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The transgenic expression of LARGE exacerbates the muscle phenotype of dystroglycanopathy mice.

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Abstract.

Mutations in fukutin related protein (FKRP) underlie a group of muscular dystrophies associated with the hypoglycosylation of α-dystroglycan (α-DG), a proportion of which show central nervous system involvement. Our original FKRP knock down mouse ($FKRP^{KD}$) replicated many of the characteristics seen in patients at the severe end of the dystroglycanopathy spectrum but died perinatally precluding its full phenotyping and use in testing potential therapies. We have now overcome this by crossing $FKRP^{KD}$ mice with those expressing Cre recombinase under the Sox1 promoter. Due to our original targeting strategy this has resulted in the restoration of Fkrp levels in the central nervous system but not the muscle, thereby generating a new model ($FKRP_{MD}$) which develops a progressive muscular dystrophy resembling what is observed in limb girdle muscular dystrophy. Like-acetylglucosaminyltransferase (LARGE) is a bifunctional glycosyltransferase previously shown to hyperglycosylate α-dystroglycan. In order to investigate the therapeutic potential of LARGE up-regulation we have now crossed the $FKRP_{MD}$ line with one overexpressing LARGE and show that, contrary to expectation, this results in a worsening of the muscle pathology implying that any future strategies based upon LARGE up-regulation require careful management.

Introduction.

Dystroglycan (DG) forms the central component of the dystrophin associated protein complex and has been attributed with a primary role in the deposition, organisation and turnover of basement membranes (1-7). It is composed of two subunits, both of which are encoded by the *DAG1* gene: β-DG, a transmembrane protein and α-DG, a highly glycosylated peripheral membrane protein (l). The primary sequence of α-DG predicts a molecular mass of 72kDa: however, due to extensive post-translational glycosylation the final molecular weight is around 156kDa in skeletal muscle (8). The O-linked glycan chains of the central mucin domain of α-DG mediate binding to basement membrane ligands including laminin (9), perlecan (10) agrin (11-13), neurexin in the brain (14), pikachurin in the eye (15) and Slit (16) by interaction with the laminin LG (laminin globular) domains (17,18) and their loss from the central mucin domain of α-DG is considered to be central to the pathogenesis of a subgroup of congenital muscular dystrophies (CMDs), the dystroglycanopathies (7,19-21).

To date at least 15 genes have been implicated in the glycosylation and/or processing of αDG, which now include *POMT1 (PROTEIN O-MANNOSYL-TRANSFERASE 1)* (22), *POMT2 (PROTEIN O-MANNOSYL-TRANSFERASE 2)* (23), *POMGNT1 (PROTEIN O-MANNOSE BETA-1,2-N-ACETYLGLUCOSAMINYLTRANSFERASE)* (24), *LARGE* (25-27), *FKT (FUKUTIN)* (28), *FKRP* (29,30), *DPM2 (DOLICHYL-PHOSPHATE MANNOSYLTRANSFERASE POLYPEPTIDE 2), DPM3 (DOLICHYL-PHOSPHATE MANNOSYLTRANSFERASE POLYPEPTIDE 3*) (31), *ISPD (ISOPRENOID SYNTHASE DOMAIN CONTAINING)* (32,33), *GTDC2 (GLYCOSYLTRANSFERASE-LIKE DOMAIN*

CONTAINING 2) (34), *TMEM5 (TRANSMEMBRANE PROTEIN 5)* (35), *B3GNT1 (BETA-1,3-N-ACETYLGLUCOSAMINYLTRANSFERASE 1)* (36), *DOLK (DOLICHOL KINASE)* (37), *SGK196 (SUGEN KINASE 196)* (38) and *GMPPB (GDP-MANNOSE PYROPHOSPHORYLASE B)* (39). Mutations in these genes are often associated with a wide clinical spectrum of phenotypes, including severe CMD and structural brain defects as exemplified by Walker Warburg syndrome (WWS) OMIM 236670, Muscle Eye Brain disease (MEB) OMIM 253280, Fukuyama CMD (FCMD) OMIM 253800, and congenital muscular dystrophy type 1D (MDC1D) OMIM 608840). Whilst the precise pathway in which these proteins function is unclear, recent work suggests that SGK196 is a glycosylation specific O-mannose kinase and that FKRP, FKTN, TMEM5, B3GNT1 and LARGE all contribute to the generation of an extracellular matrix binding moiety on the resulting phosphorylated core M3 glycan (38). The loss of matrix binding is associated with a profound reduction in the binding of either IIH6 and/or VIA4-1 antibodies to α-dystroglycan (40). In addition a deficiency of Dol-P-Man synthase subunit DPM2 and DPM3 indicates a possible link between the congenital disorders of glycosylation and the dystroglycanopathies. However, it is mutations in FKRP that underlie LGMD2I, which is one of the most frequent autosomal recessive forms of LGMD (Limb girdle muscular dystrophy) in the UK, reported to make up 19.1% of the total LGMD group, with a prevalence of 0.43/100 000 .

We previously generated a mouse model for FKRP related disease by inducing a knock-down in Fkrp expression via the insertion of a floxed Neomycin cassette into intron 2 of the mouse *Fkrp* gene ($FKRP^{KD}$). This resulted in perinatal lethality due to central nervous system involvement and whilst this model has proved useful in studies of disease pathogenesis, it is limited in regard to its potential for evaluating potential therapies aimed at restoring muscle function. In order to

overcome this we have now crossed the $FKRP^{KD}$ line of mice with one expressing *Cre recombinase* under the control of the Sox1 (Sex determining region Y-box 1) promoter, and show here that this strategy successfully restored Fkrp activity in the brain but not muscle. This new line, henceforth referred to as $FKRP_{MD}$, lives a normal lifespan and begins to show muscle damage at around 6 weeks of age and develops a progressive muscular dystrophy by 12 weeks, thereby representing a new mouse model of the less severe end of the dystroglycanopathy spectrum.

Whilst there have been two recent reports of the successful restoration of functional glycosylation and amelioration of the muscle pathology by AAV (Adeno-Associated Virus) vectors carrying either FKRP (41) or fukutin (42), one of the most promising forms of therapy proposed in recent years for the dystroglycanopathies is the up-regulation of LARGE; a bifunctional glycosyltransferase that alternately transfers xylose and glucuronic acid to generate a heteropolysaccharide that confers α-dystroglycan with its ligand binding properties (43). This is based on observations showing that LARGE is able to restore α-dystroglycan glycosylation and functional laminin binding to cells taken from patients with congenital muscular dystrophy (FCMD, MEB and WWS), seemingly irrespective of the gene involved (44). Whilst this response may be dependent on the availability of O-mannosyl phosphate acceptor sites (32), this strategy is still considered as being potentially useful for a wide range of patients. We have previously shown that in mice, over-expression of LARGE on a wild type background induces no overt pathology and is only associated with a minor loss of force in response to eccentric exercise in older mice, supporting the idea that increasing levels of this glycosyltransferase may represent an important therapeutic approach (45). However, it remains crucial to test such a

strategy on a disease background and in order to do this we crossed our newly generated $FKRP_{MD}$ mouse line with one of the original *LARGE* overexpressing lines *(45)*. Somewhat surprisingly we report that this fails to ameliorate the phenotype and in a proportion of mice leads to a worsening of the disease process despite a marked increase in dystroglycan glycosylation implying that any future strategies based upon LARGE up-regulation require careful management.

Results

Restoration of Fkrp levels in the central nervous system prevents the perinatal lethality of $FKRP^{KD}$ mice.

$FKRP^{KD}$ mice have a significant reduction in *Fkrp* expression in the muscle and brain, compared to wild type mice, which we attributed to the insertion of the floxed Neomycin cassette into intron 2 (46). Since we believed that central nervous system involvement was responsible for the perinatal mortality, we set out to restore *Fkrp* expression in the central nervous system by crossing the $FKRP^{KD}$ line with one expressing Cre recombinase under the Sox1 promoter. $FKRP^{KD}$ mice expressing the Sox1 Cre transgene are henceforth referred to as $FKRP_{MD}$. This cross should delete the neomycin cassette from exon 2 in neurectoderm derived tissues. Since defects in the pial basement membrane due to a loss of α-dystroglycan are thought to be central to the brain phenotype of $FKRP^{KD}$ mice (46,47) we examined paraffin wax embedded coronal sections of the cortex of newborn mice. These sections showed a clear disruption of cortical architecture in the $FKRP^{KD}$ (Figure 1B) but no overt abnormalities in the $FKRP_{MD}$ mice, the latter of which were comparable to wild type (Figure 1A and C). IIH6 immunolabelling at the

pial basement membrane of the $FKRP_{MD}$ was comparable to that of WT (Figure 1A and C) but not the $FKRP^{KD}$ (Figure 1B) and immunolabelling of frozen sections with a pan laminin antibody showed that the disorganisation of the pial basement membrane apparent in $FKRP^{KD}$ mice (Figure 1E) had been restored to that of WT in the $FKRP_{MD}$ (Figure 1D and F). In order to further confirm that this strategy was successful we undertook an analysis of muscle and brain tissue from the $FKRP_{MD}$ mice using quantitative RT PCR. This showed that whilst a marked reduction in *Fkrp* transcript levels was still apparent in the muscle of $FKRP_{MD}$ mice, levels had been restored to that of wild type in the brain (Figure 1G). We previously showed that the percentage knock-down of *Fkrp* in the $FKRP^{KD}$ mouse was similar in all tissues. Here we show that the percentage knock-down of *Fkrp* in the muscle of the $FKRP_{MD}$ was similar to that seen in the brain of the $FKRP^{KD}$ at E15.5 (Figure 1H).

Reduced *Fkrp* levels in skeletal muscle are associated with a progressive muscular dystrophy.

$FKRP_{MD}$ male mice were shown to have a 12 and 20 week body weight not significantly different from control mice although female body weight was reduced at 20 but not 12 weeks relative to controls (Figure 2A). The lifespan and general behaviour of $FKRP_{MD}$ mice was indistinguishable from their wild type littermates.

Haematoxylin and eosin stained sections of newborn $FKRP_{MD}$ muscle showed no evidence of muscle fibre necrosis either in the fore or hindlimb muscles (data not shown). However, by 6 weeks of age occasional areas of small basophilic regenerating fibres and inflammatory infiltrates could be seen in the $FKRP_{MD}$ gastrocnemius (Figure 2C). This feature was quite

variable between individuals with some mice showing only minimal evidence of any pathology at this age. By 12 weeks of age both the gastrocnemius (Figure 2E) and the diaphragm (data not shown) of all $FKRP_{MD}$ mice exhibited fibre degeneration characterised by sarcoplasmic hyalinisation, loss of cross striations, and sarcoplasmic fragmentation and frequent groups of small, regenerative myofibres, with large, centralised nuclei and a granular pale basophilic cytoplasm (Figure 2 E). Small infiltrates of macrophages, lymphocytes and rare plasma cells, were observed to invade the interstitium and infiltrate necrotic myofibres. At 30 weeks there was evidence of an attenuation of muscle fibre degeneration and regeneration with clusters of basophilic regenerative fibres being only occasionally evident together with rare, interstitial lymphoplasmacytic foci. Representative images of the diaphragm, gastrocnemius and quadriceps at 30 weeks are shown in Figure 2 G - K.

Interestingly, the soleus of the $FKRP_{MD}$ showed evidence of only minimal damage even at 30 weeks of age, reflected by the low percentage of central nucleation which is a marker of previous rounds of degeneration and regeneration (Figure 2 L-M). This parameter nonetheless increased between 12 and 30 weeks of age in all three muscles (gastrocnemius (48.9% to 57.3%), soleus (5.6% to 20.9%) and diaphragm (32.4% to 40.7%), with a Generalised Estimating Equations model showing that age, muscle and genotype were significant interacting factors affecting the percentage of centrally nucleated muscle fibres (Figure 2 L-M). In contrast to previous findings in the dystrophin deficient *mdx* mouse, the diaphragm did not seem to be more severely affected than the limb muscles. Muscle sampled from wild type littermates at either 12 or 30 week time points was histologically unremarkable, exhibiting normal histopathological changes for mice of this genetic background i.e. the presence of minimal, rare, interstitial, lymphoplasmacytic infiltrates.

A reduction in α-dystroglycan glycosylation is associated with an alteration in laminin α2 and α4 expression.

The skeletal muscle of the $FKRP_{MD}$ mice displayed a near absence of immunolabelling with the IIH6 antibody relative to wild type littermates at 30 weeks of age (Figure 6B), with Western blotting further confirming the absence of functional glycosylation as judged by the absence of the IIH6 epitope and also showed that β-dystroglycan was unchanged (Figure 3G). Immunolabelling for laminin α2 which has previously been shown to be variably reduced in cases of LGMD2I (48), was shown to be increased on some fibres but decreased on others relative to WT controls in the $FKRP_{MD}$ at 12 weeks. Those fibres with a higher level tended to be associated with areas of increased cellular activity, whilst larger fibres showed either a slight decrease or similar levels to that of WT controls (Figure 3A-F). The application of a look up table to these images shows this variation more clearly (Figure 3H,I).

Laminin α4 has previously been shown to be up-regulated in the basement membranes of blood vessels, the perineurium of intramuscular nerves, and isolated regenerating muscle fibres of laminin α2 deficient mice (*dy/dy*) (49). In wild type controls laminin α4 localised to the capillaries, nerves and neuromuscular junctions (Figure 4A,C,D), however in the $FKRP_{MD}$ at 12 weeks of age laminin α4 was additionally increased at the sarcolemma of small groups of fibres. By 30 weeks of age a higher proportion of fibres showed laminin α4 at the sarcolemma than at earlier ages (Figure 4D,F). This was the case for each of the muscles examined (diaphragm, quadriceps and triceps). This up-regulation was not specifically associated with regenerating

fibres as determined by immunolabelling with developmental myosin which only labelled very few fibres at either 12 or 30 weeks.

The overexpression of LARGE leads to a shortened lifespan and worsening of the $FKRP_{MD}$ phenotype.

Previous work indicated that the upregulation of LARGE could be beneficial in the dystroglycanopathies (44,50,51). Contrary to expectation $FKRP_{MD}$ overexpressing LARGE ($FKRP_{MD}$LARGE) had a reduced lifespan in contrast to $FKRP_{MD}$ mice - a subsequent deterioration in the overall condition of $FKRP_{MD}$LARGE mice led to some mice being culled at a humane end point which was often around 27 weeks of age. $FKRP_{MD}$LARGE displayed normal behaviour, aside from an abnormal stance and a partial collapse of the leg extensor reflex, a feature not observed in the $FKRP_{MD}$ mice.

In keeping with the more severe phenotype, $FKRP_{MD}$LARGE mice displayed a more severe pathology than age-matched $FKRP_{MD}$ controls at 12 weeks as indicated by a marked variation in fibre size, centrally nucleated muscle fibres (Supplementary Figure 1) and significant increases in the number of split fibres (Figure 7D). A Generalised Estimating Equations statistical model showed that age, genotype and muscle were significant factors affecting the percentage of centrally nucleated fibres suggesting that LARGE upregulation significantly increased the percentage of centrally nucleated fibres at both 12 and 30 weeks of age, relative to the $FKRP_{MD}$ (Figure 7). There was in addition a more pronounced expansion of the interstitium with small to moderate amounts of variably-mature fibroadipose tissue and a substantial inflammatory component, which infiltrated both the interstitium and necrotic muscle fibres and was comprised

of moderate numbers of neutrophils, macrophages and lesser numbers of lymphocytes and plasma cells (Supplementary Figure 1). This was seen in all muscles examined at 12 weeks of age, including the diaphragm, gastrocnemius, tibialis anterior (TA) and soleus (Supplementary Figure 1).

In those mice that survived to 30 weeks there was a noticeable hypertrophy of many fibres, (relative to the $FKRP_{MD}$). Individual (predominantly centrally nucleated) muscle fibres were occasionally surrounded by moderate to large amounts of compact, fibrous connective tissue and infiltrates of fat (Figure 5B, D, F, H), with some muscle fibres mineralised. At this later time point as in the $FKRP_{MD}$, there was minimal degeneration and necrosis although the inflammatory infiltration was more marked with infiltration by both neutrophils and macrophages in the diaphragm and gastrocnemius. Alizarin red staining showed a marked increase in the presence of large calcium deposits in the $FKRP_{MD}$LARGE relative to the $FKRP_{MD}$ mice (Figure 5 I-K). This was the case for all the muscles examined including the soleus. Alizarin red staining was also markedly worse in the diaphragm of the $FKRP_{MD}$LARGE mice, relative to the gastrocnemius at 12 weeks of age, a difference between these muscles that was not evident in the $FKRP_{MD}$ mice at this age.

$FKRP_{MD}$LARGE mice display an increase in IIH6 labelling and laminin deposition.

The glycosylation of α-DG as assessed by IIH6 immunolabelling was increased in all $FKRP_{MD}$LARGE muscles examined (diaphragm, gastrocnemius and soleus) relative to wild type mice (Figure 6 shows the gastrocnemius), and levels were comparable to that of the original

LARGE transgenic line (data not shown). Laminin α2 deposition at the basement membrane was increased in $FKRP_{MD}$LARGE mice relative to $FKRP_{MD}$ mice or wild type. Whilst regenerating fibres are known to express higher levels of basement membrane proteins, this increase extended beyond the clusters of small regenerating fibres. The number of fibres with sarcolemmal labelling for laminin α4 was also increased in the $FKRP_{MD}$LARGE mice relative to $FKRP_{MD}$ mice (Figure 6). Western blot analysis of muscle at 15-20 weeks showed that transgene expression in either the wild type or $FKRP_{MD}$ gives rise to an increase in laminin binding relative to wild type confirming that expression of the transgene led to the hyperglycosylation of α-DG. (Figure 7).

$FKRP_{MD}$LARGE mice show a physiological deficit relative to the $FKRP_{MD}$.

To compare the functional properties associated with a knock-down of Fkrp and how this was altered by the transgenic expression of LARGE we subjected the TA muscles of anaesthetized mice to a protocol of 10 eccentric (lengthening) contractions *in situ*. The protocol induced a 15% stretch during each of 10 maximal isometric contractions stimulated 2 minutes apart. Isometric tetanic force was measured prior to each stretch and expressed as a percentage of baseline isometric force. $FKRP_{MD}$ mice showed a similar resistance to eccentric contraction-induced injury to non-transgenic age-matched wild type controls (101.1% and 118.5% of baseline isometric force generated in the last contraction respectively) with only a small significant drop in force after contraction 10. However, $FKRP_{MD}$LARGE showed a significant reduction in their resistance (72.5% of baseline isometric force in the last contraction) relative to controls (118.1% of baseline isometric force in the last contraction) confirming that the worsened phenotype

evident on histological analysis had translated into a measurable physiological deficit (Figure 8). No significant differences were observed for the force-frequency relationship between any of the genotypes (data not shown).

Discussion.

LGMD2I is one of the most frequent autosomal recessive forms of LGMD and in the UK has been reported to make up 19.1% of the total LGMD group with a prevalence of 0.43/100 000 (52,53). Whilst the spectrum of disease in the dystroglycanopathies is wide, a significant proportion of patients are affected by relatively mild limb girdle muscular dystrophies without any central nervous system involvement, making it a good target for developing therapies. In order to gain insight into the disease pathogenesis of FKRP associated muscular dystrophy we previously generated a mouse model with a knock-down in Fkrp ($FKRP^{KD}$) that displayed a muscle eye brain phenotype. Whilst this model provided insight into the eye and brain phenotype (46), it died around the time of birth due to the severity of central nervous system involvement (47) and so was less useful for investigating the consequences of a reduction in *Fkrp* on postnatal muscle growth and function and for evaluating therapeutic strategies aimed at ameliorating the skeletal muscle phenotype.

In mammals, the cortex develops in an "inside out" manner, with migration of post mitotic neurons from the proliferative neuroepithelium (ventricular zone) to the cortical plate with each layer of post-mitotic neurons forming a more superficial layer than the last (54). The majority of neuronal migration is radial and is mediated by a scaffold of radial glial cells which extend from the ventricular zone (where their cell bodies are located) to the pial basement membrane. In the present report we addressed the perinatal lethality of the $FKRP^{KD}$ mouse by crossing it with a transgenic line in which Cre recombinase is expressed under control of the Sox-1 promoter to generate $FKRP^{KD}$/Sox1Cre mice which we refer to as $FKRP_{MD}$. The Sox1 promoter is known to drive expression in the neuroectoderm early during development (55) and this cross resulted in mice with wild type *Fkrp* transcript levels in the brain, a restoration of IIH6 immunolabelling at

the pial basement membrane and normal cortical architecture. Previous work has indicated that the restoration of glial rather than neuronal dystroglycan that plays a crucial role in forebrain development (56) and the restoration of the pial basement membrane and cortical organisation we observed was therefore consistent with these observations. As expected there remained an approximate 80% reduction of *Fkrp* transcript levels in the skeletal muscle which was associated with the loss of the laminin binding epitope IIH6 as previously reported for the $FKRP^{KD}$ line (21).

Despite the absence of the laminin binding IIH6 epitope from birth, the onset of muscle degeneration first occurred around 6 weeks of age in the majority of animals and by 12 weeks of age a marked pathology was evident in all $FKRP_{MD}$ mice. Western blot analysis confirmed that the IIH6 epitope was completely absent. This also resulted in the loss of laminin binding which is consistent with previous studies in the $LARGE_{myd,}$ $POMGnT_{null}$ and POMT1 mice that also report a reduction in the ability to bind laminin and develop a severe muscular dystrophy (57). In contrast, the muscle of a mouse model of FCMD (FCMD Hp^{-}) which contains a retrotransposal insertion in the mouse fukutin ortholog also failed to label with IIH6 but retained the ability to bind laminin albeit at 50% of the levels of controls. This mouse did not display any signs of a muscular dystrophy suggesting the existence of a threshold of glycosylation/laminin binding activity which was met in the FCMD mice (58) but not in the $LARGE_{myd}$ and $POMGnT_{null}$ or our $FKRP_{MD}$ mice.

All laminin isoforms with LN (Laminin N-terminal) domains play an integral role in basement membrane assembly by anchoring to cell surfaces, self polymerizing, and binding to nidogen and collagen IV (1). Laminin 211 is the major laminin isoform present in skeletal muscle and is reduced albeit not invariably in the skeletal muscle of dystroglycanopathy patients. A variation in

laminin α2 immunolabelling was also observed in $FKRP_{MD}$ mice at 12 weeks with higher levels of labelling apparent on small regenerating fibres whilst the majority of larger fibres either showed similar levels to that of controls or a reduction implying that a reduction in glycosylation may influence the turnover and/or stability of laminin 211 in the basement membrane of mature muscle fibres. Interestingly, there was no obvious reduction in perlecan in the $FKRP_{MD}$ mice when compared to wildtype mice (data not shown) despite previous reports of a reduction in the perlecan-binding activity and its mis-localisation in the brains of $Large_{myd}$ and dystroglycan null mice (59). However, it may be that laminin α2 rather than perlecan is the main binding partner for α-dystroglycan in muscle, indeed the binding properties of α-dystroglycan are known to be different in brain and muscle (60).

We observed a redistribution of laminin α4 in the $FKRP_{MD}$ muscle which has also been reported in laminin α2 deficient mice (49). Laminin α4 normally locates to the basement membranes of blood vessels, the endoneurium of the intramuscular nerves, and the neuromuscular junction in neonatal skeletal muscle (49). In adult muscle it locates to the perineurium of adult peripheral nerve. Laminin α4 lacks the ability to self-polymerise (61) due to the absence of the N-terminal domain, and consequently has the potential to interfere with basal lamina formation where laminin α2 is a major component. It also displays a low affinity binding for α-dystroglycan, sulfatides, and integrins α6β1 and α7β1 (62), properties that suggest it may be an ineffective substitute for laminin α2. However, the up-regulation of laminin α4, which is also a feature of laminin α2 deficiency may nonetheless be of functional significance since recent work in the zebra fish suggests that its up-regulation in damaged fibres contributes to fibre survival (63). Interestingly, an increase in the number of fibres with laminin α4 at the sarcolemma was

observed to increase with age in the $FKRP_{MD,}$ implying that a similar scenario may apply to our mice.

LARGE is a bifunctional glycosyltransferase thought to be essential for conferring α-dystroglycan with the ability to bind laminin. The over-expression of LARGE has been shown to increase-DG glycosylation in both wild type and cells from dystroglycanopathy patients, irrespective of their primary gene defect (44). Whilst more recent work now demonstrates that the ability of LARGE to hyperglycosylate α-dystroglycan is dependent on the availability of O-mannosyl phosphate acceptor sites and correlates with the severity of the clinical phenotype (32); this strategy is still considered as being potentially useful for a wide range of patients. Furthermore, the viral delivery of LARGE to skeletal muscle in animal models of dystroglycanopathy has been reported to have identical effects *in vivo*, suggesting that the restoration of functional glycosylation may be a valuable therapeutic approach in this group of disorders (44,58). We previously generated a number of transgenic lines overexpressing LARGE (45) and have now crossed one of these lines with our $FKRP_{MD}$ mice. On a wild type background the up-regulation of LARGE is not associated with any muscle fibre degeneration but there is a mild loss of force upon eccentric exercise in the TA of older animals (45), suggesting some form of subtle abnormality in basement membrane turnover occurs over an extended time period (64). However, when we crossed the $FKRP_{MD}$ line with the LARGE transgenic the pathology of the $FKRP_{MD}$ phenotype worsened with an increased percentage of centrally nucleated fibres in all the muscles examined relative to the $FKRP_{MD}$. Additional features such as calcium deposits, evidence of fibrosis and replacement of muscle fibres by adipocytes were also more evident in the presence of the LARGE transgene and the soleus muscle which was largely spared in the $FKRP_{MD}$ showed clear evidence of pathology in the

presence of the LARGE transgene. The promoter driving the LARGE transgene expresses at equivalent levels in both slow and fast fibres therefore these observations reflect a differential pattern of muscle involvement in the $FKRP_{MD}$.

Whilst there are several differences between our study and those which previously indicated that the up-regulation of LARGE would be beneficial such as the method of gene delivery, promoter used to drive expression, and animal model, the most significant difference relates to the timing over which LARGE was overexpressed, and the duration of the period of observation. For example, LARGE was introduced into the $Large^{myd}$, $POMGnT1^{-/-}$ and FCMD Hp^{-} mice via AAV vectors at postnatal data 2-4 and then evaluated 4 weeks later (44,58). More recently Yu et al (50) injected AAV9-LARGE (expression driven by the β-actin promoter) via into the newborn heart and adult tail vein of POMGnT1null and $LARGE_{myd}$ mice and looked at expression at 2 months (newborn injections) and 1 month (adult tail vein injections) later. These authors noted significant improvement of the histological appearance of the muscle and amelioration of the phenotype. Barresi et al (44) also administered *LARGE* to older $Large^{myd}$ mice, aged between 12 days and 5 weeks but reported that this was associated with muscle inflammation and a loss of IIH6 immunolabelling (*LARGE* up-regulation) as the mice aged.

These findings are in marked contrast to those of our own using transgenesis and would seem to imply that the time period over which LARGE is up-regulated and/or the stage of development that it is initiated determine the outcome. Here we have shown that despite the restoration of laminin binding in the $FKRP_{MD}$LARGE there was a significant loss of force in response to eccentric exercise which was not seen in the $FKRP_{MD}$ confirming that the overexpression of LARGE had worsened the phenotype as indicated by the histological analyses. The reasons for this remain unclear however, it is possible that on a disease background, specifically one in

which the glycosylation of α-dystroglycan is reduced or absent, LARGE not only targets additional proteins, the hyperglycosylation of which is detrimental to muscle, but may also lead to a functionally relevant alteration in the glycosylation pattern of α-DG itself. In support of these two concepts it has previously been shown that LARGE over-expression in α-DG-deficient cells leads to the expression of the IIH6 epitope (65) and that LARGE acts not only on the *O*-mannose glycans but also complex *N*-glycans and mucin *O*-GalNAc (N-Acetylgalactosamine) glycans of α-DG (66). Previous work in ES (Embryonic Stem) cells indicates that neither integrin nor dystroglycan are individually required for assembly of the basement membrane but they do regulate both their own expression and that of other basement membrane components (67). It is therefore possible that this system of regulation is perturbed by an altered pattern of α-dystroglycan glycosylation, perhaps by compromising the turnover process of the basement membrane. Finally it should also be noted that as a consequence of the transgenic approach adopted here, the $FKRP_{MD}$LARGE mice are on a different background to the $FKRP_{MD}$ mouse. However, we consider this an unlikely cause of the worsened phenotype since histological evaluation of wildtype *LARGE* overexpressing mice on this new genetic background failed to identify any evidence of a dystrophic pathology.

Our work reports the first transgenic up-regulation of LARGE on a disease background. Whilst the onset of disease in the $FKRP_{MD}$LARGE mice was not markedly different to the $FKRP_{MD,}$ suggesting that the over-expression of LARGE did not adversely affect the early stages of muscle development, several aspects of the disease process were significantly worse. The reasons for this are unclear at the present time, but emphasise the value of determining the effect of overexpression on a disease background over an extended period and suggest that any therapeutic approach involving LARGE up-regulation requires careful management.

Materials and Methods.

Generation of FKRP-Neo$^{Tyr307Asn+/+Sox1Cre}$ *mice (FKRP$_{MD}$)*

All animal experiments were carried out under license from the Home Office (UK) in accordance with The Animals (Scientific Procedures) Act 1986 and were approved by Royal Veterinary College ethical committee. The FKRP-Neo$^{Tyr307Asn+/+}$ (FKRP^{KD}) mouse colony (47) was crossed with a second transgenic line expressing *Cre recombinase* throughout the developing neural tube under the Sox1 promoter (a kind gift from Professor Liz Robertson, Sir William Dunn School of Pathology, Oxford U.K.) (68). Briefly FKRP-Neo$^{Tyr307Asn+/-}$ were crossed with Sox1Cre mice and the resulting FKRP-Neo$^{Tyr307Asn+/-Sox1Cre}$ mice were bred with FKRP-Neo$^{Tyr307Asn+/-}$ mice which generated FKRP-Neo$^{Tyr307Asn+/+Sox1Cre}$ mice, referred to as FKRP$_{MD}$. Thus, the first cross introduced the Sox1Cre transgene into the background of the FKRP^{KD} colony, while the second first generation cross generated the FKRP$_{MD}$ offspring at a frequency of approximately 7%. FKRP$_{MD}$ mice are fertile and breeding them with FKRP-Neo$^{Tyr307Asn+/-}$ mice increased the incidence of mice to approximately 14%. However, pairing two FKRP-Neo$^{Tyr307Asn+/-Sox1Cre}$ heterozygote mice resulted in high pre-weaning losses, with a number of offspring suffering from hydrocephalus.

Generation of FKRP-Neo$^{Tyr307Asn+/+Sox1CreLARGE}$ (FKRP$_{MD}$LARGE).

The following strategy was used to generate FKRP$_{MD}$, FKRP^{KD} and FKRP$_{MD}$ LARGE mice. FKRP-Neo$^{Tyr307Asn+/-Sox1Cre}$ mice were crossed with a transgenic mouse line (LV5) overexpressing human LARGE (45) to introduce the LARGE transgene into the background of the FKRP$_{MD}$

mice. FKRP-Neo$^{Tyr307Asn+/-Sox1CreLARGE}$ mice were then crossed with FKRP-Neo$^{Tyr307Asn+/-}$ to generate FKRP-Neo$^{Tyr307Asn+/+Sox1CreLARGE}$ mice, henceforth referred to as $FKRP_{MD}$LARGE mice.

Genotyping $FKRP_{MD}$ offspring

Offspring were genotyped by PCR analysis using either ear or tail biopsies. Genomic DNA from the mouse tissue was prepared by digestion in Direct PCR Lysis Ear or Tail buffer (Eurogentec) respectively containing 0.2mg/mL Proteinase K (Roche Diagnostics) at 55°C overnight. The Proteinase K was heat inactivated at 85°C for 30 minutes and PCR was performed with the crude DNA lysate using Biomix Red PCR Kit (Bioline) with the following multiplex primers for Fkrp (FKRP-F: CTAGGAGGTTGAGGATGATGG, FKRP-R: GTTGTGCTTAAACCACCTTC, and FKRP-NeoF:GGTGGATTAGATAAATGC), Cre recombinase (Cre-F: CCCAGGCTAAGTGCCTTCTC and Cre-R: CCAGGTTCGTTCACTCATGG) and *LARGE* (Large-F:TAATACGACTCACTATAGGG Large-R: AAGGTTCTCGCTGTCTCC).

Histology and Immunocytochemistry

For standard histochemistry, newborn mice were collected and fixed in Bouins (Sigma) and transferred to 70% ethanol prior to processing and embedding in paraffin wax. Samples were serially sectioned at 5µm, with sections collected onto charged slides (Superfrost Plus, VWR), rehydrated and stained with haematoxylin and eosin, using standard methods. For immunohistochemistry sections were deparaffinised and rehydrated prior to incubation with anti α-dystroglycan (IIH6, Millipore) diluted in phosphate buffered saline containing 0.05% tween 20

(Sigma) for 1 hour at room temperature. Visualisation of the IIH6 was performed using the Envision system (DAKO).

Alternatively, muscle and brain samples were frozen in isopentane cooled in liquid nitrogen and 10µm sections were cut using a Bright Cryostat. These were then stained with haematoxylin and eosin using standard methods to evaluate general tissue pathology and calculate the percentage of centrally located nuclei. Additionally, Alizarin Red staining was performed to identify calcium deposits. Images of newborn mouse heads and muscles stained with these histochemical methods were digitally captured using a DM4000B upright microscope (Leica, Germany) interfaced with a DC500 colour camera (Leica) using the Leica Application Suite (Leica Microsystems) software provided and compiled into figures using Photoshop CS4 or CS5 (Adobe, U.S.A.). Figures were compiled using Photoshop CS (Adobe, U.S.A.). All observations are based on a minimum of n=3 (of either sex), and representative images are shown. Counts of centrally nucleated muscle fibres were made across an entire section from each individual mouse muscle (n=3 for wildtype, $FKRP_{MD}$ and $FKRP_{MD}$LARGE mice at 12 and 30 weeks of age) randomly chosen from the mid-region of each muscle, with the total number of fibres counted numbering approximately 500, 1500 and 2000 muscle fibres in the soleus, diaphragm and gastrocnemius respectively. The incidence of split muscle fibres was based on counts of approximately 2000 muscle fibres across an entire section from the mid-belly region of 12 week old wildtype, $FKRP_{MD}$ and $FKRP_{MD}$LARGE gastrocnemius (n=3 for all genotypes).

For immunohistochemical analysis of muscle and brain, cryosections were immunolabelled with rabbit anti pan-laminin (Sigma-Aldrich), rat anti laminin α2 (4H8, Abcam), goat anti-laminin α4 (R&D Systems) and the IIH6 antibody against a glycosylated epitope of α-DG (Millipore). This was followed by anti-rat/rabbit/goat tagged with Alexa 488 or 594 (Molecular Probes) for 30 minutes, with the exception of IIH6 which was labelled with anti-IgM biotinylated antibody (30 minutes) followed by streptavidin conjugated with Alexa 488/594 (30 minutes). Nuclei were stained with Hoechst 33342 (Sigma-Aldrich). All dilutions and washes were made in phosphate buffered saline. Sections were mounted in aqueous mountant and viewed with epifluorescence using a DM4000B upright microscope (Leica, Germany). Images were digitally captured with an Axiovision mRM monochrome camera, (Zeiss, UK) and compiled using Photoshop CS (Adobe, U.S.A.). Where direct comparisons have been made, fluorescent images were captured with equal exposure and have had equal scaling applied. All observations are based on a minimum of n=3 (of either sex), representative images are shown.

qRTPCR analysis.

Brain and muscle were dissected out and homogenised with liquid nitrogen using a mortar and pestle and the lysate passed through a QiaShredder®(Qiagen). RNA was isolated from the homogenised tissue using an RNeasy®kit (Qiagen) and for muscle RNeasy® Fibrous Tissue Kit (Qiagen) eluted with 30µl RNase free H_2O. 1µg of RNA was reverse transcribed with Superscript®III Platinum for qRT-PCR kit (Invitrogen). qRT-PCR was performed on a 7500 FAST Real-Time PCR system (Applied Biosystems) using aFAM(tm) reporter dye system. For each reaction 0.8µl of cDNA was used as template in a PCR mix consisting of 1µl of primer mix,

10 ul TaqMan Universal PCR Mastermix (Applied Biosystems) and 8.2µl H_2O. The primers for the gene expression assays were sourced commercially from Applied Biosystems (*FKRP* Mm00557870_mL, *GAPDH* Mm99999915_gL). Each experiment represents a minimum of n=4 (of either sex) and all reactions were performed in triplicate.

Western blotting and laminin overlay assay.

Cell proteins were extracted in sample buffer consisting of 75 mM Tris–HCl, 1% SDS, 2-mercaptoethanol, plus a cocktail of protease inhibitors (Roche). 30µg of soluble proteins were resolved using a NuPage Pre-cast gel (3–8% Tris-acetate; Invitrogen, USA) and then transferred electrophoretically to nitrocellulose membrane (Hybond-PVDF, GE Healthcare, UK. Nitrocellulose strips were blocked in 5% dried non fat milk in phosphate-buffered saline buffer, and then probed with the primary antibodies: anti mouse α-DG IIH6 (Millipore UK,cat,05-593) anti-mouse β-DG (Vector Labs, UK), at room temperature for 1 hour. After washing they were incubated with the appropriate HRP conjugated secondary antibody for one hour: anti-mouse-IgM or anti-mouse-IgG (both from Jackson ImmunoResearch). After washing, membranes were visualized using chemiluminescence (ECL+Plus, GE Healthcare, UK). For the laminin overlay assay, nitrocellulose membranes were blocked for 1 hour in laminin binding buffer (LBB: 10 mM triethanolamine, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) containing 5% non-fat dry milk followed by incubation of mouse Engelbreth-Holm-Swarm laminin (Invitrogen,USA) overnight at 4°C in LBB. Membranes were washed and incubated with anti rabbit laminin (Sigma, USA) followed by HRP-anti rabbit IgG (Jackson ImmunoResearch, USA). Blots were visualized using chemiluminescence (ECL+Plus, GE Healthcare,UK).

In situ/vivo muscle electrophysiology.

Mice were surgically prepared as described previously (69,70). Contractions were stimulated in the TA muscle *in situ* via the surgically isolated common peroneal nerve. The TA muscle underwent a series of 5 submaximal isometric contractions as a warm up. Isometric force measurements were made over a range of stimulating frequencies and maximum isometric tetanic force (P_0) was determined from the plateau of the force–frequency curve (20). After completing the final isometric contraction the optimum length (Lo) was measured with digital callipers and the muscle was allowed to rest for 5 min before the eccentric contraction protocol was initiated. A tetanic contraction was induced using a stimulus of 120 Hz (the frequency that resulted in P_0 without causing fatigue during the contraction) for 700 ms. During the last 200 ms of this contraction, the muscle was stretched by 15% of L_o at a velocity of 0.75 $L_o s^{-1}$ and relaxed at $-0.75 L_o s^{-1}$. The isometric tension recorded prior to the first stretch was used as a baseline. The muscle was then subjected to 10 eccentric contractions each separated by a 2 min rest period to avoid the confounding effect of muscle fatigue. The isometric tension prior to each stretch was recorded and expressed as a percentage of the baseline tension (69). The mouse was then euthanized and the muscle was carefully removed and weighed.

Statistical analyses.

Body weights were analysed with a Linear Mixed Effects Model and central nucleation counts were analysed with a General Estimating Equations model, both performed using SPSS Statistics (IBM, U.S.A). The incidence of split fibres was analysed with a one-tailed Mann-Whitney test,

Muscle physiology data were analysed using a Repeated Measures One-way ANOVA with Tukey's *post-hoc* comparison.

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Figure Legends.

Figure 1 Real time gene expression analysis of FKRP in brain and muscle and histological evaluation of $FKRP_{MD}$ brain. (A) IIH6 immunolabelling of haematoxylin stained coronal sections of $FKRP^{KD}$ heterozygote (A), $FKRP^{KD}$ (B) and $FKRP_{MD}$ (C) brains at P0. The cortical disorganisation evident in the $FKRP^{KD}$ is no longer evident in the $FKRP_{MD}$ which now reflects that seen in the $FKRP^{KD}$ heterozygote control. IIH6 immunolabelling can be seen at the pial basement membrane of the $FKRP^{KD}$ heterozygote and $FKRP_{MD}$ but not the $FKRP^{KD}$. Immunolabelling of the pial basement membrane with a pan laminin antibody (D-F) shows the

disorganisation at the intrahemispheric fissure in the $FKRP^{KD}$, whereas in the $FKRP_{MD}$ (F) organisation is comparable to that of heterozygote $FKRP^{KD}$ control (D). Scale bar in images A-D are 50µm. Relative expression of FKRP (G,H). Taq man (Applied Biosystems) RT-PCR probes were used to measure relative FKRP mRNA expression in brain and skeletal muscle of $FKRP_{MD}$ mice compared to age-matched wild type controls. Expression levels were normalised against endogenous GAPDH mRNA expression. The percentage knock-down evident in the $FKRP^{KD}$ has been maintained in the $FKRP_{MD}$ as a comparison with the levels in the $FKRP^{KD}$ brain at E15.5 show. Error bars represent SEM (n = 4). All samples were analysed as triplicate data sets. * P value = < 0.05 (two tailed t-test) ** P value < 0.005. Error bars represent ± SEM (n = 4).

Figure 2. Body weight and histological analysis of muscle at 6, 12 and 20 weeks. (A) Mean body weights ± SEM of male and female wildtype (white) and $FKRP_{MD}$ (patterned) at 6 weeks (male WT n=8, male $FKRP_{MD}$ n=29; female WT n=14, female $FKRP_{MD}$ n=12), 12 (male WT n=10, male $FKRP_{MD}$ n=17; female WT n=14, female $FKRP_{MD}$ n=24) and 20 weeks of age (male WT n=7, male $FKRP_{MD}$ n=13; female WT n=6, female $FKRP_{MD}$ n=12). Data were analysed with a Linear Mixed Effects Model performed using SPSS Statistics (IBM Corporation, U.S.A). This model showed that age was a significant factor affecting mouse body weight in both male and female mice, but genotype was not a significant factor affecting male body weight (p=0.245) although it was with respect to female body weight, with significance shown as follows: *0.01 $\leq p<0.05$, ** $0.001 \leq p<0.01$. (B-K) Digital images of haematoxylin and eosin stained cryosections from wildtype (B,D,F,H,J) and $FKRP_{MD}$ (C,E,G,I,K) gastrocnemius (B-E, H-I) diaphragm (F-G) and quadriceps (J-K) at 6 (B-C), 12 (D-E) and 30 (F-K) weeks of age. $FKRP_{MD}$ muscle shows evidence of inflammatory infiltrates and muscle fibre degeneration at 6 weeks of age (C), with

muscle fibre regeneration seen at 12 weeks of age (D). At 30 weeks of age, centrally nucleated muscle fibres indicating previous regeneration cycles are seen in the $FKRP_{MD}$ diaphragm (G) and gastrocnemius (I). Counts of central nucleation were carried out on transverse 10µm muscle cryosections of wildtype and $FKRP_{MD}$ diaphragm, gastrocnemius and soleus at 12 and 30 weeks of age - the muscle fibres with central nuclei were counted and expressed as a percentage of the total number counted (approximately 500 for the soleus, 1500 for the diaphragm and 2000 for the gastrocnemius). The histogram in L shows the mean percentage of centrally nucleated muscle fibres ± SEM of wildtype (white) and $FKRP_{MD}$ (patterned) in the diaphragm, gastrocnemius and soleus at 12 and 30 weeks of age as indicated. This data was analysed using a General Estimating Equations Model performed using SPSS statistics which showed that age, muscle and genotype were significant factors affecting the percentage of centrally nucleated muscle fibres. The output of this model is shown as a multiple line graph in M. Scale Bars represent 50µm in B-K.

Figure 3. Immunolabelling and Western blot analysis of $FKRP_{MD}$ muscle.

Transverse 10µm cryosections from 12 week old wildtype (A,C,E) and $FKRP_{MD}$ (B,D,F) mouse triceps were labelled with Hoescht 33342 to visualise the nuclei (A-B) and an antibody against laminin α2 (C-D), with a colour composite shown in E-F. Laminin α2 immunolabelling was variable in the $FKRP_{MD}$ with small clusters of fibres showing an increase relative controls whilst the majority of other fibres displayed either similar levels to controls or a slight decrease. Scale bar represents 50µm. Western Blotting analysis (G) of quadriceps from wildtype and $FKRP_{MD}$ mice shows IIH6 labelling, present in WT mice (lanes 3,4 and 5) was absent in $FKRP_{MD}$ mice

(lanes 1,2,6 and 7). Each lane contains an extract from individual animals. β-DG (43kDa) which also acts as a loading control is shown to be unchanged in the $FKRP_{MD}$. The images shown in C and D) are shown using a look up table from Image J to emphasise the variation in intensity of laminin α2 immunolabelling across the section. J shows the scale used.

Figure 4. Laminin α4 immunolabelling of $FKRP_{MD}$ and wild type controls.

Transverse 10µm cryosections from 12 week (A-B) and 30 week (C-F) old wildtype (A,C,E) and $FKRP_{MD}$ (B, D, F) diaphragm (A-D) and rectus femoris (E,F) were immunolabelled with an antibody against laminin α4. Immunolabelling with this antibody was confined to the capillaries and nerves of wild type muscle, whereas immmunolabelling was observed at the basement membrane of small diameter fibres in the $FKRP_{MD}$ diaphragm and rectus femoris at 12 and 30 weeks of age. By 30 weeks of age laminin α4 was also evident at the basement membrane of larger diameter fibres. Scale bar represents 50µm.

Figure 5. $FKRP_{MD}$ LARGE histology. 10µm cryosections from $FKRP_{MD}$ (A,C,E,G) and $FKRP_{MD}$LARGE (B,D,F,H) diaphragm (A-B), soleus (C-D) gastrocnemius (E-F), and tibialis anterior (G-H) at 30 weeks of age stained with haematoxylin and eosin (A-H). All muscles showed a marked variation in fibre size, the presence of degenerative fibres infiltrated with macrophages and an increase in centrally nucleated fibres relative to the $FKRP_{MD.}$ I-K show Alizarin Red labelling of wildtype (I), $FKRP_{MD}$ (J) and $FKRP_{MD}$LARGE (K) diaphragm, with a greater incidence of calcium deposits (red) observed in the $FKRP_{MD}$LARGE relative to $FKRP_{MD}$. (L) is an image of a 30 week old $FKRP_{MD}$LARGE gastrocnemius, showing an example of split

muscle fibres. In images A-H and L scale bar represents 50µm. In I-K scale bar represents 100µm.

Figure 6. Immunolabelling showing a decrease in IIH6, a reduction in laminin α2 and increase in laminin α4.

Transverse 10µm cryosections of the gastrocnemius (A-D) and diaphragm (E,F) of wildtype (A,C,E) and $FKRP_{MD}$LARGE (B,D,F) mice, immunolabelled with IIH6 (antibody against glycosylated α-DG) (A,B), laminin α2 (C,D) and laminin α4 (E,F). IIH6 and laminin α2 immunolabelling was increased in the $FKRP_{MD}$LARGE mice relative to wildtype mice. Laminin α4 can be seen at the muscle fibre basement membrane of $FKRP_{MD}$LARGE mice whilst it is confined to the capillaries of wild type mice. Scale bar represents 50µm.

Figure 7. Ligand binding and quantitative analysis of split fibres and central nucleation. Western blot of α and β DG of WT, $FKRP_{MD,}$ $FKRP_{MD}$LARGE (A) and laminin overlay assay of wild type, wild type overexpressing LARGE, $FKRP_{MD}$ and $FKRP_{MD}$LARGE (B). As can be seen transgene expression in either the wild type or $FKRP_{MD}$ gives rise to an increase in laminin binding relative to wild type confirming that expression of the transgene led to the hyperglycosylation of α-DG. The number of split fibres in the gastrocnemius of 12 week old wildtype (n=3), $FKRP_{MD}$ (n=3) and $FKRP_{MD}$LARGE (n=3) was quantified and is shown in a histogram (C). The results of a one-tailed Mann-Whitney test are shown * $0.01 \leq p < 0.05$, illustrating a significant increase with the overexpression of LARGE on the $FKRP_{MD}$ background. (D) Counts of central nucleation were carried out on transverse 10µm cryosections

of 12 and 30 week wildtype, $FKRP_{MD}$ and $FKRP_{MD}$LARGE diaphragm, gastrocnemius and soleus. Muscle fibres with central nuclei were counted and expressed as a percentage of the total number counted (approximately 500 for the soleus, 1500 for the diaphragm and 2000 for the gastrocnemius). The results of the Generalised Estimating Equations Statistical test are shown as a multiple line graph. This model shows age, muscle and genotype are significant interacting factors on the percentage of centrally nucleated muscle fibres. The $FKRP_{MD}$ data shown in Figure 2 has been included to facilitate comparisons between the $FKRP_{MD}$ and $FKRP_{MD}$LARGE models.

Figure 8. *In vivo* assessment of muscle force production following eccentric contractions. Tibialis anterior muscles from 20 to 22 week old female $FKRP_{MD}$ (n=8), $FKRP_{MD}$LARGE (n=5) and wild-type (non-transgenic) mice (n=7) underwent a series of 10 eccentric contractions *in situ* utilising a stretch of 15% of optimum muscle length. The force produced by $FKRP_{MD}$ mice was not significantly different to that of wild-type animals and showed no significant drop from baseline. However, $FKRP_{MD}$LARGE mice showed a significant drop in force compared to the value at contraction 2 ($P<0.05$ for contraction 8, $P<0.01$ for contraction 9 and $P<0.001$ for contraction 10). $FKRP_{MD}$LARGE mice were significantly weaker than wild-type at contractions 7 to 10 (blue asterix symbols, * $P<0.05$, ** $P<0.01$) and weaker than $FKRP_{MD}$ mice (red asterix symbol, * $P<0.05$) after the tenth eccentric contraction. The mean force produced after 10 eccentric contractions was 118.46%, 105.83%, and 72.47 % of baseline for WT, $FKRP_{MD}$, and $FKRP_{MD}$LARGE mice respectively. Values are presented as mean and S.E.M and data are analysed using a Repeated Measures One way ANOVA with Tukey's *post-hoc* comparison.

Supplementary Figure 1

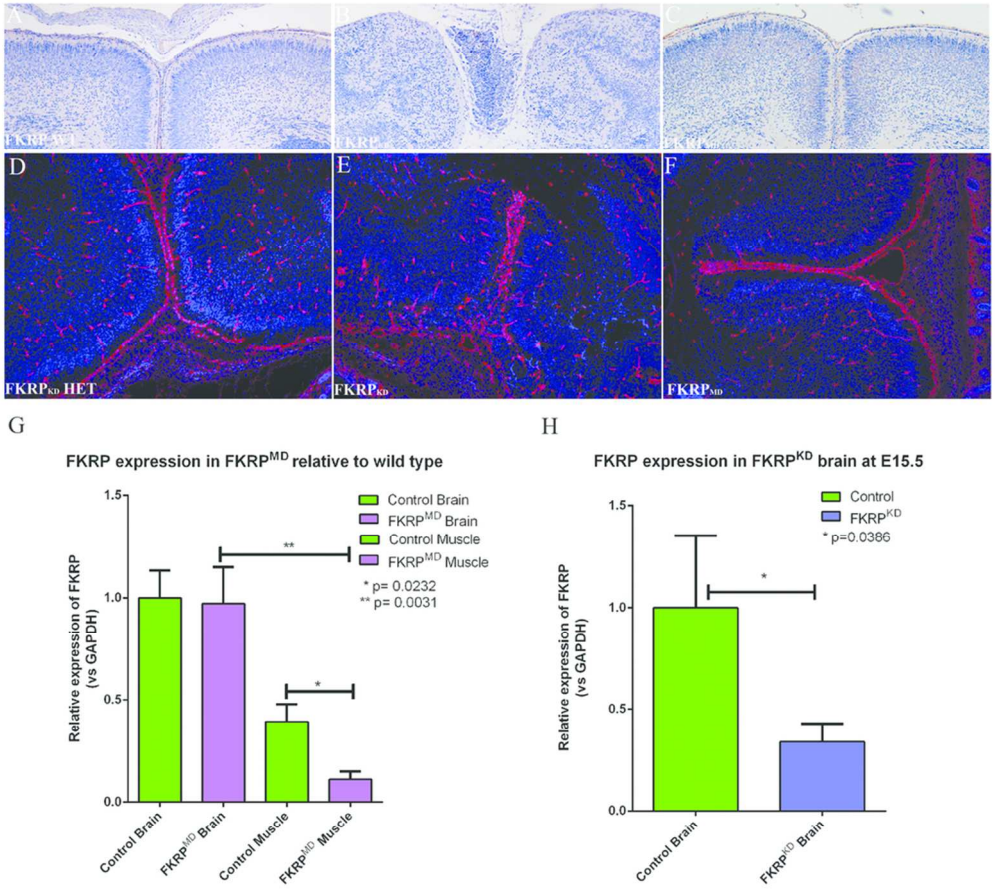
10µm cryosections from $FKRP_{MD}$ (A,C,E,G) and $FKRP_{MD}$LARGE (B,D,F,H) diaphragm (A-B), soleus (C-D) gastrocnemius (E-F), and tibialis anterior (G-H) at 12 weeks of age stained with haematoxylin and eosin. All muscles showed a marked variation in fibre size and an increase in centrally nucleated fibres relative to the $FKRP_{MD}$

Abbreviations

α-DG	α-dystroglycan
AAV	Adeno-Associated Virus
B3GNT1	Beta-1,3-N-Acetylglucosaminyltransferase 1
CMD	Congenital Muscular Dystrophy
DG	Dystroglycan
DOLK	Dolichol Kinase
DPM2	Dolichyl-Phosphate Mannosyltransferase Polypeptide 2
DPM3	Dolichyl-Phosphate Mannosyltransferase Polypeptide 3
ES	Embryonic Stem

FCMD	Fukuyama Congenital Muscular Dystrophy
FKT	Fukutin
FKRP	Fukutin Related Protein
$FKRP^{KD}$	FKRP knock down
$FKRP_{MD}$	FKRP muscular dystrophy
GalNAc	N-Acetylgalactosamine
GMPPB	GDP-mannose pyrophosphorylase B
GTDC2	Glycosyltransferase-Like Domain Containing 2
ISPD	Isoprenoid Synthase Domain Containing
LARGE	Like-acetylglucosaminyltransferase
LG	Laminin globular
LN	Laminin N-terminal
LGMD	Limb girdle muscular dystrophy
MDC1D	Congenital Muscular Dystrophy Type 1D
MEB	Muscle Eye Brain Disease
POMT1	Protein O-mannosyl-transferase 1
POMT2	Protein O-mannosyl-transferase 2

POMGnT1	Protein O-mannose beta-1,2-N-acetylglucosaminyltransferase
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
Sox1	Sex determining region Y-box 1
SGK196	Sugen Kinase 196
TA	Tibialis Anterior
TMEM5	Transmembrane Protein 5
WWS	Walker-Warburg Syndrome



Real time gene expression analysis of FKRP in brain and muscle and histological evaluation of FKRPMD brain.
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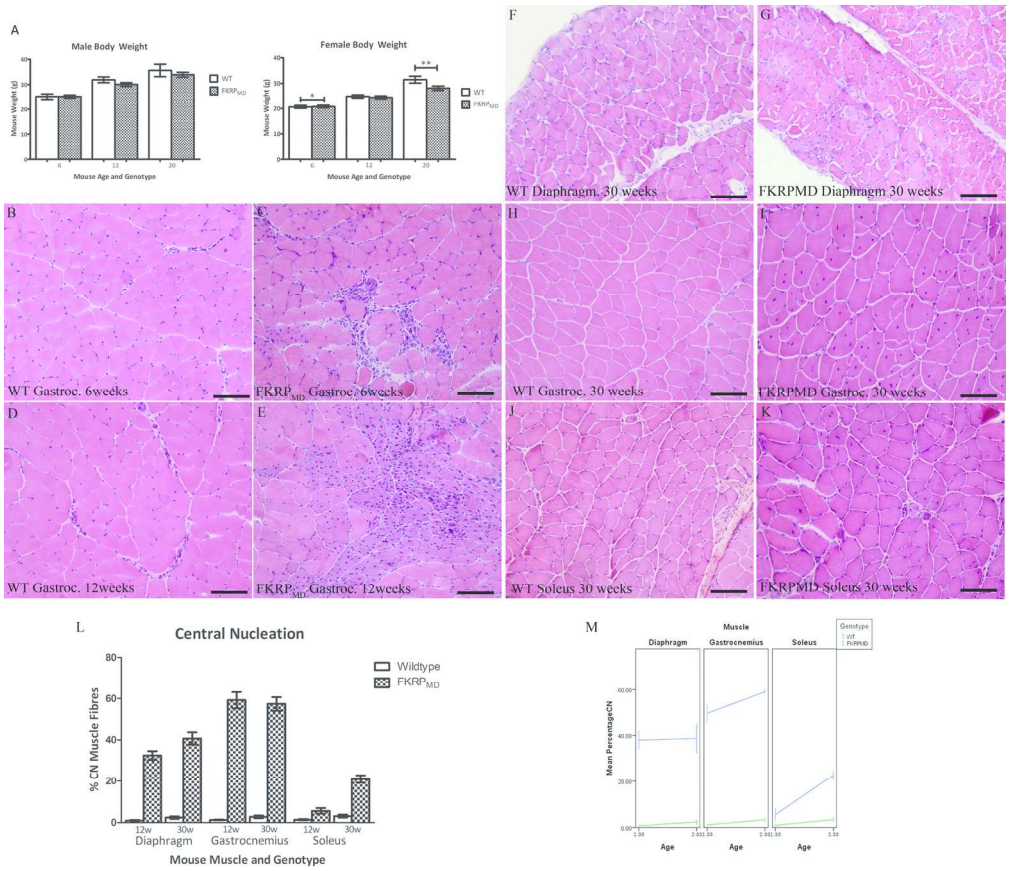

Body weight and histological analysis of muscle at 6, 12 and 20 weeks.
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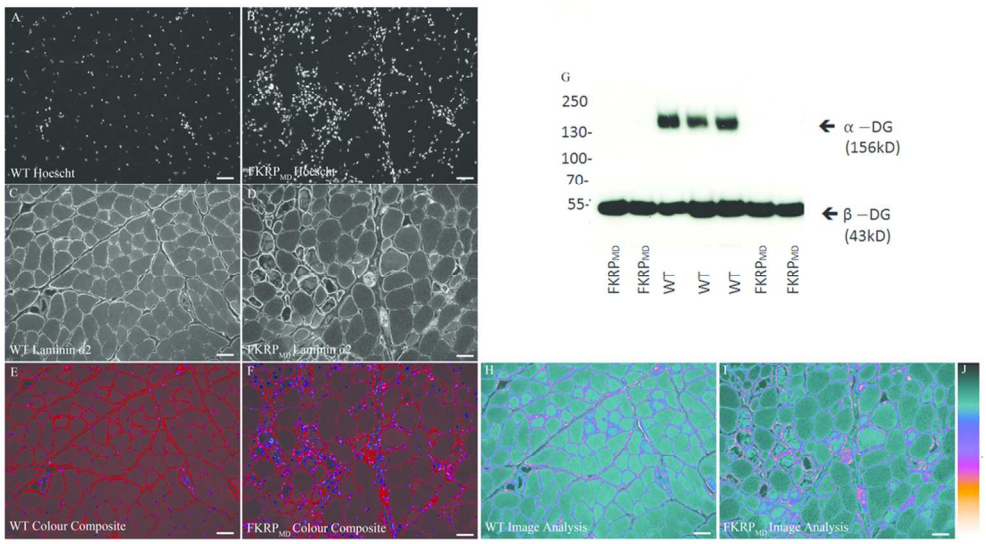

Immunolabelling and Western blot analysis of FKRPMD muscle.
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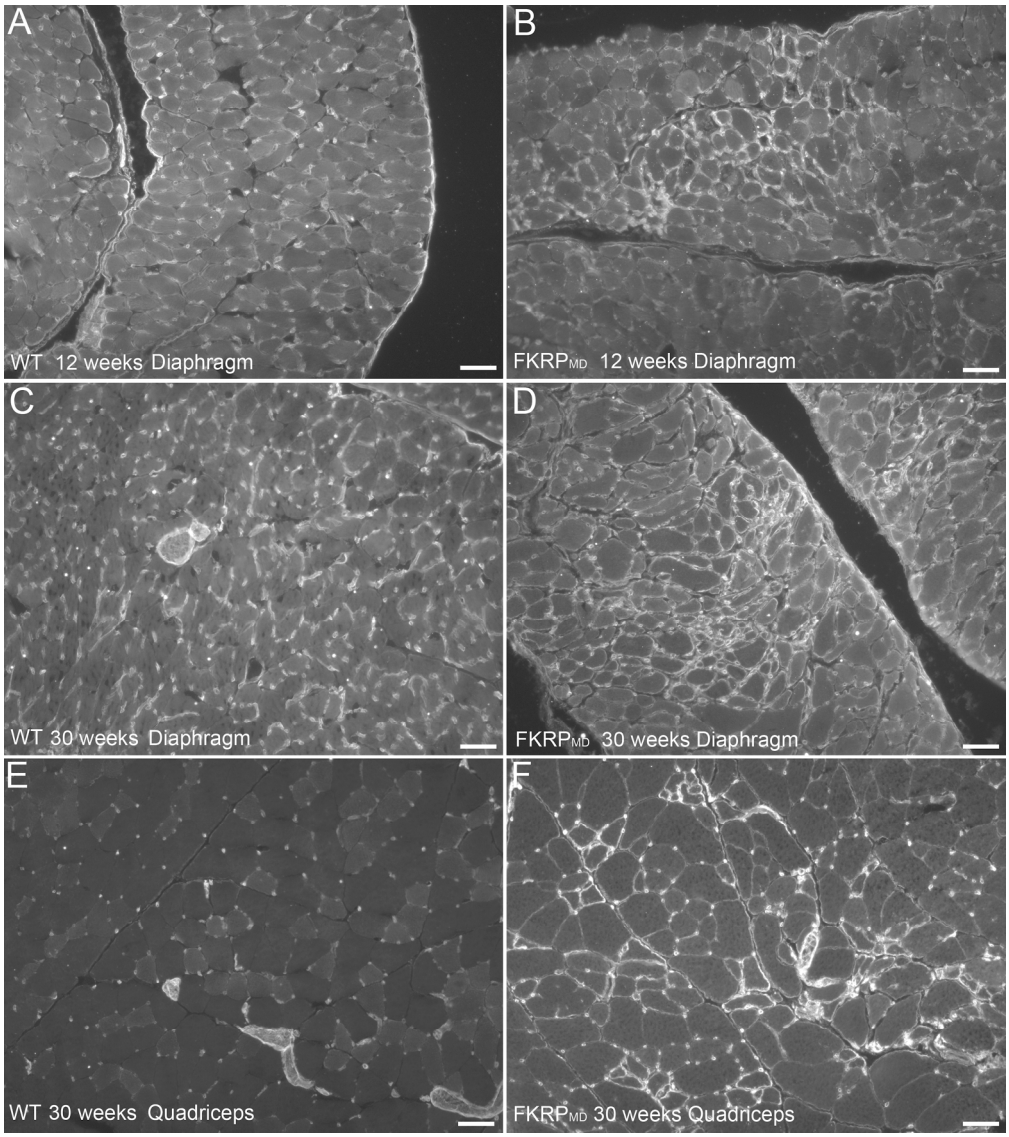

Laminin α4 immunolabelling of FKRPMD and wild type controls.
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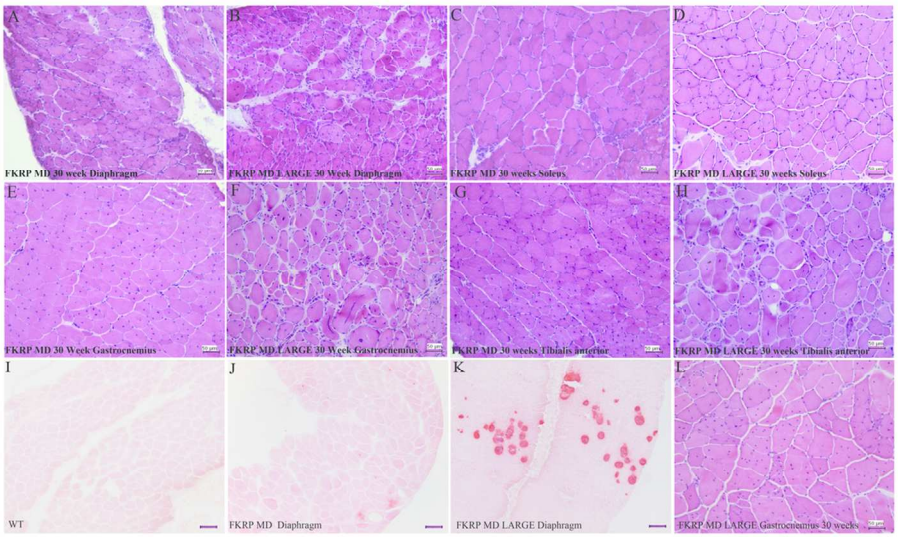

FKRPMD LARGE histology.
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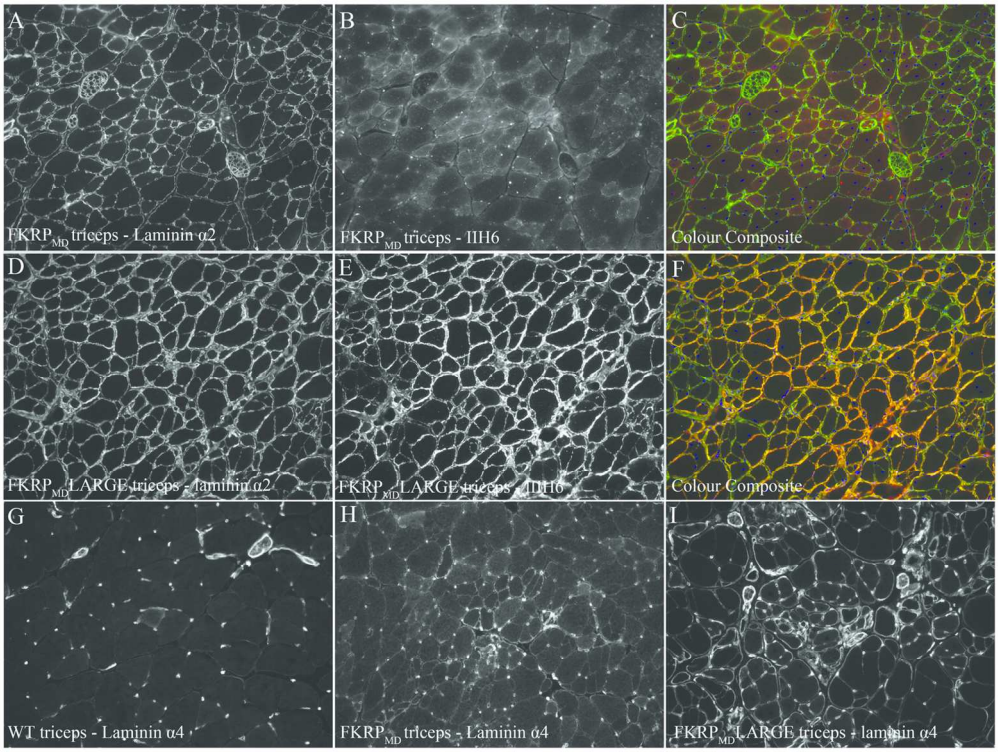
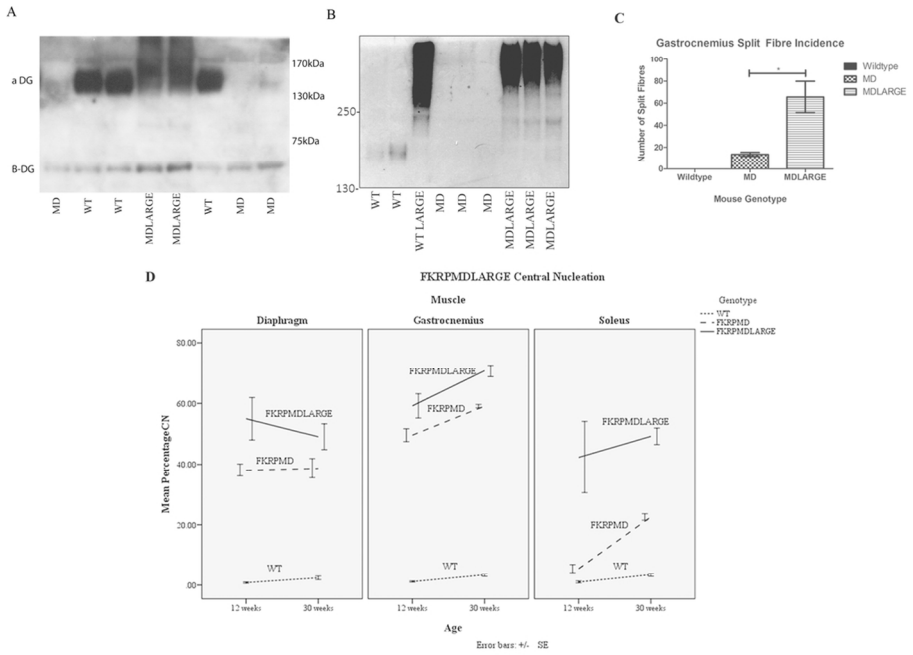
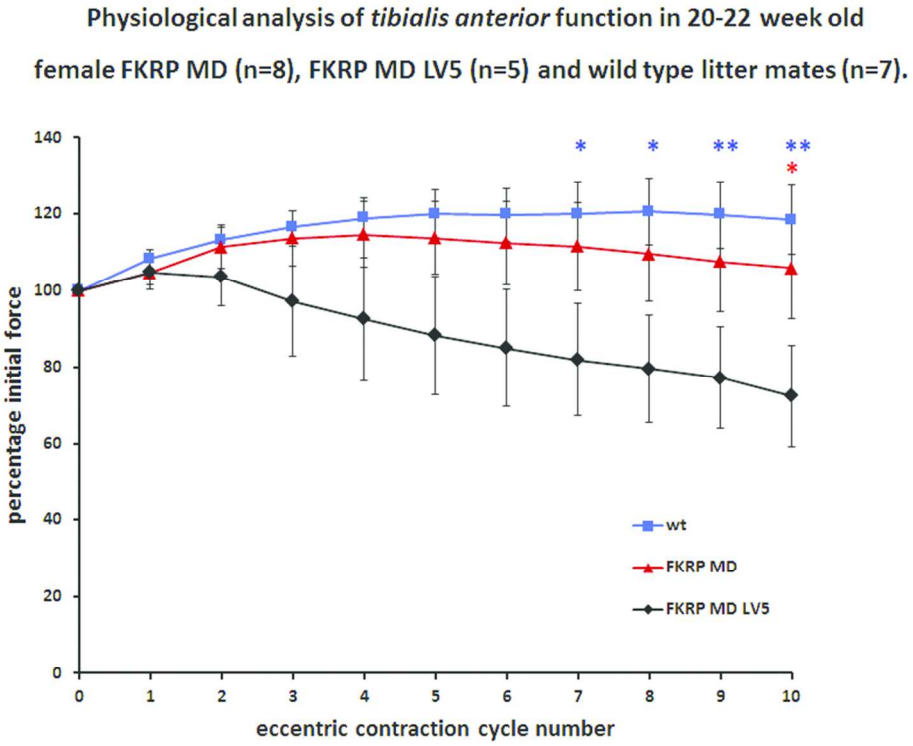


Immunolabelling showing a decrease in IIH6, a reduction in laminin α2 and increase in laminin α4.
135x101mm (300 x 300 DPI)



Ligand binding and quantitative analysis of split fibres and central nucleation.
108x78mm (300 x 300 DPI)



In vivo assessment of muscle force production following eccentric contractions.
72x56mm (300 x 300 DPI)

Reviewer: 1

This is an interesting paper in which the authors create a new FKRP disease model for the dystroglycanopathies and then test the therapeutic efficacy of LARGE overexpression in that model using transgenic mice. Brown and colleagues had previously made an FKRP knockdown mouse model by inserting a floxed neo cassette into intron 2 of the mouse FKRP gene. This led to reduced FKRP gene expression and reduced functional glycosylation of dystroglycan, causing defects in cortical migration akin to human disease with perinatal lethality. Here, they have deleted the floxed allele that knocks down FKRP expression in the brain using Sox1-Cre to rescue knockdown in neuroectoderm. This allows for normal brain development yet maintains an LGMD2I-like muscular dystrophy. This alone is worthy of publication, but the authors then go on to cross this model to a constitutively driven LARGE transgenic mouse line that they previously showed has relatively normal muscle function but can increase alpha DG glycosylation. There are at least three groups of studies suggesting that LARGE overexpression may increase aDG glycosylation in various forms of dystroglycanopathy, including some where LARGE mutations are not the cause of the disease. Unlike those studies, the work here suggests that overexpression of LARGE makes muscle disease worse, leading to increased histopathology, particularly intracellular calcium deposits, and increase lethality (around 27 weeks). The author's argue that the timing of LARGE overexpression may be important, as may other matters, and so one must think more carefully about ideas utilizing LARGE for therapy in these diseases. The concepts presented here are very worthy of publication. The work, however, would need to be significantly revised prior to being found acceptable for HMG. The issues involved include data presentation, data interpretation and lack of data.

We thank the reviewer for their positive and constructive comments regarding our manuscript and have addressed their comments as follows:-

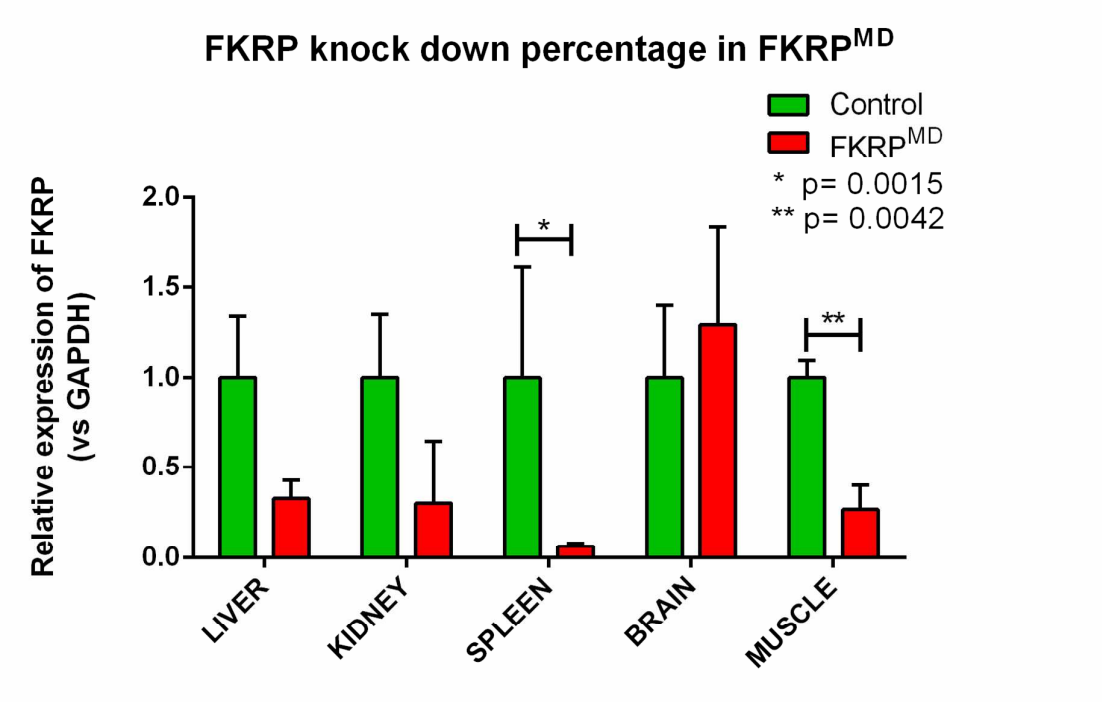
1. Each figure is unnecessarily difficult to read. The muscles, genotypes, and ages should be labeled on the panels to allow an easier understanding of what is presented.

We have now added the muscle, genotype and ages to each of the panels in Figures 2, 3, 4 and 5 and agree that this makes these figures much easier to understand.

2. For Figure 1, we should see much more data on the relationship between gene knockdown and phenotype. Is FKRP expression in FKRP MD muscle lower, greater, or the same as expression in FKRP KD muscle? This needs to be shown. Is the Sox1-Cre rescue of FKRP expression specific to brain (what about spinal cord, heart, liver, kidney, etc)? This is particularly important, given that the authors will later show that a Tg mice constitutively overexpressing LARGE makes disease worse.

We have now added a histogram depicting a QPCR of *Fkrp* in the brain at E15.5 in the $FKRP^{KD}$ mouse so that the percentage knock-down can be seen to be similar in the $FKRP_{MD}$ and $FKRP^{KD}$ mice. We have also carried out similar analyses in other tissues including liver, kidney and spleen, the results of which are shown below. As can be seen this is statistically significant in spleen, brain and muscle but there is a trend for lower levels in the other tissues which requires more analyses if it is to be verified given the size of the error bars. For this reason we would prefer not to include these preliminary analyses in the paper. We have also observed that IIH6 is reduced in the heart of

the $FKRP_{MD.}$ Overall these data are consistent with what is known about the pattern of activity of the Sox1.



If LARGE is overexpressed everywhere, how will we know its relationship to FKRP in other organs if we don't know if FKRP is lower or not.

Whilst we agree with the referee that the knock-down of *Fkrp* in other tissues is of relevance given that LARGE was expressed constitutively, the focus of our study was the up-regulation of LARGE on a background of FKRP deficiency in skeletal muscle, and our paper specifically focuses on the worsening of the skeletal muscle phenotype. The overexpression of LARGE was achieved under the CAAGs promoter which gave rise to varying levels of LARGE in each tissue, with the highest levels evident in skeletal muscle - in comparison to skeletal muscle levels were approximately 4x lower in the heart and 10x lower in brain, kidney and liver (Brockington et al. PLoS One. 2010 Dec 28;5(12). The overexpression of *LARGE* in other organs relative to the levels of Fkrp is of obvious interest to the disease phenotype, but is beyond the remit of the present study which as the reviewer notes already includes the generation of a new model for this group of diseases and the testing of a possible therapeutic strategy.

The staining for IIH6 in the pial basement membrane needs to be more convincing. Previously, the authors have used double IF with laminin and IIH6, and laminin and beta DG, to show localization at the pial BM. Why not here as well? Also, just showing FKRP MD and not other genotype for this just isn't convincing. Please show FKRP KD and WT for comparison, just as in other parts of the figure.

We have now generated a new figure which includes WT, FKRP^{KD} and FKRP$_{MD}$ immunolabelled for IIH6. We hope that the staining at the pial basement membrane is clearer as we have chosen to zoom in on the cortical surface in all these images. We have used paraffin embedded sections for this part of the figure due to IIH6 showing more clearly using the Dako Envison kit than it does with immunofluorescence which we have used previously. As can been seen the FKRP^{KD} shows a disruption of neuronal layering and an absence of IIH6 labelling at the pial basement membrane. Pan laminin immunolabelling shown in the lower half of the new figure is on frozen sections of FKRP^{KD} heterozygote (heterozygotes display no disease phenotype), FKRP^{KD} and FKRP$_{MD}$. This shows clearly that the continuity of the pial basement membrane has been restored in the FKRP$_{MD}$ such that it is indistinguishable from the FKRP^{KD} heterozygote (which shows no disease phenotype). We hope that this figure is now acceptable to the reviewer.

3. In Figure 2, it would be better to show all of the data at different ages in the same graph, with statistical comparisons between ages for each muscle described within the figure, and also with statistical comparisons between genotypes.

We have now revised the graph so the figure now contains one combined bar graph with the data from both ages. Before our first submission we consulted a statistican and analysed our data with a Generalised Estimating Equations model. This showed that age, muscle and genotype were all interacting factors significantly affecting the percentage of centrally nucleated fibres. We thank the reviewer for highlighting that the results of this analysis were not presented clearly so we have revised the text and included a line graph, which we were advised is the best way to present this analysis.

4. The magnifications in Figure 3 are oddly chosen. A and B show a very low power images, where the soleus shows IIH6 staining but the rest of the gastroc does not? This is not helpful and likely not true. Ideally, even in the FKRP MD muscles, at high power, one should be able to show IIH6 staining in an intramuscular peripheral nerve to demonstrate that the antibody worked. There are no clear positive controls for staining shown here.

The images we previously showed were an accurate reflection of the difference in intensity that we always see in the soleus compared to the gastrocnemius. We have nonetheless removed this image from Figure 3 and instead showed an image of the WT and FKRP$_{MD}$ triceps in Figure 6 labelled with both laminin α2 and IIH6. Two nerves can clearly be seen with the laminin α2 immunolabelling but are barely detectable with IIH6 (some faint labelling is visible around the endoneurium) suggesting that this is not an ideal internal control as *Fkrp* expression may not have been restored in the intramuscular nerves. All sections included in this Figure were labelled at the same time, collected using the same exposure times and include sections of FKRP$_{MD}$LARGE which we hope convinces the reviewer that our staining has worked.

5. The laminin alpha 2 staining is just not convincing. The authors suggest that FKRP MD muscle has altered laminin alpha 2 staining, with higher expression in regenerating fibers and lower expression on mature fibers. This just cannot be ascertained from what is shown. One would need co-stains (markers of regenerating versus mature muscle) and blots to further this conclusion. Also, the title of

this section refers to laminin "deposition", which is only a component of overall expression shown by staining.

We have now immunolabelled for developmental myosin as the reviewer suggests and find that very few fibres express developmental myosin. Instead those groups of fibres showing an apparent increase in laminin alpha 2 expression are associated with an increase in cellular activity. We have prepared a new figure showing the Hoechst labelled nuclei together with the laminin alpha 2 which clearly illustrates this. We have also added images using a look up table which provides a more clear illustration of variations across the section which are much less apparent in the control muscle. Western blots would not add any useful information regarding this variation in laminin alpha 2 labelling due to it necessarily being based on extracts of the entire muscle. We do nonetheless thank the reviewer for his/her comments regarding this aspect as the changes we have undertaken have greatly improved the clarity of this figure.

6. Similarly, the laminin alpha 4 staining in Figure 4 is unconvincing. It would not be surprising to see increased laminin alpha 4 staining in regenerating muscle that is unrelated to dystroglycan. Dystroglycan deficient dystrophic muscles show tons of laminin alpha 4 staining. Some co-staining to define myofiber subsets and also blots would be needed to assess increased or decreased matrix expression.

Dystrophic muscle, including laminin α2 deficient muscle (as referenced in the paper) shows an altered deposition of laminin α4, and we accept that this feature is not unique to disorders linked to dystroglycan. In addition we do not claim that overall levels of laminin alpha 4 are dramatically increased in the $FKRP_{MD}$ but rather that in specific areas its distribution pattern is altered.

Further work has now shown that in the $FKRP_{MD}$ laminin α4 immunolabelling is identified at the basement membrane of muscle fibres in discrete focal areas and that these are not necessarily those which are regenerating (as defined by the distribution of developmental myosin). Instead these areas tend to be associated with increased cellular activity as with laminin α2 immunolabelling. The images shown of the $FKRP_{MD}$ are representative of these areas. We have now modified Figure 6 to show that these areas are greatly increased as is the intensity of labelling in the $FKRP_{MD}$LARGE relative to the $FKRP_{MD}$. Western blotting is not sufficiently sensitive to be useful to document such changes in distribution given that it is based on whole protein extracts.

7. Figure 5 would be much more convincing if FKRP MD and FKRP MD LARGE muscles were shown together, size- and age-matched. The figure needs to be labeled so that it can be read without having to go to the legend. This is the most important result in the paper, yet it is not as convincing as it might be. It is very interesting that Alizarin red labeling is dramatically increased in the one FKRP MD LARGE image shown in the diaphragm. The extent of this finding should be more carefully explained, as it may be highly significant. Is this true in all muscles? Is it true only in regenerating fibers?

In order to allow for an easier comparison of the $FKRP_{MD}$ and $FKRP_{MD}$LARGE muscles we have now changed this image to show size and age matched images of each genotype and labelled the panels to facilitate comparisons. For the sake of clarity we show the muscle at 30 weeks of age and have moved all images at 12 weeks to supplementary data.

We agree with the reviewer that the increased Alizarin Red staining in the $FKRP_{MD}$LARGE mice is interesting. This increased Alizarin Red staining was observed in the 4 $FKRP_{MD}$LARGE mice and 3 $FKRP_{MD}$LARGE mice examined at 12 and 30 weeks of age respectively. Both the diaphragm and the gastrocnemius were examined and age-matched $FKRP_{MD}$LARGE mice had larger deposits than $FKRP_{MD}$ mice. The deposits in the diaphragm also appeared larger than those in the gastrocnemius. Alizarin Red staining detects calcium precipitates, so it is not a phenomenon that we would expect be restricted to regenerating fibres. Instead we view it as another histological indicator of muscle fibre degeneration which appears worse with the up-regulation of LARGE.

8. Figure 7 is confusing. In A, the LN overlay, what is the difference between the last three lanes? Where is the IIH6 and beta DG blot that should go along with the ligand binding data?

We have now included Western blot of IIH6 and β dystroglycan which shows α-dystroglycan hyperglycosylation in the $FKRP_{MD}$LARGE in the absence of any change in the molecular weight of β-dystroglycan. Regarding the laminin overlay the last three lanes represent three different $FKRP_{MD}$LARGE mice and show how consistent the laminin binding is between individuals.

Why is LN binding lower in some of the FKRP MD-LARGE lanes than in WT LARGE?

$FKRP_{MD}$ mice will have an altered glycosylation pattern of α-dystroglycan relative to wildtype mice. Although an up-regulation of LARGE has, as shown increased α-dystroglycan glycosylation over and above that of wildtype mice, the precise arrangement of these sugar groups may be different between WT LARGE and $FKRP_{MD}$LARGE, due to the reduction in *Fkrp* expression in the latter but not the former. This may account for the altered laminin binding when comparing WT LARGE to $FKRP_{MD}$LARGE animals on the laminin overlay.

Equally important here is to confirm that the FKRP MD-LARGE line made does not show an increased reduction in FKRP expression relative to FKRP MD. This data is not in the paper, but one assumes these are not pure-bred lines from a Tg cross.

We can confirm that these mice were not pure-bred lines from a transgenic cross, they were maintained on a mixed background. The knock-down in the $FKRP_{MD}$ is achieved via the insertion of a neomycin resistance cassette in intron 2 of the Fkrp gene. Unpublished data from our laboratory suggests that incorporation of the neo cassette into the Fkrp transcript leads to nonsense mediated decay. Whilst it is conceivable that the percentage knock-down might differ as a consequence of the genetic background; we believe that any differences between the different genetic backgrounds would not be detectable in view of the very low levels of endogenous Fkrp. Nonetheless we have added a sentence in the discussion to highlight this possibility.

9. The functional data leaves a lot to be desired. Figure 9 shows force drop during eccentric contractions in the TA muscle measured in situ. FKRP MD and FKRP MD LARGE mice are compared to WT, with FKRP MD LARGE showing a mild (ca. 20%) decline by the 10th contraction. As WT LARGE mice have been reported to have some mild physiological deficits, not showing data on these mice here is will raise suspicion. The n also needs to be raised beyond 5 for the FKRP MD LARGE group.

Given the errors, the entire change here could be due to one animal. Some explanation is warranted as to distribution by animal to better understand this data. It is also just odd to show physiology data on a muscle where no pathology data are shown.

We previously tested the possibility that LARGE overexpression may alter the resistance of TA muscles to contraction-induced injury which is the work that the reviewer refers to. In this study the TA muscles were subjected to a series of lengthening contractions and the impact of these repeated lengthening contractions on force generation measured over time (Brockington et al., PLoS One. 2010 Dec 28;5(12):Transgenic overexpression of LARGE induces α-dystroglycan hyperglycosylation in skeletal and cardiac muscle). We reported no significant difference in resistance to contraction-induced injury in 2 month old LARGE transgenic mice compared to control mice although at 8 months of age, LARGE transgenic mice developed a significant susceptibility to contraction-induced injury, as demonstrated by a 30% greater decline in force generation compared to controls following 8 successive lengthening contractions. This work is published, moreover the deficit was only seen in older animals and so we felt no requirement to repeat these analyses in the present study which was based on animals at 20 weeks.

Regarding the possibility that with these error values one animal could have contributed to the statistical significance of this result; we have now re- analysed the data removing one of the animals in which the response was more marked relative to the others. However, there is still a significant difference between wild type and $FKRP_{MD}$LARGE animals at eccentric cycle 9 (p=0.042) and at eccentric cycle 10 (p=0.019). Our experience with these animals at this age and also data we have at other ages indicates that the response is variable between individuals and that this should be reported as such.

The physiological data was performed on the TA muscle and quite rightly the reviewer draws attention to the fact that we failed to present the pathology of this muscle, we have therefore now included images of this muscle in Figure 5.

Minor:

1. The authors need to state that Campbell and colleagues have shown that LARGE is a Xyl GlcA tandem glycosyltransferase. They have also recently shown that SGK196 is the phosphor-mannose transferase, not LARGE, as stated here.

In response to this very important point we have modified the paragraph on pages 3-4 to read: "Whilst the precise pathway in which these proteins function is unclear, recent work suggests that SGK196 is a glycosylation specific O-mannose kinase and that FKRP, FKTN, TMEM5, B3GNT1 and LARGE all contribute to the generation of an extracellular matrix binding moiety on the resulting phosphorylated core M3 glycan (38). The loss of matrix binding is associated with a profound reduction in the binding of either IIH6 and/or VIA4-1 antibodies to α-dystroglycan (40)".

and on page 5:
"Whilst there have been two recent reports of the successful restoration of functional glycosylation and amelioration of the muscle pathology by AAV vectors carrying either FKRP (41)

or fukutin (42), one of the most promising forms of therapy proposed in recent years for the dystroglycanopathies is the up-regulation of LARGE; a bifunctional glycosyltransferase that alternately transfers xylose and glucuronic acid to generate a heteropolysaccharide that confers α-dystroglycan with its ligand binding properties (43).”

2. Page15, Introduction. Willers et al 2012 have shown that LARGE does not affect glycosylation of some POMT1, FKRP and FKTN mutations. This should be cited and stated.

We have now altered the paragraph on page 5 to read as follows:-
“This is based on observations showing that LARGE is able to restore α-dystroglycan glycosylation and functional laminin binding to cells taken from patients with congenital muscular dystrophy (FCMD, MEB and WWS), seemingly irrespective of the gene involved (43). Whilst this response may be dependent on the availability of O-mannosyl phosphate acceptor sites (32), this strategy is still considered as being potentially useful for a wide range of patients.”

and on page 15:
“Whilst more recent work now demonstrates that the ability of LARGE to hyperglycosylate α-dystroglycan is dependent on the availability of O-mannosyl phosphate acceptor sites and correlates with the severity of the clinical phenotype (32); this strategy is still considered as being potentially useful for a wide range of patients”.

The Willer paper has now been added to the bibliography (32).

3. Page 18 Title, “deposition” should be replaced by “expression”.

This change has now been made to the manuscript.

4. Page 27. The authors have not shown a reduction in O-mannosylation in their mouse model. Best to eliminate that sentence.

We have now changed the sentence on page 27 to read “a reduction in α-dystroglycan glycosylation”.

Reviewer: 2

The manuscript “The transgenic expression of LARGE exacerbates the muscle phenotype of dystroglycanopathy mice” by Charlotte Whitmore et al describes some very interesting observation in the FKRP mutant mouse model. The lack of FKRP expression in the CNS cause embryonic lethality, but this is rescued by the removal of the neo cassette specifically in CNS, leading to the restoration of functional glycosylation of alpha-DG in the CNS. The mild dystrophic phenotype in skeletal muscles of the FKRPMDis a small surprise as the mice apparently completely lack functional glycosylated alpha-DG. The demonstration that LARGE over-expression rescues the expression of functional glycosylated alpha-DG, but results in worsening of muscle pathology is significant, implying that the strategy of LARGE upregulation as a therapy to dystroglycanopathies requires careful reconsideration. This is perhaps not surprising as the functions of LARGE remains poorly understood.

The data presented in the manuscript are valid and mostly convincing. However, several issues need the authors' attention.

We thank this reviewer for the positive comments on our manuscript and address his/her specific concerns as follows:-

1) Demonstration the restoration of functional glycosylation of α-DG by western blots is important. Such results can provide vital information to assess the type of glycosylation on the α-DG by the LARGE over-expression. However, the only wb data in figure 7 is the laminin O/L. In the Figure, signals for laminin binding can hardly be observed in WT muscles and there is no molecular marker to define sizes of the signal. The quality of the western blot should be improved and a western blot with IIH6 should be provided for readers to understand the changes with the expression of the transgene in comparison to the wild type functionally glycosylated α-DG.

We have now included a IIH6 Western Blot to match the overlay and annotated the figure to show the sizes.

2) The author described the pathology of the new FKRPMD in skeletal muscles, but there is no description of the cardiac muscle. This is important for assessing the impact of LARGE over-expression on lifespan. The mice as described by the authors have quite mild pathology in diaphragm. It is therefore possible that a shortened lifespan of the FKRPMD/LARGE is the result of transgene expression on cardiac muscle. The authors must have examined the cardiac muscle, and if so, should present the data.

We have made preliminary studies of the cardiac muscle and found no histopathological abnormalities in the $FKRP_{MD}$. In some of the $FKRP_{MD}$LARGE we did see more evidence of pathology at 30 weeks. However, in the absence of functional data, which we considered outside of the remit of the present paper, we did not feel that we had sufficient evidence to attribute such changes with a role in the shortened lifespan of these mice. We agree with the reviewer that this is nonetheless an important issue and we intend to follow this up in a subsequent study.

3) The reasons for FKRPMD-LARGE mice present worse phenotype than the FKRPMD mice do are not clear. The authors put up two hypotheses. However, genetic background affects phenotype of FKRP mutation as well. The authors should mention that the genetic background of the hybrid FKRPMD-LARGE could also be a factor for the severer phenotype even without LARGE over expression.

Whilst the $FKRP_{MD}$LARGE transgenic has a different background to the $FKRP_{MD}$ mouse histological evaluation of wildtype *LARGE* overexpressing littermates on this new genetic background failed to identify any signs of a muscle pathology, suggesting that it is an interaction between a deficiency of Fkrp and overexpression of LARGE that is the underlying cause of the worsened phenotype.

We have therefore added the following sentences to the manuscript:

"Finally it should also be noted that as a consequence of the transgenic approach adopted here, the $FKRP_{MD}$LARGE mice are on a different background to the $FKRP_{MD}$ mouse. However, we consider this an unlikely cause of the worsened phenotype since histological evaluation of wildtype LARGE

overexpressing mice on this new genetic background failed to identify any evidence of a dystrophic pathology".

For the Figure 6, the title sentence needs to be corrected by adding a , and an and. "Immunolabelling showing a decrease in IIH6, reduction in laminin α-2, and increase in laminin α-4".

We have now altered the figure title as suggested by the reviewer.