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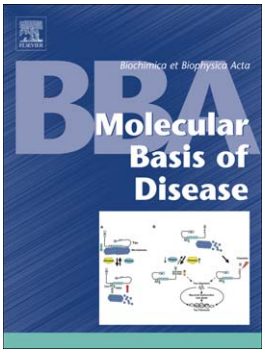

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Abnormal glycosylation of dystroglycan in human genetic disease

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Abbreviations: WWS, MDC1C, congential muscular dystrophy type 1C; WWS, Walker-Warburg syndrome; CMD, congenital muscular dystrophy; CNS, central nervous system; DG, dystroglycan; DAPC, dystrophin associated glycoprotein complex; DGC, dystrophin glycoprotein complex; α-DG, alpha-dystroglycan; β-DG, beta-dystroglycan; ECM, extracellular matrix; PAGE, polyacrylamide gel electrophoresis; kDa, kilodaltons; POMT1, Protein *O*-mannosyltransferase 1; POMT2, Protein *O*-mannosyltransferase 2; MIR, mannosyltransferase-IP3R-RyR; ER, endoplasmic reticulum; POMGnT1, protein *O*-linked mannose β1, 2 *N*-acetylglucosaminyltransferase 1; MEB, muscle–eye–brain disease; FCMD, Fukuyama congenital muscular dystrophy; UTR, untranslated region; FKRP, Fukutin related protein; LGMD2I, limb girdle muscular dystrophy type 2I; CHO, Chinese hamster ovary; ES, embryonic stem; *myd*, myodystrophy; LCMV, lymphocytic choriomeningitis virus.

Abstract

The dystroglycanopathies are a group of inherited muscular dystrophies that have a common underlying mechanism, hypoglycosylation of the extracellular receptor α-dystroglycan. Many of these disorders are also associated with defects in the central nervous system and the eye. Defects in α-dystroglycan may also play a role in cancer progression. This review discusses the six dystroglycanopathy genes identified so far, their known or proposed roles in dystroglycan glycosylation and their relevance to human disease, and some of animal models now available for the study of the dystroglycanopathies.

1. Introduction

Within the last eight years, primarily from genetic studies in humans, it has become clear that a subset of muscular dystrophies arise due to abnormal glycosylation of dystroglycan, an essential protein present in the muscle sarcolemmal membrane. Although it was first identified in skeletal muscle, dystroglycan has important roles in other tissues including the central and peripheral nervous system and epithelial cells [1]. Therefore, it is not surprising that the number of diseases known to be associated with aberrant glycosylation of dystroglycan is increasing, and I will also give an overview of other consequences of disrupting this post-translational modification.

2. The dystroglycanopathies and dystroglycan glycosylation

In humans, recessive mutations in at least six genes result in the failure of dystroglycan to be properly glycosylated, leading to genetic forms of muscular dystrophy. Deficiency in dystroglycan glycosylation is common to all these muscular dystrophies and underlies the pathogenic mechanisms; hence these disorders are collectively termed dystroglycanopathies. The dystroglycanopathies range in severity from Walker-Warburg syndrome (WWS), a

severe form of congenital muscular dystrophy (CMD) that is also associated with ocular abnormalities and CNS defects [2], to milder forms of limb girdle muscular dystrophy with no CNS involvement [3]. There is little correlation between the particular gene mutated and the clinical picture, instead all the mutations appear to act in a common pathway and the disease severity is likely to be related to the consequences of a particular mutation on dystroglycan function.

Dystroglycan (DG) is a core member of the dystrophin associated glycoprotein complex (the DAPC or DGC), which links the muscle cell cytoskeleton to the extracellular matrix via dystrophin [4]. The DAPC acts as a mechanical "shock absorber" and also has a role in cell signalling [5]. DG is synthesised as a precursor molecular that is post-translationally cleaved into α- and β- subunits [6]. α- and β-DG remain non-covalently associated within the DAPC; β-DG is a transmembrane protein that interacts directly with dystrophin via its intracellular domain [5], while α-DG is located outside the membrane and binds the extracellular matrix (ECM) protein laminin [4]. α-DG has a number of additional ECM ligands, including agrin, perlecan, neurexin and pikachurin [7-10], all of which bind α-DG via laminin-G domains [8-10].

α-DG is a heavily (and heterogeneously) glycosylated protein and it has been known for many years that this glycosylation is necessary for ligand binding [11]. The heterogeneity, combined with the low abundance of the protein, has hindered biochemical analysis of the glycans attached to the protein. The glycobiology of dystroglycan has recently been detailed in an excellent review [12] and only the key aspects will be covered here.

Although it has a predicted mass of 72kDa, α-DG migrates on PAGE gels as a heterogeneous band of much larger mass due to the extensive glycosylation [6]. There are tissue-specific differences in both the extent and nature of this glycosylation, resulting in the apparent mass of the protein varying from 120kDa in brain and peripheral nerve to more than

150 kDa in skeletal and cardiac muscle [11, 13-15]. The majority of the glycans are O-linked and attached to a central region of the protein (the mucin-like domain) that is rich in serine and threonine residues. By electron microscopy, α-DG is a dumbbell-shaped molecule (Fig 1) with two globular domains separated a rod-like domain corresponding to this mucin domain [16].

Several groups have used mass spectrometry techniques to sequence glycans on α-DG purified from sheep brain [17], cow peripheral nerve [18] and rabbit skeletal muscle [19]. All these studies identified *O*-mannosyl structures that are rare on mammalian proteins and are believed to be functionally important for α-DG ligand-binding activity. One particular sialylated *O*-linked α-mannose structure [Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Man-Ser/Thr]; Fig 1) has been proposed to be directly involved in laminin binding [18]. A number of variants of this structure have also been identified or implicated on α-DG [17, 20, 21]. In combination with the genetic evidence discussed below, the biochemical data support an essential role for these *O*-mannosyl glycans in laminin binding. However, it should be noted that not all the *O*-linked sugars on α-DG are attached via mannose, a substantial proportion contain the more common core-1 glycan structure Gal(β1-3)GalNAc-Ser/Thr [18, 19].

The deficiency in α-DG glycosylation in dystroglycanopathies is observed as a loss of immunoreactivity with one or other of two commercially available monoclonal antibodies, $VIA4_1$ and IIH6 [22-27]. A polyclonal antibody raised against a hypoglycosylated form of α-DG identifies a reduced molecular weight form of the protein in skeletal muscle from dystroglycanopathy patients [28], supporting the idea that α-DG is hypoglycosylated in these disorders.

Both $VIA4_1$ and IIH6 recognise epitopes that are present only on the fully glycosylated form of α-DG, although whether the epitopes themselves are carbohydrates is unclear. In both *in vivo* and *in vitro* studies, immunoreactivity for both mAbs correlates with laminin binding

[28-31], while IIH6 (but not VIA4$_1$) blocks this activity [11, 32]. These findings suggest that IIH6 and VIA4$_1$ recognize distinct, glycan-dependent epitopes and demonstrate that there is a close relationship between these epitopes and the laminin-binding site. However, whether laminin binding is mediated directly by glycans is still unknown.

3. Dystroglycanopathy genes involved in *O*-mannosylation

3.1 POMT1 and POMT2

Protein *O*-mannosyltransferase 1 (POMT1) and 2 (POMT2) proteins are type III membrane ER-resident proteins [33, 34]. They are related to the yeast Pmt (protein *O*-mannosyltransferase) proteins that add a mannose from a dolichol phosphate donor to a Ser/Thr residue of a protein via an O-linkage [27, 34]. The protein *O*-mannosyltransferase (PMT) domain responsible for catalytic activity is located in the N-terminal regions of POMT1 and POMT2. POMT proteins also contain several conserved mannosyltransferase-IP3R-RyR (MIR) domains, which are thought to be involved in ligand binding [35]. *POMT1*/ *POMT2* are two of four *Pmt*-related genes identified in humans. However, the other two genes (*SDF2* and *SDF2L1*) do not encode Pmt catalytic domains [35]. Together with the genetic evidence, this supports the idea that POMT1 and POMT2 add O-linked mannose directly to α-DG in the ER and are responsible for the initiation of *O*-mannosyl glycan formation [36].

Co-expression of both *POMT1* and *POMT2* and formation of a complex between the two proteins is necessary for *O*-mannosyltransferase activity in mammalian cells [37] and both genes are broadly expressed in human tissues [33, 34], explaining why mutations in one or other gene gives essentially the same phenotype. Mouse *Pomt1* is expressed at highest levels in the developing nervous system, muscle and eye [38, 39], tissues that are the most severely affected in WWS.

O-mannosylation is rare in mammals and has only been identified in a limited number of glycoproteins (including α-DG) in brain, nerve and skeletal muscle [40]. Mutations in *POMT1* were first identified in Walker-Warburg Syndrome [WWS; 27], a very severe, recessive form of CMD that is associated with ocular and retinal abnormalities and brain defects including type II lissencephaly [2, 41]. Subsequently, mutations in *POMT2*, an obvious candidate gene, were also shown to be causative for WWS [42, 43]. However, WWS is genetically extremely heterogeneous; only a minority of patients have point mutations in either *POMT1* or *POMT2* [44, 45]. Although mutations in the additional dystroglycanopathy genes described below have been identified in some of the remaining patients, at least several WWS loci remain uncharacterized [35].

While the majority of mutations in *POMT1* or *POMT2* produce severe, WWS type phenotypes, mutations in these genes can also been responsible for milder forms of disease including muscle-eye-brain disease [46, 47] and limb girdle muscular dystrophy [48, 49]. A *POMT1* mutation has recently been identified in a patient with both WWS and cleft lip and palate [50], further expanding the phenotypes that could be associated with mutations in this gene. Although most WWS cases have missense mutations or deletions within the conserved PMT or MIR domains [35], it is difficult to define clear genotype/phenotype correlations [51]. Three *POMT1* mutations that are causative for WWS, including two missense mutations G76R and V428D, have been demonstrated to result in loss of enzymatic activity in cellular expression assays [52]. However, several mutations leading to milder forms of CMD or limb girdle muscular dystrophy (G65R, A200P, and W582C) also show complete loss of enzymatic activity in this cellular assay [53, 54]. Thus, it seems that the phenotypic variation between *POMT1* disease mutations may not be simply due to levels of residual enzymatic activity and it is possible that genetic variation at other loci may influence disease severity.

3.2 POMGnT1

This gene encodes the Golgi-resident protein *O*-linked mannose β1, 2 *N*-acetylglucosaminyltransferase 1, which catalyzes the transfer of *N*-acetylglucosamine from UDP-GlcNAc to *O*-mannosyl glycoproteins [55-57]. *POMGnT1* therefore acts downstream of *POMT1/2* in the synthesis of *O*-mannosyl structures on α-DG (Fig 1). *POMGnT1* is widely expressed in heart, placenta, lung, liver, kidney and pancreas, with highest mRNA levels in skeletal muscle and brain [56]. Mutations in the human were first shown to be causative in muscle–eye–brain disease (MEB) [56, 58]. While MEB is ess severe than WWS, it is also characterized by congenital muscular dystrophy, brain malformations and ocular abnormalities. As with POMT1/2 mutations in WWS, MEB mutations produce POMGnT1 proteins that are non-functional when assayed *in vitro* [56].

The high prevalence of mutations in *POMT1/2* or *POMGnT1* in the dystroglycanopathies is strong evidence for an essential role of *O*-mannosyl structures for functional α-DG. However, it is still unclear if laminin binds directly to these O-glycans. It is possible that they are necessary for additional glycosylation events or they may have a more structural role, such as enabling α-DG to fold into an appropriate conformation.

4. Genes of unknown function

This far, the three genes described above are the only dystroglycanopathy loci for which biochemical activities of the encoded proteins have been identified. Three additional genes required for functional glycosylation of α-DG (*fukutin*, *FKRP* and *LARGE*) have all been identified from genetic studies in humans and mice. Although sequence homologies for all the proteins encoded by these genes suggest a role in protein glycosylation, their enzyme activities (if any) have not yet been defined.

4.1 Fukutin

Mutations in the *fukutin* gene are mainly associated with Fukuyama congenital muscular dystrophy (FCMD) [59, 60]. In addition to severe muscle weakness, patients with FCMD almost always have severe mental retardation, with seizures occurring in about half the cases [60]. The disease is most prevalent in Japan, where patients carry at least one copy of an ancestral founder mutation with integration of a 3kb retrotransposon element into the 3' UTR that results in a reduction in *fukutin* mRNA levels [60]. Individuals who are homozygous for this mutation are less severely affected than compound heterozygotes with a point mutation on the other allele, presumably because they have higher residual levels of functional fukutin [60]. Consistent with this, individuals who are homozygous or compound heterozygotes for point mutations usually have a more severe WWS-like disease [61-65]. In a study of 43 WWS patients, *fukutin* mutations were found to be the most common cause of WWS in European/American cases [45].

Fukutin is a Golgi-resident protein [59, 66]. The presumed catalytic domain shows similarity to phosphoryl-sugar transferases and includes a DXD motif that is involved in the coordination of divalent cations and is characteristic of many glycosyltransferases [67]. However, no enzymatic activity has yet been demonstrated for the protein. Using co-IP, a physical interaction between fukutin and POMGnT1 has been reported, suggesting a possible role as a chaperone or a modifier of POMGnT1 activity [68].

4.2 FKRP

Fukutin related protein (FKRP) was identified through homology searches using the Fukutin amino acid sequence [69]. By Northern blot analysis, *FKRP* is expressed in a wide range of tissues with highest levels in skeletal muscle, placenta and heart [69]. As with Fukutin, no specific enzymatic activity has been demonstrated and its role in α-DG glycosylation is unknown. Mutations in *FKRP* were originally identified in a form of CMD (MDC1C) and in

a clinically-defined group of limb girdle muscular dystrophy patients [25, 69]. However, it soon became apparent that mutations in this gene could also give rise to much more severe forms of CMD, including WWS and MEB [70].

Some interesting genotype/phenotype correlations have come from the study of *FKRP* mutations. A missense mutation L276I is particularly common in a form of limb girdle muscular dystrophy (LGMD2I), which has no CNS involvement [25]. This mutation appears to be hypomorphic and is associated with a relatively mild phenotype when present in the homozygous state [71, 72]. The frequency of this allele has been estimated to be as high as 1 in 200 in UK and Danish populations, and 1 in 600 in Germany [25, 73]. Such a high frequency is suggestive of positive selection pressure in heterozygotes and may be related the fact that α-DG can act as a viral co-receptor (discussed below).

The effect of MDC1C-associated mutations in FKRP on the protein function is unclear. Overexpression of FKRP protein constructs containing MDC1D mutations mislocalized to the endoplasmic reticulum in Cos-7 cells [74], indicating that ER retention may play a role in the pathogenesis of this disease. However, in patients carrying the same mutations, studies of skeletal muscle biopsies found no difference in the localization of FKRP compared to control muscle [75]. These conflicting results may represent a difference in cellular localization of FKRP in differentiated muscle compared to other cell types or simply reflect the difficulty in replicating the disease situation in cultured cells. Without knowledge of the function of FKRP, and therefore an assay of biological activity, it is difficult to draw strong conclusions about the effects of particular mutations.

In cell culture models, forced co-expression of FKRP and dystroglycan in CHO cells appears to alter the post-translational processing of α-DG. This, undefined, processing event was abolished by co-expressing dystroglycan with a mutant form of FKRP [66]. This is consistent with a role for FKRP in modification and possibly glycosylation of α-DG.

However, despite the high frequency of mutations in this gene and its clinical importance, the role of the protein is currently little understood.

4.3 LARGE

LARGE encodes a putative, bi-functional glycosyltransferase [76, 77]. A role for *LARGE* in dystroglycan glycosylation was first demonstrated by the identification of a loss of function mutation in this gene in the myodystrophy (*myd*) mouse mutant [22]. The name *LARGE* derives from the fact that this gene, at 650kb, is the largest on human chromosome 22 [76]. Indeed, it is one of the largest genes in the human genome, even though the coding region covers only 2 kb and is contained within only 14 exons.

Perhaps in part as a consequence of the size of the gene, only a few mutations in *LARGE* have been reported in human dystroglycanopathy patients making it difficult to draw conclusions about genotype/phenotype correlations. A homozygous intragenic deletion predicted to result in a loss of function was identified in a patient with WWS [78], while a less severely affected patient was found to be a compound heterozygote for a missense and a truncating mutation [79]. Very recently, a dystroglycanopathy patient was reported to be homozygous for the missense mutation W495R [80]. This residue lies within a putative catalytic domain and is absolutely invariant in vertebrate and invertebrate LARGE proteins. Therefore, this mutation is likely to disrupt the function of the protein significantly.

Vertebrate genomes contain a closely related paralogue of *LARGE* called *LARGE2* [77, 81]. Although overexpression studies indicate that *LARGE2* is likely also to be involved in dystroglycan glycosylation [81-83], the mRNA has a very different tissue distribution to *LARGE* with little or no expression in muscle and brain [81]. Thus, *LARGE2* appears not to be a good candidate for involvement in muscular dystrophy and mutations have not yet been identified in this gene.

The LARGE protein is predicted to contain an N-terminal transmembrane anchor, typical

of glycosyltransferases. It also has a predicted coiled coil motif and two presumptive catalytic regions [22, 76]. These two domains are not closely related to each other, each showing homology to a different family of glycosyltransferases [84]. LARGE is highly conserved, with orthologues in almost all animal genomes, including sponges and cnidarians, *Drosophila* being a notable exception [85].

Although the nature of the enzyme activity remains elusive, a number of groups have shown that over expression of *LARGE* can induce expression of IIH6 and VIA4$_1$ epitopes on dystroglycan and that this is concomitant with induction of laminin binding activity [30, 81-83, 86]. This activity of LARGE requires a direct interaction with the N-terminal domain of α-DG [31]. Co-expression of LARGE and tagged α-DG deletion constructs showed that amino acids 313-408 within the mucin domain are also necessary (but not sufficient) for induction of the IIH6-positive glycan [31].

The use of Chinese hamster ovary (CHO) cell lines with mutations in glycosyltransferase pathway genes showed that neither *O*-mannose nor high mannose N-glycans were required as a prerequisite for LARGE activity [86]. In mutant CHO cells in which transfer of galactose, fucose or sialic acid to glycoconjugates is compromised, LARGE was still able to induce IIH6-positive dystroglycan glycosylation [86]. Additional data supporting the conclusion that N-glycan structures are not required comes from a study showing that tunicamycin has no affect on the activity of exogenously expressed *LARGE2* and that *N*-glycanase treatment does not reduce the mass of *LARGE* glycosylated α-DG [83]. Treatment with glycosaminoglycan-degrading enzymes also failed to remove the IIH6-reactive LARGE dependent α-DG glycosylation [83]. Thus, the nature of the "IIH6" glycan and the function of LARGE remain frustratingly elusive.

A remarkable finding was that even in cells carrying loss of function mutations in other genes in this pathway such as *POMT1* or *POMGnT1* over expression of *LARGE* can induce

IIH6-positive glycosylation on α-DG [30]. This result has a number of important implications. It suggests that, at least when over expressed, *LARGE* acts in a different pathway to *POMT1* and *POMGnT1*. This is consistent with the data from the studies on glycosylation-defective CHO cells [86]. This ability of LARGE might simply reflect promiscuity and an ability to modify (either directly or indirectly) a wide range of glycan acceptors. Alternatively, LARGE may induce a rare, uncharacterised glycan structure on α-DG that is not absolutely dependent on the presence of *O*-mannosyl glycans. In either case, upregulation of LARGE could be a therapeutic strategy for a wide range of dystroglycanopathies [30, 87]. *In vivo* studies support this by showing that electroporation of *LARGE* into skeletal muscle can at least partially rescue α-DG glycosylation and function in *fukutin* or *POMGnT1* mutant mice [88].

5. The relationship between α-DG glycosylation and laminin binding activity

The genetic data discussed above demonstrate an essential role for *O*-mannosyl glycans on α-DG *in vivo*. This is in agreement with biochemical data implicating a role for sialylated *O*-linked sugars in the laminin-binding activity of dystroglycan [89]. The POMT1/2 complex is believed to catalyse the initial attachment of the mannose to dystroglycan, while POMGnT1 adds the subsequent GlcNAc residue [55, 56]. Whether fukutin, LARGE and FKRP are also involved in generation of *O*-mannosyl structures is unclear.

A elegant study interrogated the glycan structures on purified skeletal muscle α-DG through a combination of enzymatic deglycosylation followed by analysis of lectin and antibody binding to the product [90]. Digestion was carried out with a cocktail of glycosidases that were predicted to reduce the structure Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Manα-O-Ser/Thr to Manα-O-Ser/Thr and completely remove the core-1 glycan structure Gal(β1-3)GalNAcα-O-Ser/Thr. Surprisingly, this enzymatic treatment did not abolish laminin-1, IIH6 and $VIA4_1$ binding, but rather enhanced it.

This result raises a paradox. The Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-2)Manα-O-Ser/Thr glycan requires POMGnT1 activity and is presumably absent in dystroglycanopathy patients. This structure has also been postulated as the structure necessary for the binding of laminin-1 [18]. However, although there is clear evidence for an *in vivo* requirement of this glycan for normal ligand binding, removal by enzymatic digestion *in vitro* does not lead to loss of this activity.

In the same study, treatment of α-DG with enzymes that remove α-linked galactose, *N*-acetylgalactosamine and fucose, β-linked glucuronic acid, α- and β-linked mannose also did not reduce laminin-1 binding or IIH6/VIA4$_1$ immunoreactivity. The reactivity of the remaining epitope with the ConA lectin and its partial resistance to periodate oxidation treatment led the authors to suggest that a terminal α-linked GlcNAc is present in the population of glycans on IIH6-reactive α-DG [90].

Therefore, it is clear that α-DG glycosylation has more complexity and detail to be uncovered. Meanwhile, the lack of knowledge about the IIH6 epitope and the biochemical activity of LARGE are serious impediments to further development in this field.

6. Animal models for the study of dystroglycanopathies

A number of animal models are now being developed and investigated that should provide insights into the molecular, biochemical and developmental pathways that are disrupted in the dystroglycanopathies. Dystroglycan and most of the genes known to play a role in glycosylation of the molecule are highly conserved, allowing both vertebrate and invertebrate organisms to be used.

6.1 Mouse

Dystroglycan (*Dag1*) null mice are embryonic lethal due to very early defects in basement

membrane assembly [91]. While chimeric mice can be generated from *Dag1*$^{-/-}$ ES cells and develop a progressive severe myopathy [92], Cre-LoxP technology has now been used extensively by Kevin Campbell's group in Iowa, USA to produce conditional tissue-specific knockouts of dystroglycan. Depletion of *Dag1* in differentiated skeletal muscle produced a relatively mild dystrophic phenotype as satellite cells were still able to express dystroglycan and regenerate muscle fibres [93]. However, elimination of dystroglycan from the CNS produced a phenotype with striking similarities to WWS, MEB, FCMD and the *myd* mouse, with disruption of cortical layering, fusion of cerebral hemispheres and aberrant migration of granule cells [94].

More recently, selective deletion of dystroglycan in the epiblast (using a Mox2-Cre transgene driver that maintained expression of dystroglycan in extraembryonic structures) has allowed the generation of a complete DG-null mouse [95]. A careful and detailed study of this mouse model showed it to be an excellent model for WWS, including the ocular pathology [95]. Together, these mouse studies implicate dystroglycan hypoglycosylation as the major, if not only, factor underlying the dystroglycanopathy phenotypes.

Not surprisingly, mutation of the mouse orthologues of dystroglycanopathy genes rather that dystroglycan itself also leads to recapitulation of many of the features of these diseases and there are several spontaneous or engineered mouse models. The myodystrophy (*myd* or Large^{myd}) mouse was first noted due to its muscular dystrophy phenotype [96], and later shown also to show myelination defects [97]. The *myd* mutation was shown to be a loss of function mutation in the *Large* gene [22]. Subsequently, the *myd* phenotype was found also to include neuronal migration abnormalities, retinal and peripheral nerve defects [28, 29], which have subsequently been studied in more detail [98-102]. Two additional alleles of *Large* that result in the same phenotype have also been identified [103, 104].

Two strains of *POMGnT1* null mice have been produced. One model was created by a

gene trap disruption of the exon 2 [105], the other was generated by targeting exon 18 with a neomycin replacement cassette [106]. Both models have a similar phenotype with myopathy (which was much milder in the exon 18 targeted mouse), aberrant glycosylation of dystroglycan and CNS abnormalities. Like the Large^{myd} and *Fukutin* chimeric mice, they exhibit the phenotype of clasping limbs close to the body when suspended by the tail, show a reduction in cerebellum size and have neuronal migration defects. Despite a milder muscle phenotype, the targeted mutant generated by Miyagoe-Suzuki *et al.* was associated with a much lower postnatal survival rate than the exon 2 disruption mutation, with more than 60% homozygotes dying within 3 weeks of birth [106]. This may reflect differences in genetic background, as each strategy appears to have generated a complete null of *POMGnT1*.

Fukutin-deficient chimeric mice, generated using embryonic stem (ES) cells targeted for both *fukutin* alleles, have a phenotype that resembles WWS [107]. These mice are viable, but show significant muscle weakness. The mutants also display brain anomalies including disorganized laminar structures and fusion of the cerebral cortex hemispheres. They have eye defects reminiscent of those in FCMD, including abnormal lens development, retinal detachment and retinal degeneration. A problem with analysis of this model is that the extent of chimerism is variable between individual mice. Therefore, a knock-in mouse was created that contains the human FCMD-associated retrotransposon targeted into the mouse fukutin 3' UTR [88]. Although these mice show reduced levels of fukutin mRNA (5-10% of normal levels) and hypoglycosylation of the majority of skeletal muscle α-DG, they exhibit no signs of muscular dystrophy, even when adult. This study demonstrates that in mice at least, only a small amount of functional α-DG is sufficient for normal muscle function.

In contrast to the Large and PomGnT1 mutants, for most of the dystroglycanopathy genes knock-out or knock-down strategies produce a very severe phenotype. Similar to *Dag1* knockouts [91], *Fukutin* and *POMT1* null mice have a very early embryonic lethality due to

abnormal basement membrane formation [38, 108]. This is in contrast to the Fukutin-deficient chimeric mice described above. Ackroyd *et al.* recently generated two strains of *Fkrp* mutant using a knock-in strategy to replicate a severe human mutation (Tyr307Asn) [109]. Homozygous Fkrp-Neo^{Tyr307Asn} mice showed a severe phenotype, dying soon after birth, with reduced (but not absent) α-DG glycosylation in muscle and brain. In the neonates, this reduction in glycosylation was associated with abnormal neuronal migration within the CNS and reduced muscle mass, although there was no evidence of dystrophic processes. The reduction in *Fkrp* mRNA levels appears to be due to the presence of the neomycin cassette in intron 2 as homozygous Fkrp^{Tyr307Asn} mice showed no phenotype up to 6 months of age. Presumably the Tyr307Asn change, which produces a severe MEB phenotype in human [70] does not affect the function of the mouse protein. This study demonstrates the difficulty of generating mouse models of the dystroglycanopathies that accurately reflect the human disorders.

6.2 Zebrafish

The zebrafish is an attractive alternative model system for functional studies of dystroglycanopathy genes. One advantage of zebrafish, especially for developmental studies, is the translucent embryo. In addition, zebrafish embryos have a high proportion of muscle tissue [110]. An exciting potential of the zebrafish is that it is suited to large-scale screens for modifier genes and testing of potential therapeutic reagents.

All the dystroglycanopathy genes identified so far are conserved in zebrafish and are present as single copy genes. Furthermore, the glycan epitopes on α-DG appear also to be conserved [111, 112]. Knockdown of *FKRP* in the zebrafish embryo using morpholino technology produced muscle, neuronal and eye abnormalities [112].

6.3 Drosophila

POMT1 is the human homologue of the *Drosophila rotated abdomen* (*rt*) gene [33], while POMT2 is the orthologue of *twisted*; a locus that shows a genetic interaction with *rt* [113]. *Drosophila POMT1* or *POMT2* mutants show abnormal embryonic muscle development [114, 115] and synaptic defects [116]. Co-expression of the *Drosophila POMT1* and *POMT2* genes is required for glycosylation of the corresponding dystroglycan orthologue (Dg) *in vitro* [117] and *in vivo* [116]. The similarities in dystroglycan mannosylation between human and flies and the ability to carry out large-scale modifier screens using *Drosophila* make this organism an attractive model for identification of additional players in this *O*-mannosylation pathway. One difference with this species is that there is no *LARGE* orthologue, even though they exist in other invertebrates and in some insects such as bees [81]. Therefore, the post-translational modification of α-DG in *Drosophila* is unlikely to be completely analogous to that in humans.

7. Relevance of α-DG glycosylation to other human diseases

7.1 Viral infection

α-DG has previously been identified as a cellular receptor for several arenaviruses including lymphocytic choriomeningitis virus (LCMV) and the human pathogen Lassa fever virus [118]. In these studies it was observed that binding did not occur on recombinant proteins but only native proteins, suggesting that posttranslational modification such as glycosylation was required. More recently, it was demonstrated that the virus binding activity of α-DG is dependent on amino acids 313-408 within the mucin-like domain and that overexpression of *LARGE* in cell lines increases LCMV binding and infectivity [119]. This, viral binding appears to correlate with laminin binding. Further, virus infectivity was reduced by pre-incubation with IIH6. This suggests that a common IIH6 and laminin reactive epitope produced by LARGE is also important for LCMV infectivity [119].

O-mannosylation of α-DG is also important for LCMV infection [120]. Fibroblasts derived

from Large^{myd} mice or from MEB patients were less susceptible to infection than control fibroblasts. Cell lines deficient in general *O*-mannosylation also showed a reduced infectivity.

This relationship between α-DG glycosylation and viral co-receptor activity may be an explanation for the high frequency of some dystroglycanopathy mutations in the general population such as the L276I mutation in *FKRP* in Caucasian populations [25] and the *fukutin* retrotransposon insertion in the Japanese population [60]. It may be significant that both these mutations appear to be hypomorphic rather than null alleles. There is also evidence from HapMap data for positive selection acting on the *LARGE* gene during human evolution [121].

7.2 Cancer

Dystroglycan plays an important role in basement membrane and epithelial assembly via cell contact with the ECM [91, 122]. Changes in α-DG glycosylation may also play a role in cancer progression [123]. A number of cancers of epithelial and neural origin show an association between loss of α-DG and tumour progression, while β-DG expression is normal indicating that this loss of immunoreactive α-DG might also reflect changes in glycosylation [124-127].

Several lines of evidence indicate that LARGE may have an important anti-tumorigenic function. *LARGE* was originally identified as a gene present in a region of human chromosome 22q frequently deleted in meningioma tumours [76]. This locus has also been reported to be a common fragile site region [128], thus breaks and deletions may often occur within *LARGE* in tumour cells providing a possible mechanism for loss of IIH6/VIA4$_1$ reactivity within tumours. A number of epithelial cancer cell lines show silencing of *LARGE* by epigenetic mechanisms [129]. Moreover, forced expression of *LARGE* in one of these cell lines rescued α-DG glycosylation and inhibited both cell migration and anchorage-dependent growth [129]. There is a high density of CpG nucleotides in the promoter and 5’ UTR regions

of both the human and mouse *LARGE* genes [81] and thus epigenetic silencing may be a common silencing mechanism for this locus in cancer cells.

8. Conclusions

A large number of mutation studies have now been performed for dystroglycanopathies and it is clear that there is no direct correlation between the clinical disease presentation and the particular gene mutated. It is likely that most important factor will be the impact of the particular mutation on the function of the protein product of that gene and the downstream consequences for α-DG glycosylation. In one large study of genotype-phenotype correlations there appeared to be a general correlation between the extent of α-DG glycosylation and disease severity [47]. However, this does not hold for all patients [130] and other factors are clearly important.

Thus far only six dystroglycanopathy genes have been identified. Molecular genetic studies have identified many patients that do not harbour mutations in any of these genes and therefore other proteins in this pathway remain to be identified. It is probable that not all of these will be related to glycosylation processes, as some may be important for processing or stability of α-DG. For example a chicken mutant with muscular dystrophy and loss of α-DG was recently shown to have a mutation in the ubiquitin ligase gene WWP1 [131], while the Sphynx cat model shows a reduction in the overall levels of dystroglycan, but no changes in glycosylation or ligand binding activity [132].

The identification of the elusive LARGE induced glycan(s) has thus far proved to be a more difficult and frustrating problem than initially expected. It is to be hoped that further genetic studies of dystroglycanopathy patients will identify additional genes may help to provide insights into the possible function of *LARGE* and its role in post-translational modification of α-DG.

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Fig 1 O-glycan structures on α-dystroglycan

The diagram represents the structures of O-glycans known to be present on α-dystroglycan. **A**) Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα-O-Ser/Thr, **B**) Galβ1-4(Fucα1-3)GlcNAcβ1-2Manα-O-Ser/Thr and **C**) Galβ1-3GalNAcα-O-Ser/Thr. The steps in which glycosyltransferases discussed in this review are involved are indicated with arrows. A key to sugar residues is shown and anomeric configuration and linkage are marked on bonds.