletal muscles, thus recapitulating moderate LGMDR9 to mild MDC1C (Figure 3) [43].

Several mouse models carrying the common L276I mutation associated with LGMDR9 have been generated [44–47]. Knock-in, shRNAs and homologous recombination strategies have been used to alter FKRP expression. All these strains have phenotypes resembling the features of mild LGMDR9 with late onset. To study ocular diseases, the FKRP<sup>tvrm53</sup> mouse line was generated by chemical mutagenesis using N-ethyl-N-nitrosourea to produce a missense mutation (I356T). These mice display a severe MDC1C phenotype with ocular and muscle defects [48]. Recently, we documented the generation of an immunodeficient FKRP mutant mouse model [49]. This strain was generated by crossing the FKRP<sup>P448L</sup> mouse [43] with the NSG immunodeficient mouse model [50]. Similar to the immunocompetent counterpart, the FKRP<sup>P448L</sup>-NSG mice show hypoglycosylation of  $\alpha$ -DG in muscle cells, early signs of regeneration, and weaker muscles compared to wild type, recapitulating a moderate LGMDR9 phenotype. This is the first and only FKRP mutant mouse model suitable for the transplantation of human cells [49].

## Human models

Studies involving animal models are informative and have been vital for expanding our understanding on the mechanisms underlining FKRP's function and pathogenesis, but they do not recapitulate all the aspects of human disease, and therefore, promising treatments tested solely *in vivo* could fail when translated to patients due to differences in physiology. To address this, some investigators have used muscle biopsies from patients with FKRP mutations, but the difficulty of obtaining these samples is a limiting factor [28, 51, 52]. To bypass these hurdles, several groups have used patient fibroblast [53, 54] or modified haploid cell lines, such as the HAP1 overexpressing FKRP variants [55]. Although informative, due to differences in tissue type, the pathophysiology of the disease may not always be recapitulated. A powerful tool for generating large numbers of patient-specific cell types is the reprogramming of somatic cells into iPSCs using the expression of pluripotency-associated transcription factors *OCT3/4*, *SOX2*, *KL4* and *MYC* [56]. Using

lineage-specific *in vitro* differentiation, iPSCs can give rise to desired cell types, allowing for disease modeling and drug screening [57].

Several iPSC patient-specific lines have been established for LGMDR9 and MDC1C, among them, an iPSC line from a LGMDR9 patient with the L276I mutation [58]. Cardiomyocytes derived from this iPSC line showed ion channel dysfunction and abnormal calcium dynamics, providing a platform to further elucidate the role of FKRP in cardiac function [58]. Recently, the derivation of iPSCs from an MDC1C patient with CNS involvement was reported [59]. These cells were differentiated into cortical neurons and used for drug screening. LGMDR9 patient-specific iPSCs have also been generated to study ECM defects *in vitro* [60]. Both these studies demonstrated reduced functional  $\alpha$ -DG glycosylation, indicating that iPSC-derivatives can be used to study FKRP-associated diseases.

## Experimental Treatments

At present, there are no active clinical trials for FKRP-associated dystroglycanopathies, but several promising therapies are underway (Figure 4). Here we describe therapeutic strategies that show rescue of the dystrophic pathology in experimental models. Detailed information is provided in Table 1.

### Small molecules

The combinatorial use of corticosteroids and bisphosphonates have been tested in the FKRP<sup>P448L</sup> mouse model [61, 62]. Corticosteroids are anti-inflammatory drugs used as a palliative treatment for Duchenne MD (DMD) as they enhance muscle strength and function [63], whereas bisphosphonates prevent bone density loss, a well-known side effect of extended corticosteroid treatment [64]. Results in FKRP<sup>P448L</sup> mice showed decreased muscle degeneration, but no improvement in functional outcomes [61]. Later on, the same group tested the therapeutic effect of the selective estrogen receptor modulators tamoxifen and raloxifene [62], which have been shown to inhibit fibrosis and improve muscle strength in a DMD mouse model [65]. One-year treatment in FKRP<sup>P448L</sup> mice resulted in decreased infiltration and muscle degeneration, as well as improved muscle and respiratory function. While the dose for raloxifene was above that recommended in humans, tamoxifen dosage was consistent with upper levels currently used in the clinic for other diseases.

Several compounds [39] that had previously been shown to ameliorate the dystrophic pathology in DMD models were tested in the zebrafish L276I model. Among these, the authors found pentetic acid, a calcium and magnesium chelator, to be the most efficacious in rescuing the FKRP zebrafish pathology (Table 1). Using a library of FDA approved drugs, they identified 20 different compounds that attenuated the MD pathology by decreasing eye and head malformations, pericardiac edema, and increasing muscle birefringence and overall viability. Most of these hits were steroids and nonsteroidal anti-inflammatory drugs, or antibacterial compounds [39].

The recent identification of FKRP as a ribitol-5-phosphate transferase opened the possibility of manipulating the levels of CDP-ribitol and ribitol-5-phosphate (Figure 1; Box 1) to increase  $\alpha$ -DG glycosylation [10–12]. Studies by Cataldi *et al.* investigating the effect of

oral **ribitol** supplementation in FKRP<sup>P448L</sup> mice showed increased levels of CDP-ribitol and ribitol-5-phosphate in the heart and quadriceps, confirming the capacity of ribitol to increase downstream substrates [66]. Of note, continuous ribitol treatment from the start of pregnancy, led to increased  $\alpha$ -DG functional glycosylation in all analyzed muscles, which was accompanied by reduced fibrosis and improved muscle function [66]. Recently, ribitol was also reported to enhance IIIH6 immunoreactivity in a human iPSC-derived basal lamina embryoid body model, but no significant changes were noted in laminin binding [60].

Due to the reported capacity of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) to improve dystrophic pathology in a DG model [67], the effect of this coenzyme was tested in FKRP zebrafish **morphants** [36]. The study reported decreased muscle degeneration and improved muscle organization and function when treatment occurred at gastrulation. However, there were no improvements in the vascular system and brain.

To identify new drugs, a drug screening was performed on immortalized mouse myoblasts [59]. They identified 4-(4-bromophenyl)-6-ethylsulfanyl-2-oxo-3,4-dihydro-1H-pyridine-5-carbonitrile (4BPPNIt) as a candidate due to its capacity to increase IIIH6 immunoreactivity. Using MDC1C iPSC-derived cortical neurons, the group confirmed 4BPPNIt increased  $\alpha$ -DG functional glycosylation and laminin binding.

### Gene therapy

Gene therapy has been extensively investigated for the treatment of MDs [68]. Several preclinical studies have investigated the use of recombinant adeno-associated virus (AAV) to deliver functional FKRP as well as other genes involved in glycosylation, such as *LARGE1* and *Beta-1,4 N-acetylgalactosaminyltransferase 2* (B4GALNT2 previously GALGT2) [69–71] (Figure 4).

Initial studies tested intraperitoneal delivery of the human *FKRP* gene using an AAV9 vector with a non-muscle specific promoter (AAV9-*FKRP*) in FKRP<sup>P448L</sup> mice (Table 1). After 4 months, the authors observed increased IIIH6 immunoreactivity and laminin binding, reduced muscle degeneration, and enhanced muscle function [69]. Similar rescue was observed when AAV9-*FKRP* was delivered to neonate and adult FKRP L276I<sup>KI</sup> mice [45]. Subsequent studies demonstrated that systemic delivery of AAV9-*FKRP* in FKRP<sup>P448L</sup> mice promoted long-term one year rescue of  $\alpha$ -DG functional glycosylation. Moreover, the authors observed improvement in respiratory function, muscle performance, and partial correction of metabolic alterations [72, 73]. Another group investigated delivery of the mouse *FKRP* gene using a muscle-specific promoter in FKRP<sup>L276I</sup> mice (Table 1). Intramuscular (IM) and systemic delivery resulted in increased IIIH6 immunoreactivity, laminin binding, and decreased muscle regeneration. Because IM delivery of high doses of the vector led to decreased  $\alpha$ -DG functional glycosylation, the group concluded that gene therapy could be effective, but that controlled expression of *FKRP* is critical to avoid toxicity [47].

Several investigators have explored the overexpression of other genes involved in  $\alpha$ -DG glycosylation as a strategy to rescue the FKRP phenotype. As described above, *LARGE1* is a key enzyme involved in  $\alpha$ -DG glycosylation (Figure 1). Previous studies



provided evidence that LARGE1 overexpression can compensate for the loss of other glycosyltransferases, such as POMGNT1, restoring  $\alpha$ -DG glycosylation *in vitro* and *in vivo* [74, 75]. In the context of FKRP deficiency, overexpression of LARGE1 using an AAV9 vector in FKRP<sup>P448L</sup> mice led to increased  $\alpha$ -DG functional glycosylation, but levels were higher than those observed in wild type mice [70]. While treated mice showed less muscle degeneration, no changes were detected in CK levels. Because LARGE1 overexpression resulted in hyperglycosylation of  $\alpha$ -DG, further studies are necessary to understand the implications in muscle function.

Another approach is overexpression of B4GALNT2, a glycosyltransferase involved in the generation of cytotoxic T-cell glycan in adult skeletal muscle [76], which had provided encouraging results in other forms of MD, including DMD,  $\alpha$ -sarcoglycan-associated LGMD, and laminin  $\alpha$ 2-deficient CMD [77–79]. One study assessed B4GALNT2 overexpression using an AAVrh74 vector with a muscle creatine kinase (MCK) promoter [71], which following IM delivery, resulted in improved muscle pathology and increased number of embryonic myosin heavy chain positive fibers in treated mice when compared to untreated counterparts, suggesting that B4GALNT2 overexpression may enhance regeneration [71] (Table 1). In any case, no striking increase in  $\alpha$ -DG glycosylation was observed and therefore, it remains to be defined the mechanism underlining these effects and whether B4GALNT2 overexpression improves muscle function.

More recently, investigators have begun exploring gene therapy combined with the use of small molecules. Specifically, FKRP<sup>P448L</sup> mice were subjected to overexpression of isoprenoid synthase domain-containing (ISPD), which is involved in the generation of CDP-ribitol (Figure 1), along with ribitol treatment [80]. This combinatorial treatment did not result in functional improvement when compared to ribitol alone, but a significant increase in  $\alpha$ -DG functional glycosylation was observed in the heart.

### Cell therapy

Since skeletal muscle is a highly regenerative tissue, cell-based therapeutic approaches focusing on the delivery of muscle stem cells/early progenitor cells to replace diseased muscle tissue with healthy myofibers as well as **satellite cells** are highly attractive. To date, two studies have been reported on the use of cell transplantation for FKRP-associated dystroglycanopathies (Figure 4). One utilized transplantation of satellite cells isolated from FKRP L276I<sup>KI</sup> mice engineered to overexpress wild type human FKRP [81]. Following IM injection of these cells into the tibialis anterior (TA) muscles of FKRP L276I<sup>KI</sup> mice, the authors confirmed increased levels of FKRP protein, which were accompanied by enhanced  $\alpha$ -DG functional glycosylation in the TA, however there was no evidence of cell engraftment. The group also documented rescue of  $\alpha$ -DG glycosylation in the heart of treated mice. These counterintuitive results led the authors to hypothesize that FKRP may circulate in exosomes, therefore having the capacity to contribute to  $\alpha$ -DG glycosylation at distant sites [81]. Nevertheless, the mechanism, as well as the long-term effects for this strategy remain to be further elucidated.

The second study used PSC-derived **myogenic progenitors**, which have been extensively validated for their *in vivo* regenerative capacity in several MD mouse models [82–86]. To

determine the ability of these progenitors to treat FKRP-associated dystroglycanopathies, Azzag and colleagues injected mouse and human iPSC-derived myogenic progenitors into TA muscles of FKRP<sup>P448L</sup>-NSG mice [49]. These transplantation studies revealed robust myofiber engraftment that was accompanied by increased  $\alpha$ -DG functional glycosylation, and improved muscle function compared to untreated controls. Importantly, the authors reported the presence of donor-derived satellite cells, which correlated with the persistence of donor-derived fibers 4 months post-transplantation, indicating the long-term contribution of these cells, which is critical to ensure long-lasting therapeutic benefit [49].

## Concluding Remarks

Significant progress has been achieved in recent years, but several questions remain to be addressed as well as challenges to be circumvented for the development of therapies. For instance, studies to date have focused on the activity of FKRP glycosyltransferase on  $\alpha$ -DG, but the recent discovery of ribitol-5-phosphate post-translational modifications in mammals [11, 12] may allow for the identification of other proteins that might be modified by FKRP (see Outstanding Questions). While weak ECM binding is a well-established hallmark for disease progression, other mechanisms have been proposed to contribute to FKRP pathogenesis, including endoplasmic reticulum stress and unfolded protein response [34, 36, 39, 87]. Moreover, certain mutations have been associated with FKRP mislocalization [55, 88, 89]. Increased understanding of the pathophysiological mechanisms underlining FKRP-associated dystroglycanopathies will be essential for the development of effective therapies.

Several experimental studies have shown improvement of disease phenotype. These include the use of small molecules, gene delivery and cell therapy (Figure 4). Although each of these treatments have the potential to treat FKRP-associated dystroglycanopathies, all have specific challenges to be circumvented. The small molecules discovered in zebrafish models should be further validated in mouse and/or human models. Ribitol treatment holds promise as it could be an economically accessible treatment. One aspect to consider is that FKRP residual functional activity is required, warranting further investigation of its therapeutic effect on patients carrying different mutations. FKRP gene replacement has been extensively studied, however challenges remain to be addressed, among which i) the immune response associated with pre-existing immunity, ii) the immunotoxicity associated with large vector doses [90] and iii) the timing of treatment since several studies suggest that age may potentially influence efficiency, with earlier interventions being more efficacious [91, 92]. Importantly, as discussed above, FKRP expression may have to be controlled, as high levels might be deleterious. For cell therapy, challenges include efficient cell delivery to all target muscles as well as high cost at present, in particular if one considers the use of an autologous cell product, which would involve gene editing of patient-specific iPSC prior to the generation of myogenic progenitors and subsequent transplantation. One option would be the transplantation of allogeneic iPSC-derived myogenic progenitors, which would allow the same iPSC muscle product to be used for multiple patients. Several institutions worldwide have established universal donor iPSC banks, also referred as the HLA haplobank model [93–96], which allows for the selection of matched donors to generate graft material that will require only limited immunosuppression.



To date, two interventional clinical trials involving patients with FKRP mutations have been conducted. Ten years ago, the myostatin inhibitor MYO-029 was tested<sup>ii</sup> on a subset of adult MD patients, including LGMDR9, but this trial did not progress beyond phase II. More recently, a trial for the glucocorticoid deflazacort, used for DMD patients, was registered<sup>iii</sup> for LGMDR9, but this trial is no longer recruiting. Most of the treatments discussed here have been established recently, and although no new interventional clinical trials are currently underway, initiatives like the Global FKRP registry<sup>iv</sup> intend to facilitate and accelerate this process. The potential displayed by all these treatments to rescue  $\alpha$ -DG functional glycosylation offers hope for the development of effective treatments for FKRP-associated dystroglycanopathies.

## ACKNOWLEDGMENTS

R.C.R.P. is supported by the National Institutes of Health (NIH) grants R01 AR055299 and R01 AR071439. C.O.C. was supported by PINN MICITT Costa Rica. We are thankful to Cynthia Faraday for graphical design.

## GLOSSARY

### **Agtrin**

extracellular matrix proteoglycan involved in synaptic formation and maintenance in the brain and muscle.

### **Creatine Kinase (CK)**

is an enzyme expressed in various tissues, like brain and muscle. High levels of CK in the blood can be used as an indirect indicator of muscle damage.

### **Extracellular matrix (ECM)**

network of proteins and polysaccharides that provide structural scaffolding and biochemical cues to support surrounding cells.

### **Frameshift mutations**

mutations that add or delete extra bases of DNA causing a shift in the reading frame. This leads to the DNA sequence after the mutation to be disrupted or read incorrectly.

### **Glycosylation**

is a common modification in which a carbohydrate is attached to an organic molecule donor. This commonly occurs on proteins and is important for protein function and stability.

### **Missense mutations**

mutations in which there is a change in a single base pair causing a substitution of a different amino acid in the resulting protein.

### **Morphants**

organisms treated with morpholinos to modify gene expression.

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<sup>ii</sup> <https://www.clinicaltrials.gov/ct2/show/study/NCT00104078>

<sup>iii</sup> <https://clinicaltrials.gov/ct2/show/NCT03783923>

<sup>iv</sup> <https://www.fkrp-registry.org/>

**Morpholino**

synthetic nucleotides that recognize and bind short sequences (18–25 nucleotides) of RNA commonly used to prevent splicing or translation of RNA.

**Myogenic progenitors**

cells which can differentiate to form muscle *in vitro* and *in vivo*.

**Nonsense mutations**

mutations in which the substitution of a single base pair causes the change of a normal codon into a stop codon, resulting in a truncated protein.

**Perlecan**

proteoglycan found in the extracellular matrix of muscle and other tissues.

**Pluripotent stem cells (PSCs)**

undifferentiated cells endowed with the ability to self-renew as well as to differentiate into any cell type of the body.

**Ribitol**

sugar alcohol formed by the reduction of ribose.

**Sarcolemma**

specialized plasma membrane surrounding skeletal and cardiac muscle fibers.

**Satellite cells**

adult muscle stem cells. These cells regenerate muscle in response to injury or disease.

**TALENs**

engineered enzymes that can be used to cut specific DNA sequences for gene editing.

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**Box 1.****Secondary dystroglycanopathies and M3 O-mannose glycosylation**

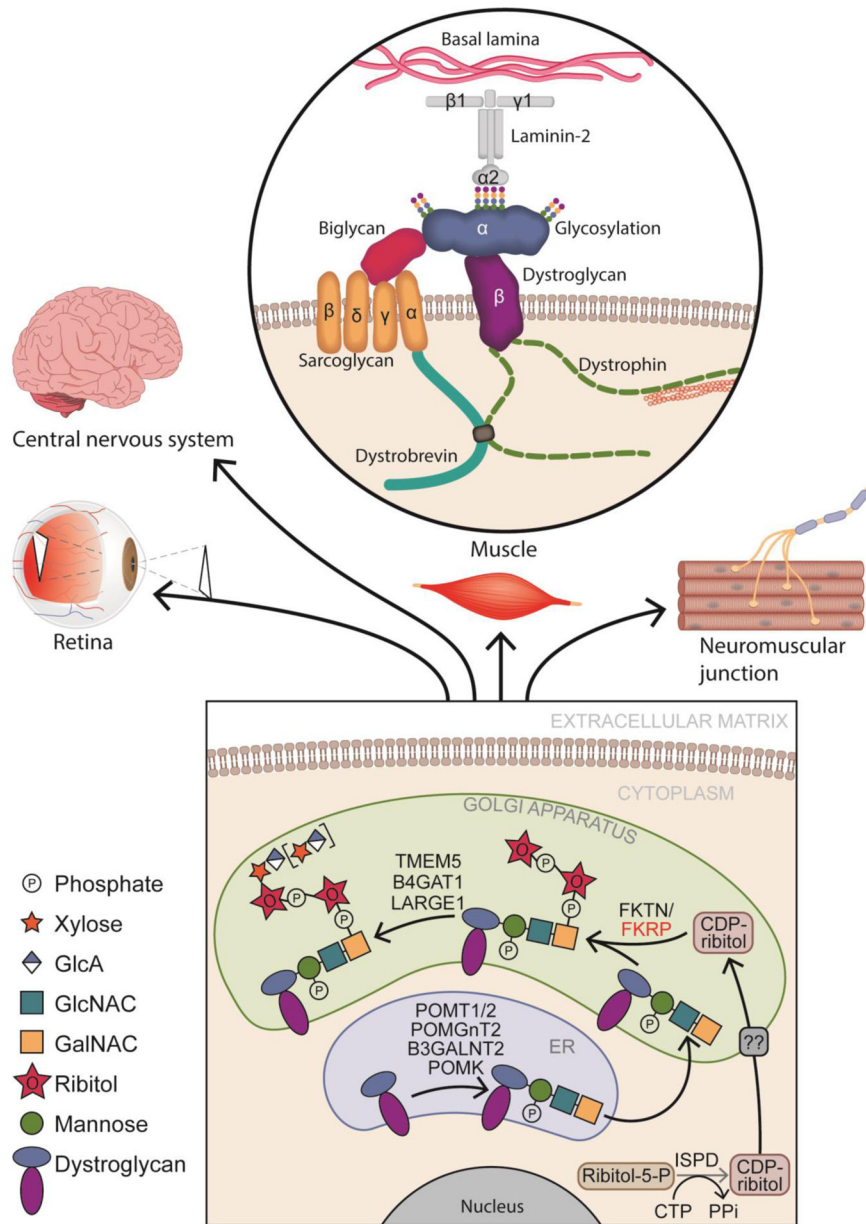
MDs associated with aberrant O-mannosylation of  $\alpha$ -DG are called secondary or tertiary dystroglycanopathies [1]. DG is encoded by the DAG1 gene, which by post-translational processing is cleaved into  $\alpha$ -DG and  $\beta$ -DG, a transmembrane protein that binds to dystrophin [97].  $\alpha$ -DG is heavily glycosylated, with N-linked and O-linked glycosylation, including O-mannosyl glycans. Among the several types of  $\alpha$ -DG O-mannose glycans, FKRP is involved in M3 O-mannose glycosylation [4]. The M3 glycan core begins with protein O-mannosyl transferase 1/2 (POMT1/2) complex catalyzing the addition of a mannose group to serine/threonine residues on  $\alpha$ -DG [98] (Figure 1). N-acetylglucosaminyltransferase 2 (POMGNT2) and  $\beta$ 1,3-N-acetylgalactosaminyltransferase 2 (B3GALNT2) carry out further modification by adding a  $\beta$ 1,4 linked N-acetylglucosamine (GlcNAc) and a  $\beta$ 1,3-N-acetylgalactosamine (GalNAc), respectively. This O-mannosyl trisaccharide is the substrate for protein-O-mannose kinase (POMK) that phosphorylates the initial mannose at the 6-position [99]. Isoprenoid synthase domain-containing (ISPD) modifies ribitol-5-phosphate by adding a cytidine 5'-diphosphate (CDP) group. Once in the Golgi apparatus, CDP-ribitol is then used by FKTN and FKRP to transfer a tandem ribitol-5-phosphate onto the 3-GalNAc- $\beta$ 3-GlcNAc- $\beta$ 4-(6-P)-Man-O-(Ser/Thr) modification on  $\alpha$ -DG [10–12]. Finally, transmembrane protein 5 (TMEM5) and  $\beta$ -1,4-glucuronyltransferase 1 (B4GAT1) will sequentially transfer a  $\beta$ 1,2-Xylose (Xyl) and a  $\beta$ 1,4 glucuronic acid (GlcA) that will serve as a primer for like-acetylglucosaminyltransferase (LARGE1) to add  $\alpha$ 1,3-Xyl- $\beta$ 1,3-GlcA repeats allowing  $\alpha$ -DG to bind to ECM ligands [100–103].

### OUTSTANDING QUESTIONS

- Does FKRP participate in the glycosylation of other glycoproteins? If so, is this function tissue specific?
- Are there other mechanisms underlining LGMDR9 pathophysiology in patients displaying high IIH6 immunoreactivity?
- Do FKRP mutations trigger endoplasmic reticulum stress and unfolded protein response? Are these involved in disease progression?
- Will ribitol treatment be effective for the treatment of all *FKRP* pathological mutations?
- Is it possible to enhance the production of CDP-ribitol and ribitol-5-phosphate by using additional small molecules?

**HIGHLIGHTS**

- FKRP is a ribitol-5-phosphate transferase involved in  $\alpha$ -DG glycosylation. Mutations in *FKRP* have been associated with a broad spectrum of dystroglycanopathies.
- Several FKRP mutant models have been generated to better understand disease pathogenesis of FKRP-associated dystroglycanopathies as well as to develop therapeutics.
- Several strategies, including the use of small molecules, gene therapy and cell therapy, have shown improvement of disease phenotype as evidenced by rescue of  $\alpha$ -DG functional glycosylation.



**Figure 1: Core M3 α-DG glycosylation process and ECM binding.**

In the endoplasmic reticulum (ER), the glycosylation process begins when protein O-mannosyl transferase 1/2 (POMT1/2) complex catalyzes the addition of a mannose group to serine/threonine residues on α-dystroglycan (DG). Subsequently N-acetylglucosaminyltransferase 2 (POMGNT2) and β1,3-N-acetylgalactosaminyltransferase 2 (B3GALNT2) attach sequentially a N-acetylglucosamine (GlcNAc) and a N-acetylgalactosamine (GalNAc). Protein-O-mannose kinase (POMK) phosphorylates mannose. Once in the golgi apparatus, fukutin (FKTN) and fukutin related protein (FKRP) will use cytidine 5-diphosphate (CDP)-ribitol produced by isoprenoid synthase domain-containing protein (ISPD), to transfer tandem ribitol-5-phosphate onto the 3-GalNAc-β3-GlcNAc-β4-(6-P)-Man-O-(Ser/Thr) modification on α-DG. Afterwards, transmembrane

protein 5 (TMEM5) and b-1,4-glucuronyltransferase 1 (B4GAT1) add a  $\beta$ 1,2-Xylose (Xyl) and a  $\beta$ 1,4 glucuronic acid (GlcA) allowing like-acetylglucosaminyltransferase 1 (LARGE1) to add  $\alpha$ 1,3-Xyl- $\beta$ 1,3-GlcA repeats. Glycosylated  $\alpha$ -DG will bind to laminin  $\alpha$ -2 in the muscle sarcolemma, as well as to other components of the extracellular matrix (ECM) in the retina, the central nervous system and at the neuromuscular junction.

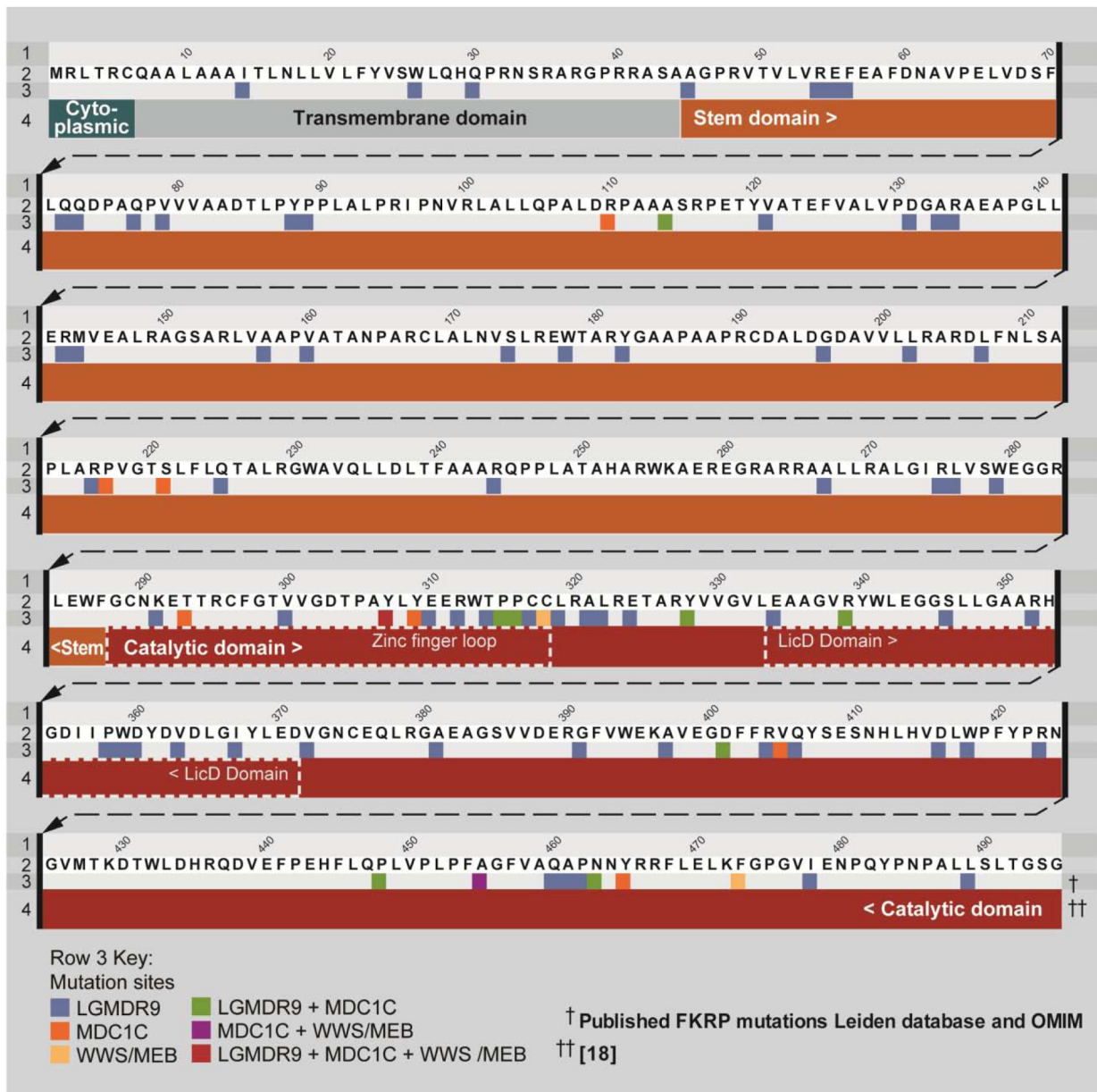
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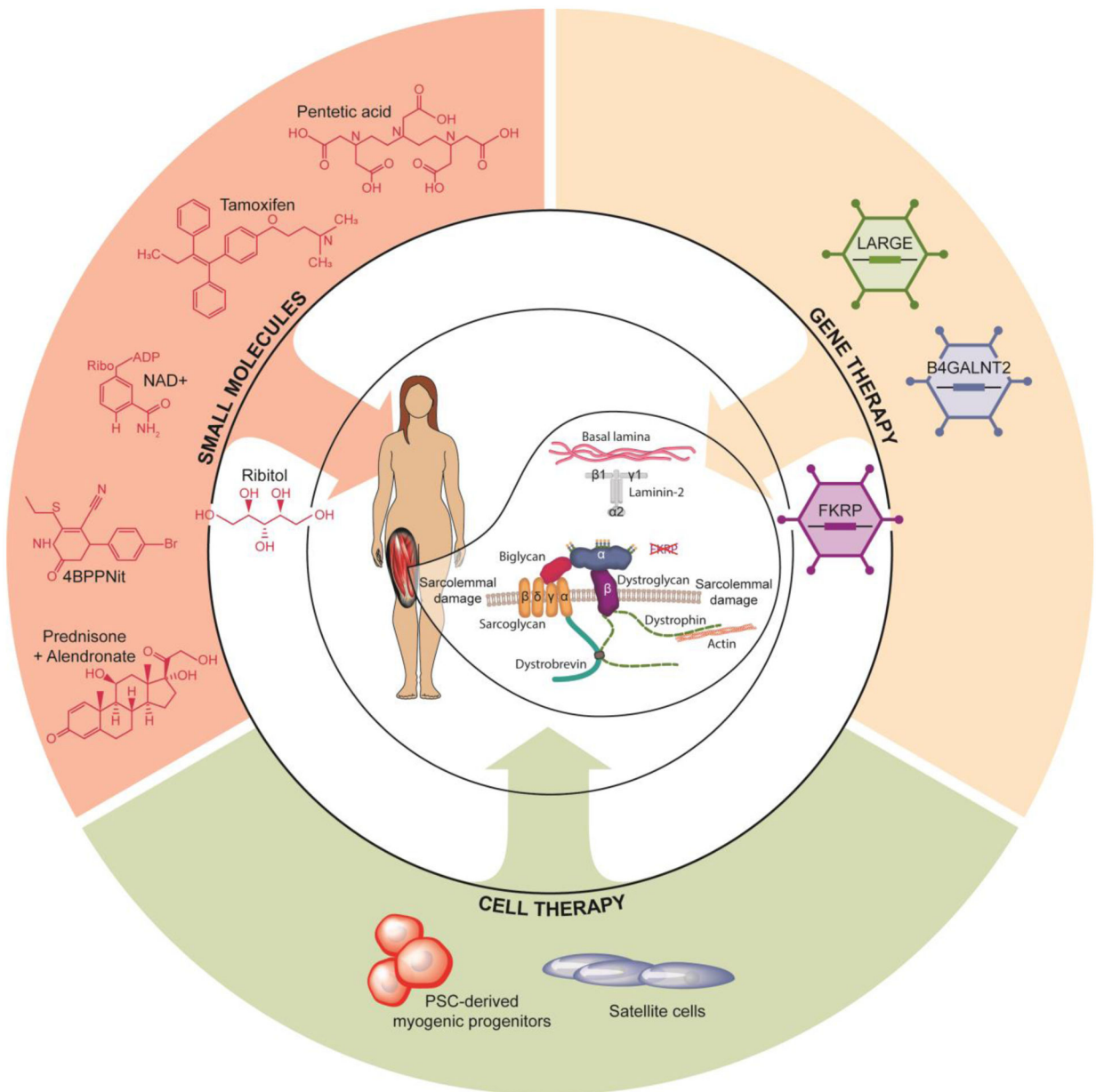
**Figure 2: FKRP mutations associated with dystroglycanopathies.**

Linear representation of fukutin related protein (FKRP) protein and different unique pathological amino acid variants associated with missense or frameshift mutations as described in the Leiden database. FKRP-related limb-girdle muscular dystrophy (LGMDR9) only in blue, FKRP-related congenital muscular dystrophy (MDC1C) in orange, Walker-Warburg Syndrome (WWS)/muscle eye brain disease (MEB) in yellow. Green shows mutations associated with LGMDR9 and MDC1C, purple mutations associated with MDC1C and WWS/MEB and the red residues are associated with LGMDR9, MDC1C and WWS/MEB.

PHENOTYPES	Asymptomatic					Mild LGMDR9	Moderate LGMDR9	MDC1C	WWS/MEB	Embryonic Lethal
	MODELS									
<i>Danio rerio</i> 						FKRP morphants [31-36]				
						FKRP TALENs 13bp del + rescue [37]			FKRP TALENs 13bp del [37]	
<i>Mus musculus</i> 	FKRP <sup>Y307N</sup> [38]	FKRP L276I <sup>KI</sup> [43]		FKRP <sub>MD</sub> [39]		FKRP <sup>lvrm53</sup> [46]		FKRP <sup>KD</sup> [38]		
			FKRP <sup>L276I</sup> [44, 45]		FKRP <sup>P448L</sup> [41]		FKRP-neo <sup>P448L</sup> [40]		FKRP <sup>E310del</sup> [40]	
			FKRP shRNAs [42]		FKRP <sup>P448L</sup> -NSG [47]					
<i>Homo sapiens</i> 						HAP1 FKRP mutated cells [53]				
			iPSC-derived EBs [58]		iPSC-derived cardiomyocytes [56]		iPSC-derived neurons [57]			

**Figure 3: Available *in vitro* and *in vivo* models to study *FKRP* mutations.**

Biological models of fukutin related protein (*FKRP*)-associated dystroglycanopathies have been developed using zebrafish (*danio rerio*), mouse (*mus musculus*), and human cells (*homo sapiens*). Several zebrafish and mouse *FKRP* models recapitulate the large spectrum of diseases. The recent use of patient-specific induced pluripotent stem cells (iPSCs) to recapitulate *in vitro* disease phenotype allowed for the generation of LGMDR9 iPSC-derived EBs and cardiomyocytes, as well as MDC1 iPSC-derived-neurons.



**Figure 4: Potential treatments for FKRP-associated dystroglycanopathies.**

Preclinical studies aiming to develop treatments for fukutin related protein (FKRP)-related disorders include small molecules, gene and cell therapy. Small molecules, like ribitol, nicotinamide adenine dinucleotide (NAD<sup>+</sup>), 4-(4-bromophenyl)-6-ethylsulfanyl-2-oxo-3,4-dihydro-1H-pyridine-5-carbonitrile (4BBNIt), pentetic acid, prednisolone combine with alendronate and the use of tamoxifen have shown the ability to improve disease phenotype. Gene therapy studies with *FKRP*, *like-acetylglucosaminyltransferase (LARGE1)* and *Beta-1,4 N-acetylgalactosaminyltransferase 2 (B4GALNT2)* have shown promising results. Cell therapy studies using engineered satellite cells and the transplantation of pluripotent

stem cell (PSC)-derived myogenic progenitors have also successfully rescued the FKRP pathology.

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**Table 1.**

Reported therapeutic strategies for FKR- associated dystroglycanopathies.

Treatment	Model	Results	Refs
Prednisolone + Alendronate	FKRP <sup>P448L</sup> mouse	<ul style="list-style-type: none"> <li>Decreased CK levels, infiltration (TA), degeneration and CNF (TA)</li> <li>Increased functionally glycosylated <math>\alpha</math>-DG (TA)</li> </ul>	[61]
		<ul style="list-style-type: none"> <li>Decreased infiltration (TA), CNF (TA), CK levels and collagen accumulation (HA-QUA-TA-DIA)</li> <li>Improved skeletal muscle and respiratory function</li> </ul>	[62]
Pantetic Acid	Zebrafish Tg [hsp70l-hFKRP(L276I)-IRES-eGFP]	<ul style="list-style-type: none"> <li>Decreased pericardiac edema</li> <li>Increased life span and muscle birefringence</li> </ul>	[39]
Small molecules and metabolites	FKRP <sup>P448L</sup> mouse	<ul style="list-style-type: none"> <li>Decreased fibrosis (HA-TA-DIA)</li> <li>Increased functionally glycosylated <math>\alpha</math>-DG (HA-TA-DIA)</li> </ul>	[66]
	Ribitol	<ul style="list-style-type: none"> <li>Improved skeletal muscle and respiratory function</li> </ul>	
NAD <sup>+</sup>	iPSC-derived human embryoid bodies	<ul style="list-style-type: none"> <li>Increased functionally glycosylated <math>\alpha</math>-DG</li> <li>Decreased muscle degeneration</li> </ul>	[60]
	Zebrafish FKR morphants	<ul style="list-style-type: none"> <li>Increased concentration of laminin at the myotendinous junction</li> <li>Improved muscle fiber organization, skeletal muscle function and myotendinous junction</li> </ul>	[36]
4BPPN <sup>it</sup>	iPSC-derived cortical neurons	<ul style="list-style-type: none"> <li>Increased functionally glycosylated <math>\alpha</math>-DG</li> </ul>	[59]
AAV9-CAG-FKR	FKRP <sup>P448L</sup> mouse	IP and IV injection: <ul style="list-style-type: none"> <li>Decreased CNF, CK, fibrosis (HA-DIA)</li> <li>Increased functionally glycosylated <math>\alpha</math>-DG and LOA (HA-TA-DIA-GAS)</li> <li>Improved skeletal muscle and respiratory function</li> </ul>	[69, 72, 73]
		IV injection: <ul style="list-style-type: none"> <li>Partial recovery of metabolic alterations</li> </ul>	
Gene Therapy	FKRP L276I <sup>K1</sup> mouse	<ul style="list-style-type: none"> <li>Decreased CK, CNF</li> <li>Increased in functionally glycosylated <math>\alpha</math>-DG and cardiac function (EF and FS)</li> <li>Improved skeletal muscle function</li> </ul>	[45]
	rAAV2/9-Des-mFkrp	FKRP <sup>L276I</sup> mouse	<ul style="list-style-type: none"> <li>Decreased CNF (PSO)</li> <li>Increased functionally glycosylated <math>\alpha</math>-DG and laminin binding (GAS-GLU-pSo)</li> </ul>
AAV9-CAG-LARGE1	FKRP <sup>P448L</sup> mouse	IP injection:	[70]

Treatment	Model	Results	Refs
		<ul style="list-style-type: none"> <li>Decreased CNF</li> <li>Increased functionally glycosylated <math>\alpha</math>-DG and laminin binding (HA-TA-DIA)</li> </ul>	
AAVrh74-MCK-GALGT2	FKRP <sup>P448L</sup> mouse	IM injection: <ul style="list-style-type: none"> <li>Decreased CNF (QUA-TA-GAS), membrane instability (TA) and muscle loss (QUA-TA-GAS)</li> <li>Increased regenerating fibers</li> </ul>	[71]
pLenti-CAG (hFKRP)-Rsv satellite cells	FKRP L276I <sup>KI</sup> mouse	TA IM injection of cells: <ul style="list-style-type: none"> <li>Increased functionally glycosylated <math>\alpha</math>-DG (TA-HA)</li> <li>Improved skeletal muscle function</li> </ul>	[81]
Cell therapy		TA IM injection of cells: <ul style="list-style-type: none"> <li>Decreased CNF</li> <li>Increased functionally glycosylated <math>\alpha</math>-DG</li> <li>Improved skeletal muscle function</li> </ul>	[49]
Pax3/7 iPSC-derived myogenic progenitors	FKRP <sup>P448L</sup> -NSG mouse		

Abbreviations: CNF, centrally nucleated fibers; DIA, diaphragm; EF, ejection fraction; FS, fractional shortening; GAS, gastrocnemius; GLU, gluteus; IP, intraperitoneal; IV, intravenous; TA, tibialis anterior; HA, heart; QUA, quadriceps; PSO, psoas.