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The Dystroglycanopathies: The New Disorders of O-Linked Glycosylation

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Abstract

It has become clear in the past half decade that a number of forms of congenital muscular dystrophy are in fact congenital disorders of glycosylation. Genes for Walker Warburg syndrome, muscle-eye-brain disease, Fukuyama congenital muscular dystrophy, congenital muscular dystrophy 1C and 1D, and limb girdle muscular dystrophy 2I have been identified, and gene mutations resulting in these diseases all cause the underglycosylation of α dystroglycan with O-linked carbohydrates. Unlike congenital disorders of glycosylation involving the N-linked pathway, these O-linked disorders possess distinctive muscle, eye, and brain phenotypes. Studies using mice and patient tissues strongly suggest that underglycosylation of dystroglycan inhibits the binding extracellular matrix proteins, effectively divorcing this important cell adhesion molecule from its extracellular environment. Moreover, defects in dystroglycan alone can account for most, if not all, cellular pathology. Thus, these disorders are now collectively referred to as dystroglycanopathies.

O-linked glycosylation typically occurs via an alpha linkage of the glycan to the hydroxyl group of a serine or threonine residue on a protein. The most common glycan linked in this manner is N-acetylgalactosamine or GalNAc.¹ O-linked GalNAc can then be further modified by subsequent glycosylation events to generate more elaborate carbohydrate structures. The class of enzymes that is responsible for synthesizing O-GalNAc contains a large number of genes.¹ As a consequence, there exists a high degree of functional redundancy such that null mutations in individual enzymes do not cause a glycosylation defect.² Thus, although defects that lead to a surplus of O-linked GalNAc are known to cause disease (Tn syndrome, Schindler disease³), defects in O-GalNAc synthesis are not. Null mutations in enzymes that create less common types of O-linkages, however, result in embryonic lethality.^{4,5} Protein O-fucosyltransferase I (*Pofut1*) is 1 of 2 glycosyltransferases that place O-linked fucose on biologically important glycoproteins, including Notch,⁶ while O-GlcNAc transferase (*OGT*) glycosylates nuclear and cytoplasmic proteins on serines and threonines that are also regulated by phosphorylation.⁷ Indeed, embryonic stem cells lacking O-GlcNAc do not even achieve 1 cell division.⁵ Thus, the importance of O-linked glycosylation in human disease is obscured to some degree by the relative extremes of its phenotypes; mutations in the enzymes that begin O-linked pathways are either lethal or do not cause disease because of functional redundancy. One exception to this generalization is the O-linked mannose pathway. Defects in genes that modulate this pathway have recently been shown to cause multiple forms of congenital muscular dystrophy, many of which share common brain, eye, and muscle pathology. Remarkably, in each instance, cellular pathology appears to arise from glycosylation defects in a single glycoprotein-dystroglycan. As such,

these disorders are now referred to as the dystroglycanopathies. In aggregate, these genes now account for fully half of the genes known to cause congenital muscular dystrophy.

Genetics and Clinical Findings

Mutations in 6 genes are now known to give rise to forms of congenital or limb-girdle muscular dystrophy where defects in O-linked glycosylation are responsible (Table 1). All are autosomal recessive disorders. Fukuyama congenital muscular dystrophy (FCMD), a common form of congenital muscular dystrophy in Japan, was the first dystroglycanopathy identified.^{8,9} FCMD is caused by defects in the expression of *Fukutin*. Mutations in fukutin-related protein (*FKRP*), a gene with sequence similarity to *Fukutin*, can give rise to congenital muscular dystrophy 1C (MDC1C).¹⁰ Other *FKRP* mutations, most commonly L276I, yield Limb Girdle muscular dystrophy 2I (LGMD2I), a disorder with typically milder clinical findings than MDC1C.¹¹ Mutations in the *LARGE* gene can cause congenital muscular dystrophy 1D (MDC1D).¹² *LARGE* was first cloned by Hewitt and colleagues¹³ as the gene deleted in the myodystrophy mouse, *LARGE^{myd}*, the best studied animal model for the dystroglycanopathies. Mutations in the UDP-GlcNAc:protein-O-mannose N-acetylglucosaminyl transferase (*POMGnT1*) give rise to muscle-eye-brain disease (MEB),^{14,15} whereas mutations in protein-O-mannosyltransferase I¹⁶ and II¹⁷ (*POMT1*, *POMT2*) as well as *Fukutin*,¹⁸ *FKRP*,¹⁹ and *POMGnT1*²⁰ can give rise to Walker Warburg syndrome (WWS), the most severe of the dystroglycanopathies.^{21,22}

Originally, mutations in each dystroglycanopathy gene were thought to give rise to a distinct disorder that could be defined solely by clinical findings.²³ This, however, has given way in the past year or 2 to the realization that mutations in almost all of the dystroglycanopathy genes can yield overlapping phenotypes, both with regard to the clinical spectrum of findings and to their severity.^{21–23} Thus, the notion that each disorder is clinically distinct and is caused by a mutation in a distinct gene, a theme prevalent in the early literature, is incorrect. Some generalities, however, remain. For example, Walker Warburg syndrome (WWS), MEB, and FCMD all share common brain malformations in addition to muscular dystrophy, whereas brain malformations are far less common in MDC1C and are not present in LGMD2I. There is only 1 patient identified in the literature as having MDC1D, making generalities about this disorder impossible. Brain involvement, however, as evidenced by mental retardation and white matter changes, was present in the 1 MDC1D case report.¹² The most common brain finding in WWS, FCMD, and MEB is type II (cobblestone) lissencephaly. In WWS, the brain can be almost completely agyric. Brain ultrastructure shows a cobblestone pattern of cortical neurons in lieu of the normal 6-layered pattern in the cortex. This is most likely caused by the aberrant migration of neurons through gaps in the glia limitans-basement membrane during cortical development. Other brain findings can include dilation of the cerebral ventricles, flattened brainstem, absent corpus collo-sum, aberrant myelination, and occasional occipital encephalocele. Ocular findings are common in WWS and MEB and less so in FCMD and MDC1C. These include myopia, cataracts, retinal detachment, microphthalmia, buphthalmus, persistent hyperplastic primary vitreous, Peters anomaly, and congenital glaucoma. Muscular dystrophy is present in all of the dystroglycanopathies, as evidenced by elevated serum creatine kinase levels, which can be elevated by several orders of magnitude, and by dystrophic muscle pathology on biopsy, including evidence of necrosis, fibrosis, and/or muscle regeneration. Hypotonia is a common clinical finding in more severe forms, and cardiac involvement is also common in FCMD, MDC1C, and LGMD2I after the first decade. The average life expectancy in WWS, the most severe dystroglycanopathy, is less than 1 year,²² whereas LGMD2I, the least severe form, may not manifest clinically until the second decade of life in some instances. Patients with MEB or FCMD, which are in the middle of the clinical spectrum, have an average life expectancy of 10 to 30 or 10 to 20 years, respectively.²²

Given this large spectrum of clinical findings, it should not be surprising that the relationship between mutations in the dystroglycanopathy genes and clinical findings can be highly variable. For example, mutations in *FKRP* originally identified as causing MDC1C suggested that this disease had no brain involvement,¹⁰ but subsequent *FKRP* mutations (V405L and A455D) were linked with mental retardation, microcephaly, and cerebellar cysts.²⁴ Yet, further mutations are now known that present as MEB or WWS.¹⁹ Clinical variability of all of these disorders is likely modulated by secondary genetic factors. For example, a group of LGMD2I patients from a consanguineous Bedouin tribe share the same *FKRP* missense mutation but have an age of disease onset that varies from birth to the second decade.²⁵ Indeed, some inherited mutations in *FKRP* give no abnormalities at all.²⁶ It is also becoming increasingly clear that mutations in each of the dystroglycanopathy genes give rise to a wider spectrum of clinical findings than was previously thought. For example, FCMD was originally not thought to give rise to ocular findings. This was because of the fact that most FCMD patients of Japanese descent share the same defect in the Fukutin gene. This defect, a retrotransposon insertion in the 3' untranslated region, causes reduced expression of normal fukutin protein.⁸ This has the effect of knocking down normal protein expression rather than creating a null phenotype. Once compound heterozygotes were identified in FCMD patients, however, the clinical spectrum was expanded to include ocular defects,²⁷ whereas homozygous nonsense mutations present as WWS, the most severe clinical prognosis.¹⁸ Similarly, mutations in *POMT1* were originally reported to give rise to WWS.¹⁶ A recent report, however, has identified a *POMT1* mutation (A200P) as causing a far milder limb girdle muscular dystrophy with mental retardation (LGMD2).²⁸ The clinical spectrum of *POMGnT1* mutations has also been expanded recently to include FCMD and WWS-like phenotypes in addition to MEB.²⁰ Perhaps the most intriguing development of the past year has been the finding of Topaloglu and colleagues of a patient with a defect in the *POMGnT1* gene (IVS17-2A>G) where severe autistic features were the dominant presenting sign.²⁹ This finding could significantly expand the clinical spectrum for the dystroglycanopathies.

Dystroglycan: The Target for Glycosylation Defects

All of the dystroglycanopathies have as their common molecular defect the underglycosylation of the dystroglycan protein. This lack of proper glycosylation does not inhibit the expression of the protein at the cell membrane but rather is required for the proper binding of extracellular matrix ligands such as laminin.³⁰ The dystroglycan gene (*DAG1*) encodes a single polypeptide that is posttranslationally cleaved into 2 protein chains, α dystroglycan and β dystroglycan (Fig 1).^{31,32} α Dystroglycan is a highly glycosylated peripheral membrane protein that binds tightly but noncovalently to β dystroglycan, which is a transmembrane protein.³¹ This complex of α and β dystroglycan chains serves as a vital component of the dystrophin glycoprotein complex that links the extracellular matrix that surround myofibers (and many other cell types) through the membrane to the actin cytoskeleton.³³ α Dystroglycan contributes to this complex by binding to proteins in the basal lamina, including laminins, agrin, and perlecan, as well as to neuroligins, which are transmembrane proteins. α Dystroglycan also binds to infectious agents (*Mycobacterium leprae*, Lymphocytic choriomeningitis virus, and Lassa fever virus) and may serve as a mode of entry for these agents into cells. β dystroglycan binds α dystroglycan on the extracellular face of the membrane and dystrophin (and other proteins) via its intracellular domain. Dystrophin, which is the protein absent in Duchenne muscular dystrophy, provides a vital link between the muscle cell membrane and the cytoskeleton by virtue of its ability to bind filamentous actin.³⁴

α Dystroglycan is predicted to encode a 72-kDa protein, yet native α dystroglycan protein migrates as a diffuse band centered on 160 kDa in skeletal muscle tissue.^{31,32} Thus, α

dystroglycan is over half carbohydrate by molecular weight. This is because of the presence of a serine-threonine rich mucin domain in the middle of the protein that contains up to 55 sites for O-linked glycosylation.³² O-linked carbohydrates are attached to proteins via serine (S) or threonine (T) residues. Several groups have sequenced the carbohydrates present in this region of α dystroglycan using tissues from several sources, including skeletal muscle.^{35–37} All have identified the presence of 2 types of O-linked carbohydrates. The first sequence, Gal β 1,3GalNAc α -S/T, is a common carbohydrate structure found on many proteins containing O-linked glycosylation. The second sequence, NeuAc α 2,3Gal β 1,4GlcNAc β 1,2Man α -S/T, is a structure that has only been described on α dystroglycan in mammalian tissues. The novelty of this structure arises from the O-linked mannose in this sequence. Although O-linked mannose is found on cell membranes of many lower organisms, such as yeast,³⁸ it appears to be highly specific for α dystroglycan in mammals. Indeed, loss of dystroglycan protein in brain, peripheral nerve, and skeletal muscle copies most of the cellular pathology found in the dystroglycanopathies.^{39–41} Thus, all glycosylation defects in these disorders appear to be caused by defects in the glycosylation of a single protein.

Function of Dystroglycanopathy Genes

There is good reason to believe that most of the CMD genes involved in dystroglycanopathies are involved in the synthesis of the unique O-linked mannose chains on α dystroglycan (Fig 2). Mutations in *POMT1* and *POMT2* have now been linked to Walker Warburg syndrome and are associated with hypoglycosylation of α dystroglycan.^{16,17,42} That mutations in both of these genes cause disease is consistent with the demonstration by Endo and colleagues in flies that RNAi knockdown of *POMT1* and *POMT2* yield the same phenotype (rotated abdomen)⁴³ and the fact that coexpression of *POMT1* and *POMT2* is required for O-mannosyltransferase activity in cell lines.⁴⁴ *POMT1* has also been eliminated in mice.⁴⁵ The phenotype of these animals, embryonic lethality due to disruption of Reichert's membrane, is the same phenotype found in mice lacking dystroglycan (*DAG1*).⁴⁶ Reichert's membrane is a rodent-specific structure. Therefore, it is unclear if embryonic lethality in *DAG1*-deficient mice would be reflective of a similarly lethal phenotype in humans. This would, however, be one explanation as to why *DAG1* mutations have not yet been identified in the dystroglycanopathies. At least one mutation in *DAG1*, however, does cause muscular dystrophy in mice.⁴⁷ Therefore, mutations in the dystroglycan gene may ultimately be found in these disorders. Mutations in *POMT1* that cause WWS have been shown to be defective in protein O-mannosyltransferase enzyme activity.⁴² Thus, it is likely that WWS is caused by a dearth of O-mannose linked carbohydrates on α dystroglycan, even though this has never been proven using actual patient tissue. Along with *POMT1* and *POMT2*, the third known entity in the dystroglycanopathies is *POMGnT1*. *POMGnT1* synthesizes the second carbohydrate on the O-mannose chains of α dystroglycan, transferring N-acetylglucosamine (GlcNAc) in a β 1,2 linkage to O-mannose.¹⁴ Mutations in *POMGnT1* that cause MEB have been shown to possess reduced or absent enzyme activity.¹⁵ Thus, MEB is also very likely caused by the dearth of these carbohydrates on α dystroglycan.

There are at least 3 mystery genes that remain in the CMD picture, *Fukutin*, fukutin-related protein (*FKRP*), and *LARGE*. Fukutin protein is localized to the cis compartment of the Golgi apparatus, where it would be well positioned to affect the glycosylation of α dystroglycan.⁴⁸ *Fukutin* shares mild homology with yeast proteins involved in mannosyl phosphorylation of oligosaccharides. It has been suggested based on this evidence that it is a glycosyltransferase. To date, however, there has been no demonstration of an enzyme activity for this protein. Loss of *Fukutin* in mice is lethal at an early embryonic stage.⁴⁹ Chimeric mice lacking *Fukutin* in some cells, however, show most facets of cellular

pathology found in FCMD.⁴⁹ Fukutin-related protein (*FKRP*) was named based on its shared sequence homology with *Fukutin*. Like *Fukutin*, *FKRP* has a domain structure and sequence suggestive of a glycosyltransferase, but again the activity is unknown. *FKRP* protein has been reported to be localized both to the endoplasmic reticulum and to the Golgi apparatus in different cell types.^{48,50} Esapa and colleagues⁵⁰ have shown that mutations in *FKRP* that cause MDC1C (S221R, A455D, P448L) alter *FKRP* expression from the Golgi apparatus to the endoplasmic reticulum.⁵⁰ Mutations that cause the milder LGMD2I (L276I), by contrast, do not.⁵⁰ These experiments suggest that glycosylation defects caused by mutations in *FKRP* may not be because of loss of function but to improper cellular targeting. Muntoni and colleagues have shown a correlation between the extent of dystroglycan underglycosylation in MDC1C and LGMD2I and clinical phenotype, with the L276I mutation being least severe on both fronts.⁵¹ Therefore, underglycosylation of α dystroglycan appears to be the causative factor in these disorders as well.

Perhaps the most important gene in this pathway for which the function is unknown is *LARGE*. *LARGE* was originally cloned by Hewitt and colleagues¹³ based on its deletion in the myodystrophy (*myd*) mouse, now termed *LARGE^{myd}*, which shares many features of WWS, MEB, and FCMD. Unlike *Fukutin* and *FKRP*, the primary sequence of *LARGE* is highly suggestive of a Golgi-localized tandem glycosyltransferase. Thus, *LARGE* has 2 independent domains that may possess the ability to synthesize a repeating disaccharide, much as is known to occur with enzymes that synthesize glycosaminoglycans (GAGs) such as heparan sulfate or chondroitin sulfate.⁵² There is no evidence, however, that *LARGE* synthesizes GAGs. What has become clear in the past year is what *LARGE* does not do. Using CHO cell mutants, Patnaik and Stanley⁵³ have shown that *LARGE* does not require sialic acid, galactose, or fucose to glycosylate α dystroglycan. Similarly, Combs and Ervasti⁵⁴ have shown that enzymatic removal of sialic acid, galactose, and N-acetylglucosamine actually stimulates laminin binding, much as *LARGE* overexpression does.⁵⁵ Again, this suggests that these carbohydrates are not synthesized by *LARGE*. Enzymes that remove GAGs also have no effect consistent with *LARGE* being a GAG synthase.⁵⁶ Therefore, the only logical conclusion would appear to be that *LARGE* creates a novel carbohydrate structure, and that this may be linked to structures other than, or in addition to, O-mannose.

Very recent articles have shown that there is a second gene in the *LARGE* family, *LARGE2*.^{56–58} Like *LARGE*, *LARGE2* can stimulate the glycosylation of α dystroglycan such that laminin binding is increased.^{56–58} Unlike *LARGE*, however, *LARGE2* is not appreciably expressed in brain or muscle.⁵⁷ Thus, it appears that it may not compensate for the absence of *LARGE* in these tissues and may therefore also not do so in the dystroglycanopathies. Consistent with this idea, there is no defect in α dystroglycan glycosylation in *LARGE^{myd}* kidney, where *LARGE2* is highly expressed.⁵⁷

Diagnosis

Although clinical findings (hypotonia, muscle weakness, contractures, seizures, or mental retardation coupled with elevated serum creatine kinase activity) clearly will guide neurologists toward diagnosing a dystroglycanopathy, this class of disorders requires evidence of underglycosylation of α dystroglycan protein for a definitive diagnosis. All dystroglycanopathies have the common molecular finding that α dystroglycan is underglycosylated.^{9–13,15,17,42} Underglycosylation is defined by a combination of immunostaining and immunoblotting. There are only 2 commercially available monoclonal antibodies to α dystroglycan that are commonly used for this purpose: IIH6, a laminin blocking monoclonal antibody, and VIA4-1.³¹ Both of these antibodies require proper glycosylation of α dystroglycan to bind to the protein at all, and both also suffer from significant lot to lot variability requiring their careful calibration before use. Thus, one must

use caution in their use and in their interpretation. Unlike some disorders of glycosylation, where loss of glycosylation leads to loss of protein expression or targeting, the glycosylation defects in these disorders do not prevent α dystroglycan expression at the cell surface.³⁰ Underglycosylation must be shown by showing a lowered molecular weight for α dystroglycan gels isolated from affected tissues. This usually requires antibodies made to the α dystroglycan polypeptide, although glycosylation-dependent antibodies still work in some instances (LGMD2I¹¹). Glycosylation of β dystroglycan, by contrast, is not affected in the dystroglycanopathies, and β dystroglycan is expressed normally in the membrane in all forms of the disease, regardless of the antibody used. Underglycosylated α dystroglycan protein present in FCMD, MEB, and WWS muscle binds poorly, if at all, to laminin.³⁰ Ideally, this type of biochemical evidence of defective ligand binding would be a component of the patient workup.

Biochemical findings, coupled with clinical examination, can lead one to the list of genes currently known to search for mutations; however, it is pretty clear that this list is incomplete. Thus, lack of an identified mutation in the 6 known genes does not rule out dystroglycanopathy. Indeed, it is estimated that mutations in *POMT1*, *Fukutin*, and *FKRP* account for only 20% of WWS cases,²² whereas mutations in *POMT2* have been estimated to account for 7%.¹⁷ Clearly, other genes will be identified where mutations are present. Equally difficult is linking mutations in the known genes with their loss of function. Schachter and colleagues have developed a relatively simple assay for loss of *POMGnT1* function that can be used to diagnose MEB.⁵⁹ Loss of *POMT1* or *POMT2* function is more difficult because both of these enzymes must be expressed in the same cell to show O-mannosyltransferase activity,⁴⁴ and mannosyltransferase specificity for O-linkage must be differentiated from more common alpha mannosyltransferase activities in the N-linked pathway. The function of *LARGE*, *Fukutin*, and *FKRP* have yet to be defined. Therefore, defects in these genes cannot be identified by functional biochemical assays.

Therapeutics

Currently, there are no therapies for the dystroglycanopathies that can inhibit the progression of muscle or brain pathology. There is, however, a very recent study that points toward what may ultimately become an effective therapy. Not surprisingly, given that these are disorders of glycosylation, this approach involves the stimulation of glycosylation in the affected tissues. Campbell and colleagues have shown that overexpression of *LARGE* increases α dystroglycan glycosylation in cells taken from FCMD, MEB, and WWS patients.⁵⁵ In each instance, the increased glycosylation of α dystroglycan increased its molecular weight to (or above) the level that is found in normal tissue, and this correlated with recovery of α dystroglycan's laminin binding properties.⁵⁵ This effect was specific to *LARGE*; overexpression of *POMGnT1* in FCMD cells, for example, did not have the same effect. The inference of this work is that *LARGE* is epistatic to all other genes in the O-mannose pathway and can therefore overcome all glycosylation defects caused by their absence. The possibility that *LARGE* could be used as a target for all of these diseases is very exciting and makes the definition of *LARGE* function all the more important. In fact, stimulating glycosyltransferase activity may be therapeutic in other forms of muscular dystrophy as well; overexpression of *GALGT2*, a synaptic glycosyltransferase in skeletal muscle, stimulates the glycosylation of α dystroglycan and inhibits muscular dystrophy in the *mdx* model for Duchenne muscular dystrophy.⁶⁰ Therefore, glycosylation of α dystroglycan may be a therapeutic target for many forms of the disease.

Conclusions

Work over the past half decade has shown that genes involved in glycosylation of α dystroglycan cause forms of congenital muscular dystrophy, now termed

dystroglycanopathies. The carbohydrates created by these genes are required for the binding of extracellular matrix proteins, including laminin, to dystroglycan, and this likely is the cause of all muscle, brain, and eye pathology in these disorders. In the coming years, there will be new genes identified in this pathway that cause CMDs, and much work remains on understanding the function of genes already known to cause these disorders. Defects in glycosylation are often highly pleiotropic because of the common nature of this type of posttranslational modification. Therefore, the relatively specific nature of the pathology in the dystroglycanopathies, coupled with the fact that this pathology is linked to a defect in a single glycoprotein, makes this group of disorders one of the best in which to understand the relationship between protein glycosylation and disease. The demonstration that overexpression of specific types of glycosylation can have therapeutic benefit in multiple forms of muscular dystrophy makes understanding these relationships more important.

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Figure 1.

Dystroglycan glycosylation and its place in the dystrophinglycoprotein complex. Laminin, which is present in the extracellular matrix surrounding the myofiber membrane, binds to α dystroglycan. This interaction requires the O-mannose-linked carbohydrates present in the middle third of the α dystroglycan molecule. α dystroglycan binds to β dystroglycan, which spans the muscle membrane. The intracellular domain of β dystroglycan binds to dystrophin, which links the complex both to actin and to number of other proteins. The sarcoglycans are additional transmembrane components within the dystrophin-associated glycoprotein complex.

Figure 2.

Genes affecting dystroglycan glycosylation associated with congenital muscular dystrophy. The O-mannose-linked glycans on α dystroglycan are comprised of linear chain of 4 carbohydrates. There are 6 genes known to cause congenital muscular dystrophy that alter the glycosylation of α dystroglycan. *POMT1* and *POMT2* are both required for the synthesis O-linked mannose to serines and threonines on the α dystroglycan protein, while *POMGnT1* synthesizes the second linkage, β 1,2GlcNAc, on the O-mannose chain. Three other genes, *fukutin*, fukutin-related protein (*FKRP*), and *LARGE*, also affect this pathway, although their function is not yet clear. NeuAc, N-acetylneuraminic acid (or sialic acid); Gal, Galactose; GlcNAc, N-acetylglucosamine; Man, Mannose.

Table 1

Summary of Known Dystroglycanopathy Genes and the Disorders Caused by Their Mutation

Disorder	Gene	Reference
FCMD	Fukutin	8
WWS	POMT1	16
	POMT2	17
	Fukutin	18
	FKRP	19
	POMGnT1	20
MDC1C	FKRP	10
LGMD2I	FKRP	11
MDC1D	LARGE	12
MEB	POMGnT1	14
	FKRP	19
	POMT1	28
LGMD2		
Severe autistic features	POMGnT1	29