Chapter 6
Defective Glycosylation of Dystroglycan in Muscular Dystrophy and Cancer

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6.1 Introduction

Almost all proteins present on the extracellular surface of cells or in the extracellular matrix are glycoproteins. As such, they are modified either with N-linked glycans (on asparagines), O-linked glycans (on serines or threonines), glycosaminoglycans (on serines), or with glycosylphosphatidylinositol (GPI, on variable amino acids). These glycoproteins, in combination with glycolipids present in the plasma membrane, contribute to the expression of a glycocalyx, a highly concentrated halo of glycan that surrounds the extracellular surface of all cells. It is in this intensely carbohydrate-rich environment that all ligand-receptor signaling is initiated, all infectious processes begin, and in which all cellular movements and adhesive changes take place. As such, it should not be surprising that the carbohydrate environment of proteins provides a rich and essential context in which to understand their function. There are many examples of essential roles for glycosylation in health and disease (Freeze 2006). Examples where glycosylation is directly involved include the lysosomal storage disorders, which often arise from the inability of mutant glycosidases to break down glycans on glycolipids or glycoproteins (including proteoglycans), congenital disorders glycosylation, many of which arise from failure of enzymes in N-linked glycan biosynthesis but that also include defects in enzymes regulating glycosoaminoglycan synthesis, Golgi localization of glycosyltransferases, and O-linked biosynthesis. In addition, there are myriad examples where protein glycosylation plays a secondary role in dictating disease behavior, particularly in inflammatory diseases, blood disorders, and cancer. Tumor cell extravasation from the blood to the lymph, for example, is greatly

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affected by glycan repertoire present on cancer cells and by lectins on endothelial cells that bind to them. One very important membrane glycoprotein that plays both direct roles and indirect roles in human disease is dystroglycan (Michele and Campbell 2003; Higginson and Winder 2005; Sgambato and Brancaccio 2005; Barresi and Campbell 2006; Martin 2006). It has become clear from an amazing convergence of studies utilizing human and mouse genetics as well as biochemistry and cell biology that dystroglycan is an essential protein in mammals whose glycosylation is necessary for its function and whose aberrant glycosylation can cause disease.

6.2 The Dystroglycan Glycoprotein

In mammals, the dystroglycan gene (*Dag1*) is encoded by only two exons, both of which contain protein-coding sequence (Ibraghimov-Beskrovnaya et al. 1993). As such, splicing is not a regulatory mechanism important to dystroglycan biology (though splicing is more complex in some organisms (for example *Drosophila melanogaster* (Schneider and Baumgartner 2008))). Dystroglycan contains a signal peptide and becomes modified by N- and O-linked glycosylation as it makes its way through the endoplasmic reticulum (ER) and Golgi apparatus to the plasma membrane (Ervasti and Campbell 1991; Ibraghimov-Beskrovnaya et al. 1992; Ervasti and Campbell 1993; Holt et al. 2000). It is also proteolytically cleaved into two polypeptide chains, termed α and β dystroglycan (Ervasti and Campbell 1991). α dystroglycan is a membrane-associated extracellular protein that binds tightly, but non-covalently, to β dystroglycan, a transmembrane protein. α dystroglycan contains a mucin-like domain with as many as 55 serines or threonines (Ibraghimov-Beskrovnaya et al. 1992) and becomes heavily glycosylated with O-linked glycans in the ER and Golgi (Martin 2003a). The extent of this type of glycosylation on α dystroglycan varies between different tissues and also within the same tissue during development. α dystroglycan is converted from an unglycosylated polypeptide of 72 kDa to a glycoprotein of 120 kDa in brain, 140 kDa in cardiac muscle, and 156 kDa in skeletal muscle (Ervasti and Campbell 1993; Gee et al. 1993; Ervasti et al. 1997; Barresi and Campbell 2006). The extent of glycosylation becomes higher as skeletal muscle development proceeds (Leschziner et al. 2000) and also varies with stage of pregnancy in the placenta (Santhanakrishnan et al. 2008). In most tissues, however, it ultimately becomes a glycoprotein that is, on average, half carbohydrate by molecular weight. α Dystroglycan can also be cleaved by furin in the trans-Golgi or at the plasma membrane, thereby eliminating the N-terminal third of the protein sequence (Singh et al. 2004). The extent of glycosylation on β dystroglycan, by contrast, is far less, with the protein likely only modified on several N-linked sites. The enzyme that cleaves the protein into α and β chains has not been identified. Mutation of serine 654, the first amino acid of β dystroglycan, to alanine, however, inhibits cleavage of all endogenous α/β dystroglycan protein chains and causes muscular dystrophy when overexpressed in skeletal muscle

(Jayasinha et al. 2003). Some human tissues, in particular pediatric bone, have a 160 kDa species that can be recognized by antibodies to β dystroglycan, and this is consistent with the presence of uncleaved α/β dystroglycan in this tissue (Martin et al. 2007).

Glycan sequencing of the O-linked chains on α dystroglycan, both by Smalheiser and Dell, from sheep brain (Smalheiser et al. 1998) and by Endo and colleagues, from bovine peripheral nerve (Chiba et al. 1997) and rabbit skeletal muscle (Sasaki et al. 1998), revealed a mixture of relatively common core 1 glycans (Galβ1,3GalNAcα-O-Ser/Thr or T antigen) and an unusual O-linked mannose tetrasaccharide (NeuAcα2,3Galβ1,4GlcNAcβ1,2Manα-O-Ser/Thr). Smalheiser and Dell also identified O-mannosyl-linked Lewis X (Galβ1,4[Fucα1,3]GlcNAcβ1,2Manα-O) in brain (Smalheiser et al. 1998). These studies show that α dystroglycan contains an unusual sialylated O-linked mannose structure that has not been commonly found in mammals. Such structures, however, may not be uncommon. Feizi and colleagues, for example, showed that O-linked mannose may represent as much as the third of all O-linked glycan on proteins in rabbit brain (Chai et al. 1999), an amount far in excess of the preponderance of dystroglycan protein. In addition, O-linked mannose is a relatively common cell surface modification in lower eukaryotes such as yeast (Willer et al. 2003). While these elegant glycan sequencing papers leave no doubt that dystroglycan contains both O-linked mannose and O-linked GalNAc structures, less convincing antibody or lectin blotting suggests other structures may also be present, including the HNK-1 epitope (S04-GlcAβ1,3Galβ1,4-) (Smalheiser and Kim 1995), the Sda/CT carbohydrate epitope (Neu5Ac (or 5Gc)α2,3[GalNAcβ1,4]Galβ1,4GlcNAcβ-) (Xia et al. 2002), and Tn Antigen (GalNAc-α-O) (Ervasti et al. 1997). Both HNK-1 and CT carbohydrates have the potential to be present on O-linked mannose structures, while the Tn antigen may reflect incompletely galactosylated core 1 structures. Some of these structures may be present on small amounts of the total dystroglycan protein, and as such may not have been identified in the original sequencing studies. For example, the CT carbohydrate is present in skeletal muscle only at the neuromuscular synapse (Martin et al. 1999), which comprises about 0.1% of the total muscle membrane protein.

The original studies of Ervasti and Campbell showed that removal of the N-linked chains from α dystroglycan removed only 10 kDa of glycan from the 156 kDa polypeptide (Ervasti and Campbell 1993), an amount that reflects approximately 2–3 N-linked chains, which is consistent with the number of predicted N-linked sites (Ibraghimov-Beskrovnaya et al. 1992). These N-linked glycans, however, were not required for laminin binding to α dystroglycan. By contrast, acid digestion of all glycans (in this case both N- and O-linked) led to the loss of all laminin binding, suggesting an essential role for the O-linked chains. This seminal study has now been borne out by human genetics studies showing that genes required for O-linked mannose biosynthesis on α dystroglycan are required for the binding of laminin and other ECM proteins (Michele et al. 2002). Thus, the O-linked mannosylation of α dystroglycan is essential to its function as a receptor for the extracellular matrix.

6.3 The Dystrophin-Associated Glycoprotein Complex

Dystroglycan associates with a large number of extracellular and intracellular proteins as an essential member of transmembrane protein complexes (Fig. 6.1). The composition of these complexes differs depending on the cell type and its subcellular

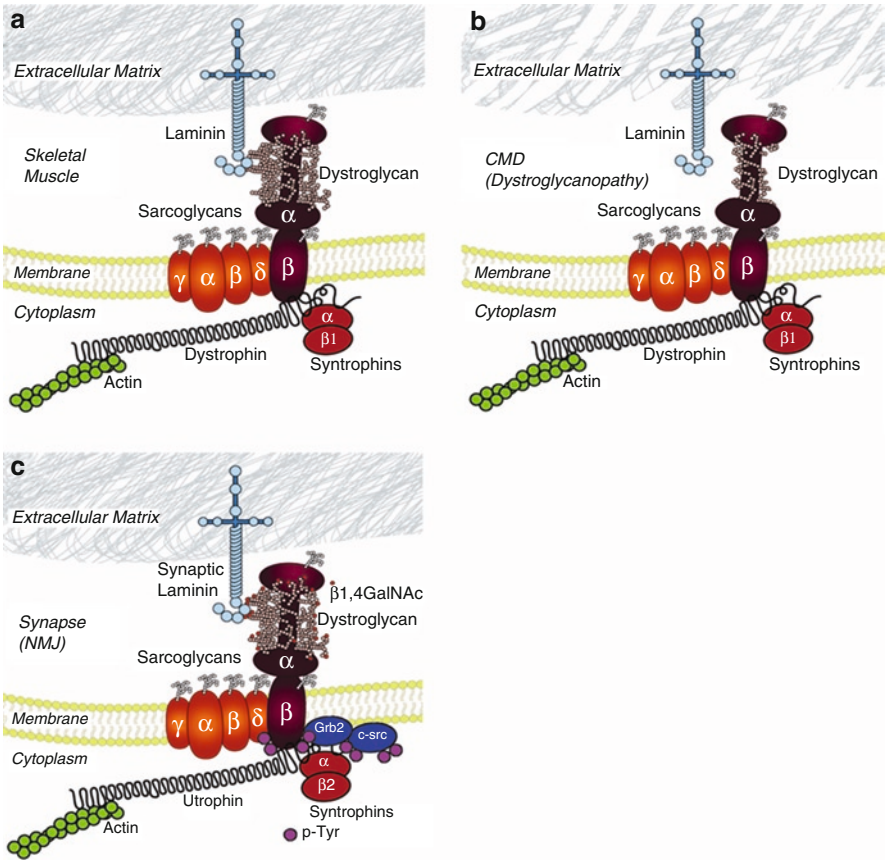

Fig. 6.1 The dystrophin-associated glycoprotein (DAG) complex in normal skeletal muscle, in skeletal muscle with dystroglycanopathy, and at the neuromuscular junction. (**a**) Normal skeletal muscle. Dystroglycan at the sarcolemmal membrane links laminin in the extracellular matrix, sarcoglycans in the membrane, and dystrophin and ultimately F-actin in the cytoplasm. α and β1 syntrophin, as well as other molecules (dystrobrevins, plectin1, ankyrin, etc., not shown), also bind dystrophin; (**b**) Patients with dystroglycanopathy (congenital muscular dystrophy, CMD) have reduced glycosylation of the O-linked structures on α dystroglycan. This can have the secondary consequence of lowering laminin expression in the extracellular matrix; (**c**) α dystroglycan at the neuromuscular junction (NMJ) is differentially glycosylated by Galgt2 with terminal β1, 4GalNAc structures. Synaptic forms of laminin bind to α dystroglycan. β dystroglycan is phosphorylated on tyrosine and interacts with synaptic linker and signaling molecules. Utrophin, a synaptic orthologue of dystrophin, is present, as is β2 syntrophin, as a uniquely synaptic form. Other synaptic proteins (e.g. α1 dystrobrevin) are not shown

localization (Martin 2003a, b). In the sarcolemmal membrane of skeletal muscle, dystroglycan is a central component of the dystrophin-associated glycoprotein (DAG) complex (Fig. 6.1a). Here, dystroglycan binds to the principal extrasyanptic muscle laminin (laminin-2 or α2,β1,γ1 laminin), and this binding requires the O-mannosyl-linked glycans present in its mucin-like domain(Ervasti and Campbell 1993; Michele et al. 2002). α/β dystroglycan interacts within the membrane with sarcoglycans, which are a four protein complex of α–δ sarcoglycan in skeletal muscle, and via the cytoplasmic domain of β dystroglycan with dystrophin, which ultimately links the complex to filamentous actin and other structural and signaling components (Fig. 6.2). The DAG complex was originally purified and

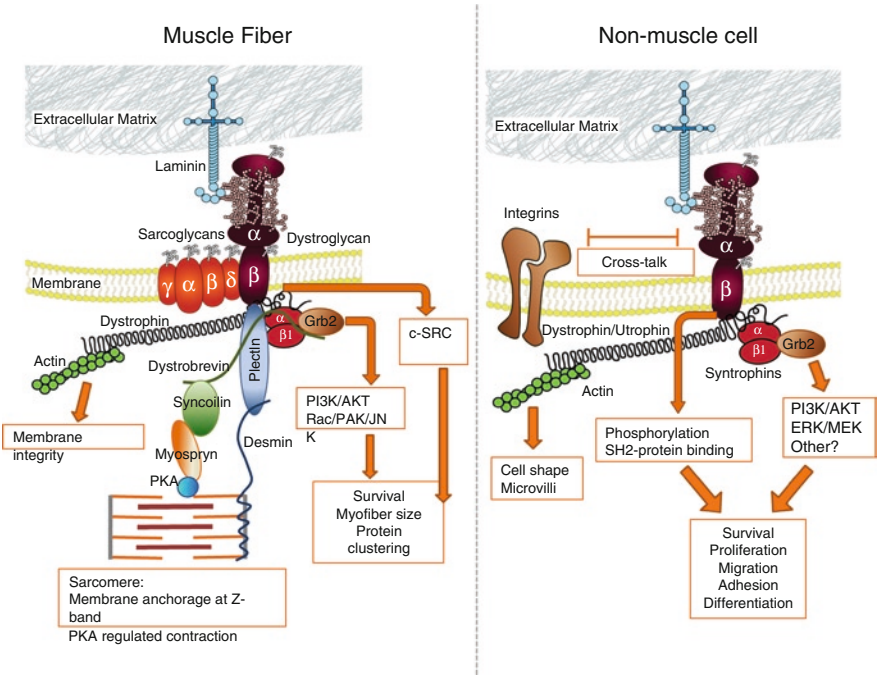

Fig. 6.2 Functions of dystroglycan in muscle and non-muscle cells. In skeletal muscle fibers, dystroglycan is part of a large complex that links laminin to the actin cytoskeleton via dystrophin. This dystrophin-associated glycoprotein (DAG) complex plays a structural role protecting the membrane from mechanical damage during repeated cycles of contraction. In addition, the DAG proteins are linked to the underlying sarcomeres at the Z-lines via desmin and regulate the phosphorylation of several sarcomeric proteins by anchoring protein kinase A (PKA). Dystroglycan also mediates activation of intracellular signaling pathways. Phosphorylation of β dystroglycan by c-src modulates its interactions with dystrophin while laminin-binding to α dystroglycan regulates activation of the PI3K/AKT and Rac/PAK/JNK pathways via the syntrophins and Grb2. In non-muscle cells and in muscle precursor cells, the dystroglycan complex antagonizes the actions of integrins. Binding of laminin to α dystroglycan regulates cell shape via re-organization of the actin cytoskeleton, and promotes differentiation and cell survival over proliferation and migration. Dystroglycan interacts with the PI3K/AKT and ERK/MEK signaling pathways, and β dystroglycan can be tyrosine phosphorylated leading to binding of SH2 domain proteins. Hypoglycosylation of α dystroglycan in cancers disrupts laminin binding and leads to loss of cell polarity, proliferation and migration

characterized by James Ervasti, Kevin Campbell, and colleagues (Ervasti et al. 1990; Ervasti and Campbell 1991).

Mutations affecting expression of almost all of DAGs cause forms of muscular dystrophy, strongly demonstrating a functional role for these protein associations. Loss of dystrophin causes Duchenne muscular dystrophy (Hoffman et al. 1987; Koenig et al. 1987), a severe X-linked myopathy, while partial loss of dystrophin causes Becker muscular dystrophy, which typically has a milder clinical progression than DMD (Love et al. 1989; Blake et al. 2002). Similarly, loss of any of the four muscle sarcoglycans (α–δ) causes Limb-girdle muscular dystrophy (LGMD2D, 2E, 2C, and 2F, respectively) (Vainzof et al. 1996; Angelini 2004; Rezniczek et al. 2007), and loss of laminin α2 causes Congenital muscular dystrophy 1A (Mendell et al. 2006). Complete loss of dystroglycan is lethal in mice from an early embryonic stage (Williamson et al. 1997), and this may explain why human *DAG1* mutations have not been identified as causing muscular dystrophy. However, loss of proteins that glycosylate α dystroglycan cause forms of congenital muscular dystrophy or limb-girdle muscular dystrophy by disrupting laminin binding (Fig. 6.1b) and will be discussed in the next section (Martin 2006; Mendell et al. 2006).

The common feature of these muscular dystrophies is a progressive degeneration of muscle fibers, often linked to increased fragility of the cell membrane in the face of repeated mechanical stress imposed by muscle contraction. All give rise to chronic cycles of muscle degeneration and regeneration, with additional immune components, that in their most severe forms lead to wasting of the skeletal muscle tissue and replacement of muscle with fat or extracellular matrix. Such muscle wasting leads to progressive muscle weakness which in the most severe forms results in wheelchair dependence and ultimately early mortality due to complications arising from respiratory and/or cardiac failure. Thus, a wealth of both biochemical and genetic data points to the importance of the DAG complex as a stabilizer of the muscle membrane.

At the neuromuscular junction, dystroglycan is differentially glycosylated with synaptic carbohydrates by Galgt2, a UDP-GalNAc:Neu5Acα2,3Galβ1,4GlcNAc-β1,4 N-acetylgalactosaminyltransferase (Nguyen et al. 2002; Xia et al. 2002). Galgt2 (Xia et al. 2002), like the CT carbohydrate it creates (Martin et al. 1999), is normally confined in skeletal muscle to the area including the neuromuscular synapse, thereby defining a uniquely synaptic glycoform of dystroglycan protein (Fig. 6.1c). At the neuromuscular synapse, there are unique isoforms of laminin (laminin 9 (α4,β2,γ1) and laminin-11 (α5,β2,γ1)) (Patton et al. 1997), now called laminin 421 and 521 respectively, that may interact with the unique glycoform of dystroglycan, and also utrophin, a synaptic orthologue of dystrophin (Ohlendieck et al. 1991). Additionally, dystroglycan may be modified by tyrosine kinases such as c-src (Sadasivam et al. 2005) via interactions with synaptic adaptor proteins, such as rapsyn (Apel et al. 1995) or Grb2 (Yang et al. 1995). Further evidence of a unique synaptic complex is the fact that transgenic overexpression of Galgt2, which creates the CT carbohydrate on α dystroglycan along the entire muscle membrane, leads to the ectopic expression of synaptic laminins and utrophin (Nguyen et al. 2002; Xia et al. 2002). The ectopic expression of this synaptic glycan further prevents dystrophin-deficient muscles from developing muscular dystrophy, which may be in part due to the extrasynaptic

expression of novel synaptic DAGs (Nguyen et al. 2002). That dystroglycan is essential for proper synaptic structure is further supported by studies where it is deleted in the skeletal muscles of mice and by studies where genes that control its O-linked mannosylation are altered (Cote et al. 1999; Saito et al. 2007). Similarly complex novel protein associations may be found in other subcellular regions of skeletal myofibers where dystroglycan is present, such as the costamere or the myotendinous junction.

The list of proteins found to interact, either directly or indirectly, with the DAG complex continues to expand. This is perhaps not surprising given the number of DAGs involved in the complex. Many interactions are mediated by dystrophin, which in addition to F-actin also binds syntrophins (Peters et al. 1997), dystrobrevins (Butler et al. 1992; Peters et al. 1998), plectin 1 (Rezniczek et al. 2007), ankyrin (Ayalon et al. 2008), and cytokeratin 19 (Ursitti et al. 2004; Stone et al. 2005). Plectin1 and ankyrin also can associate directly with the cytoplasmic face of β dystroglycan (Rezniczek et al. 2007; Ayalon et al. 2008), as can a host of signaling or adaptor proteins, including Grb2 (Yang et al. 1995; Russo et al. 2000), dynamin (Zhan et al. 2005), c-src (Sotgia et al. 2001), rapsyn (Apel et al. 1995; Cartaud et al. 1998), ezrin (Batchelor et al. 2007), and MAP kinase (Spence et al. 2004b). Additional signaling and channel proteins, including neuronal nitric oxide synthase (Brenman et al. 1995), voltage-gated Na+ channels (Gee et al. 1998), aquaporin-4 (Adams et al. 2001), TRPC1 calcium channel (Vandebrouck et al. 2007), and syncoilin (a desmin-binding protein) (Newey et al. 2001; Poon et al. 2002), may enter the complex via interactions with dystrobrevins or syntrophins. Plectin 1 and ankyrin also bind to cytoskeletal proteins including microtubules, microtubule-binding proteins, and intermediate filaments. Plectin1 also binds F-actin and loss of plectin1 causes muscular dystrophy (with epidermolysis bullosa). Filamin C, which binds sarcoglycans and F-actin, could serve similar structural roles (Thompson et al. 2000). These proteins provide additional scaffolds that may work even in the absence of dystrophin to link dystroglycan to the cytoskeleton.

Dystroglycan shows equally complex associations with the extracellular matrix proteins that reside in the basal lamina that surrounds each myofiber (Martin 2003a). Outside the cell, α dystroglycan binds to β dystroglycan, via a protein-protein interface requiring the C-terminal third of α dystroglycan, and with extracellular matrix proteins, including multiple forms of laminin, agrin, and perlecan (Henry and Campbell 1999). α Dystroglycan also interacts with specialized ECM proteins, such as pikachurin (at ribbon synpases in the retina (Sato et al. 2008)), biglycan (Bowe et al. 2000), transmembrane proteins including neurexins (in neurons) (Sugita et al. 2001), infectious agents including Lassa Fever virus, Lymphocytic choriomeningitis virus (Cao et al. 1998; Kunz et al. 2001) (but see (Imperiali et al. 2008)), and *Micobacterium leprae* (Rambukkana et al. 1998), and also Golgi proteins involved in its own glycosylation (LARGE (Kanagawa et al. 2004)). Most ECM proteins known to bind α dystroglycan contain laminin G domain motifs (Timpl et al. 2000), and these binding interactions require glycosylation of α dystroglycan with O-linked mannose structures (Michele et al. 2002). Whether these glycans mediate direct binding, however, remains a matter of debate.

6.4 The Dystroglycanopathies

Dystroglycanopathies are neuromuscular disorders defined by altered glycosylation of α dystroglycan (Martin and Freeze 2003; Jimenez-Mallebrera et al. 2005; Martin 2006; Mendell et al. 2006; Moore et al. 2008; Muntoni et al. 2008). There has been an increasing focus given to using dystroglycan glycosylation as a diagnostic, and therefore the number of patients identified continues to expand. The more severe end of the dystroglycanopathies encompasses congenital muscular dystrophies, diseases that are present at or before birth. These diseases include Walker Warburg syndrome (WWS), muscle eye brain disease (MEB), Fukuyama congenital muscular dystrophy (FCMD), and congenital muscular dystrophy 1C and 1D (MDC1C and MDC1D). These diseases arise from mutations in genes affecting dystroglycan glycosylation, including *POMT1* (Beltran-Valero de Bernabe et al. 2002), *POMT2* (van Reeuwijk et al. 2005), *POMGnT1* (Yoshida et al. 2001), *FKTN* (Kobayashi et al. 1998), *FKRP* (Brockington et al. 2001b; Topaloglu et al. 2003; Beltran-Valero de Bernabe et al. 2004), and *LARGE* (Longman et al. 2003; van Reeuwijk et al. 2007). WWS, MEB, and FCMD patients display a variety of neurological findings in the brain, including type 2-like lissencephaly (or "cobblestone cortex"), cerebellar cysts, pontocerebellar hypoplasia, hydrocephalus, reduced or absent corpus collosum, and white matter changes (hypomyelination), and also changes in the eye, including congenital glaucoma, retinal dysplasia or detachment, microopathalmia, myopia, atrophy of the optic nerve, buphthalmos, and anterior chamber defects. All also include muscle pathology consistent with severe muscular dystrophy as well as variably present cardiomyoapthy and also defects in neuromuscular synapses (Jimenez-Mallebrera et al. 2005; Taniguchi et al. 2006).

While the range of clinical presentation can be quite variable, children with WWS rarely live beyond a year of age and show severe hypotonia (Jimenez-Mallebrera et al. 2005). Children with other CMD variants can live into their teens, typically show significant muscle weakness, and can have loss of ambulation and mental retardation. These diseases differ from other muscular dystrophies involving loss of other members of the DAG complex (e.g. dystrophin and sarcoglycans) in that they show multiple brain phenotypes in addition to muscular dystrophy, arguing that dystroglycan may have unique functions in the brain that cannot be compensated for by other DAG members. In patients and in animal models where lissencephaly is present, the observed defect in neuronal migration is not cell autonomous but rather arises from defects in the formation of the glial limitans-pial membrane surface, where dystroglycan is essential for proper ECM expression and integrity (Holzfeind et al. 2002; Moore et al. 2002). This has the effect of causing a fraction of the cortical neurons to continue to migrate through holes in the pial surface, ultimately leaving the brain.

At the other end of the clinical spectrum for the dystroglycanopathies are the Limb-girdle muscular dystrophies LGMD2I, LGMD2K, LGMD2L, LGMD2M, and LGMD2N (arising from mutations in *FKRP* (Brockington et al. 2001a), *POMT1* (Balci et al. 2005; D'Amico et al. 2006), *FKTN* (Godfrey et al. 2006),

POMGnT1 (Clement et al. 2008), and *POMT2* (Biancheri et al. 2007), respectively). These diseases typically do not show brain or eye changes found in the congenital muscular dystrophies and have milder muscle and heart disease as well. There is a founder effect mutation present in the human population of *FKRP* carriers (826C > A) that leads to an increased incidence of LGMD2I relative to other LGMD dystroglycanopathies (Louhichi et al. 2004; Frosk et al. 2005). Patients with LGMD dystroglycanopathies can often ambulate as children, but muscle weakness is often progressive and can be associated with cardiomyopathy (Straub and Bushby 2006). These diseases typically show no neurological findings (Jimenez-Mallebrera et al. 2005; Muntoni et al. 2008). Because the molecular weight of skeletal muscle α dystroglycan in these LGMDs appears only to be reduced to the molecular weight normally seen in brain, brain dystroglycan function may be unaffected in these diseases.

There are at least six genes identified where loss of function mutations give rise to an aberrantly underglycosylated form of α dystroglycan with defective ECM binding properties and also cause forms of congenital or limb-girdle muscular dystrophy (Moore and Hewitt 2009; Muntoni et al. 2008). These include three genes known to be essential for the biosynthesis of O-linked mannose chains on α dystroglycan: Protein O-mannosyltransferase 1 and 2 (POMT1 and POMT2) are a dimeric protein complex required for O-linked mannose biosynthesis on α dystroglycan in mammals (Akasaka-Manya et al. 2004; Manya et al. 2004; Akasaka-Manya et al. 2006) and in flies (Ichimiya et al. 2004). POMT1 and POMT2 act to specifically glycosylate O-linked peptides from the mucin region of α dystroglycan (Manya et al. 2007). Protein O-mannosyl-β1,2-N-acetylglucosaminyltransferase (POMGnT1) synthesizes the second sugar of the O-mannosyl tetrasaccharide structure on α dystroglycan (Yoshida et al. 2001; Manya et al. 2003). The second three genes known to cause disease are fukutin (FKTN), fukutin-related protein (FKRP), and LARGE. Here again, loss of function causes underglycosylation of α dystroglycan and disease (Grewal et al. 2001; Hayashi et al. 2001; Brown et al. 2004), but the exact function of these genes is not known. FKRP, FKTN, and LARGE proteins are localized to the Golgi in muscle tissue and all three contain motifs (DxD) found in glycosyltransferases that are involved in binding sugar nucleotide substrates (Esapa et al. 2002; Brockington et al. 2005; Grewal et al. 2005; Torelli et al. 2005). Thus, they are localized and have structural motifs consistent with their either being glycosyltransferases or in mediating glycosyltransferase activity. These genes share no sequence similarity to sialyltransferases or β-galactosyltransferases that synthesize the outer glycans of the O-mannosyl tetrasaccharide on α dystroglycan.

Recent studies have shown that fukutin-deficient mice have reduced POMGnT1 activity and that fukutin can interact with POMGnT1 (Xiong et al. 2006), suggesting that fukutin may be a mediator of POMGnT1 activity or subcellular localization. Indeed, mutations in FKRP and FKTN can cause protein mislocalization from the Golgi to the ER, and FKTN mutations additionally cause POMGnT1 mislocalization (Esapa et al. 2002; Xiong et al. 2006). Further evidence of a protein targeting role is the finding that FKRP binds dystroglycan and can co-localize with it at the

sarcolemmal membrane in skeletal muscle (Beedle et al. 2007). LARGE is perhaps the most intriguing of these genes with unknown function. Campbell and colleagues have shown that overexpression of *LARGE* in cells deficient in *POMGnT1* or *FKTN* can stimulate glycosylation of α dystroglycan, converting it from its underglycosylated pathologic form to its native state. In doing so, LARGE overexpression restores laminin binding (Barresi et al. 2004). LARGE2, described by Hewitt and colleagues (Grewal et al. 2005), has a similar function, but is not normally expressed at appreciable levels in skeletal muscle. It is clear from studies using CHO cell glycosylation mutants and also from digestion of α dystroglycan with glycosidases that the type of glycosylation LARGE stimulates is not one common to other mammalian glycoproteins (Combs and Ervasti 2005; Patnaik and Stanley 2005).

Importantly, muscles from most patients with dystroglycanopathies show normal expression of α and β dystroglycan protein at the sarcolemmal membrane, only having reduced α dystroglycan glycosylation. This is typically demonstrated by showing altered migration of α dystroglycan on SDS-PAGE gels and by absent or reduced binding of carbohydrate-dependent monoclonal antibodies, such as IIH6, to α dystroglycan (using both immunostaining and immunoblotting). A defect in α dystroglycan glycosylation was first described by Hayashi et al. in FCMD patients (Hayashi et al. 2001). There are a number of patients with dystroglycanopathies where all six of the genes known to cause disease are not mutated. Thus, additional genes in this class of disorders have yet to be discovered. Indeed, there may also be patients where α dystroglycan expression is reduced without altering protein glycosylation. Such a molecular change has been recently described for a novel dystroglycanopathy in the Sphinx and Devon Rex breeds of cat (Martin et al. 2008).

Because loss of dystroglycan leads to early embryonic lethality in mice (Williamson et al. 1997), an increasing number of tissue-specific knockouts, coupled with genetic chimeras, have been created to allow viability into adulthood. These include loss of *DAG1* in skeletal muscle, astrocytes, Schwann cells, and the embryonic nervous system (Cote et al. 1999; Cohn et al. 2002; Moore et al. 2002; Saito et al. 2003; Satz et al. 2008). These valuable mouse models have shown that most of the phenotypes found in patients can be mimicked by loss of the dystroglycan protein. Work by Campbell and colleagues has conclusively demonstrated that dystroglycan can be the primary mediator of glycosylation defect because loss of dystroglycan protein in the affected tissues phenocopies loss of genes affecting its glycosylation (Cohn et al. 2002; Moore et al. 2002; Saito et al. 2003; Satz et al. 2008). Jarad and Miner (2009) used an unusual Pax3-Cre transgenic mouse that has a rostral-caudal gradient of Cre transgene expression to knock out dystroglycan specifically in caudal skeletal muscles, which allows the mice to live up to a year of age. Such animals should be especially useful in exploring the role of *DAG1* in skeletal muscle. In particular, α dystroglycan can, in some circumstances, stimulate the polymerization of proteins, in particular laminins, into the extracellular matrix (Yurchenco et al. 2004; Nishimune et al. 2008). Since laminin binding is dependent upon glycosylation, this polymerization process may be affected in dystroglycanopathy patients, where laminin α2 protein is often reduced in the

muscle basal lamina (Brockington et al. 2001a, b; Brown et al. 2004). Thus, altered α dystroglycan glycosylation may work in a bidirectional manner, leading to both loss of ECM ligand binding and ECM polymerization. Indeed, lethality in *DAG1*, POMT1, and FKTN null mice appears to be due to loss of laminin expression in the basal lamina (in particular Reichert's membrane) and resultant fracturing of surrounding basement membranes (Williamson et al. 1997; Willer et al. 2004; Kurahashi et al. 2005).

As is the case with all of the muscular dystrophies, the relative severity of dystroglycanopathies can vary between affected tissues, as can the glycosylation changes. For example, Nishino and colleagues have described patients with *FKTN* mutations that have minimal muscle weakness but with severe cardiomyopathy, much as can be seen in some Becker MD cases (Murakami et al. 2006). Likewise, Muntoni and collaborators have found several patients with *FKTN* and *FKRP* mutations where dystroglycan glycosylation is greatly reduced but that have only clinically mild forms of LGMD (Jimenez-Mallebrera et al. 2008). While such data would seem to argue that glycosylation might not be an absolute predictor of disease, the carbohydrate reagents used for such studies are often fraught with quality control issues, as the specific glycans they bind to are unknown. Toda and colleagues have made a knock-in model of the most common FCMD gene deletion in Japan, which is retrotransponsal insertion in the 3′ untranslated region of the *FKTN* gene (Kanagawa et al. 2009). This in effect knocks down FKTN mRNA expression in the affected tissues. Mice with this insertion, unlike FCMD patients with the same genetic abnormality, show only a 50% decrease in glycosylated α dystroglycan, and show no disease (Kanagawa et al. 2009). This appears to be an increasingly common theme in mouse models. In mice, quality control in protein synthesis and/or folding may be more efficient than in humans, thus leading to a relatively reduced loss of function for missense mutations or insertions. Perhaps the best recent example of this is that mice with a knock-in of the most common LGMD2D mutation in α sarcoglycan (R77C) show normal expression of the mutant protein on the sarcolemmal membrane, and thus no disease (Kobuke et al. 2008), while humans with the same mutation have reduced α sarcoglycan expression (and have LGMD2D) (Vainzof et al. 1996). It may be that fukutin protein is similarly stabilized in mice with FCMD-like insertions relative to humans, thus making the reduction in its protein levels less impact-full than would be the case in human muscle.

The secondary pathological or molecular consequence of dystroglycanopathy mutations is similarly becoming increasingly variable. For example, Reilich et al. (2006) have identified LGMD2I patients that have inclusion bodies in their muscles, a finding not present in most muscular dystrophies, including CMDs. While such changes could be secondary, for example to muscle inflammation (which can be present in LGMD2I (Darin et al. 2007)), this may also reflect the fact that the pathological consequences of FKRP mutations have not yet be fully elucidated. Similarly, Topolalglu and colleagues have identified a patient with a POMGnT1 mutation where the dominant presenting sign is severe autistic features (Haliloglu et al. 2004), a finding very different from most CMD patients. At the molecular level, the notion that α dystroglycan is normally expressed at the membrane is

questioned by the study of McNalley and colleagues where some patients with FKRP mutations have reduced expression of both α and β dystroglycan on the sarcolemmal membrane (MacLeod et al. 2007). Again, this is contrary to the original published reports showing that glycosylation did not affect membrane expression of α and β dystroglycan protein (Michele et al. 2002).

Similar findings, including reduced expression also of sarcoglycans and sarcospans, have been found in FCMD muscles (Wakayama et al. 2008). Such studies add potentially new insights to the current molecular dogmas of how these diseases are caused. In a similar vein, Hewitt and colleagues has shown that IIH6, the antibody that recognizes the laminin-binding carbohydrate epitope of α dystroglycan, shows a very restricted pattern that does not coincide with LARGE but rather with LARGE2 in zebrafish (Moore et al. 2008). As such, LARGE2 may be important for stimulating the glycosylation-dependent laminin binding epitopes on α dystroglycan, consistent with its activity in cultured cells (Brockington et al. 2005; Fujimura et al. 2005; Grewal et al. 2005). Knock-down of LARGE2 in eliminates IIH6 staining, demonstrating a clear requirement of LARGE2 for dystroglycan glycosylation in fish (Moore et al. 2008). Because LARGE2, like LARGE, can stimulate α dystroglycan glycosylation when overexpressed, it may be an important new target for therapeutic intervention in the CMDs. Additionally, fish may be used to identify genetic suppressors of LARGE that inhibit its activity.

6.5 Dystroglycan and Cancer

The development and progression of many types of cancers involves changes in cell-extracellular matrix receptors that normally function in the maintenance of normal tissue cytoarchitecture and adhesion. In the context of cancer, the most studied extracellular matrix receptors are integrins, α and β heterodimeric protein receptors involved in regulating cell adhesion, migration, survival and proliferation. A growing body of evidence, however, now also implicates dystroglycan in tumor cell biology. In fact, disruption of the dystroglycan complex appears to be a widespread phenomenon in cancers of varied cellular origins.

In vitro and in vivo studies have implicated dystroglycan in epithelial cell growth inhibition, cell polarity, tissue-specific gene expression, differentiation, basement membrane formation, and survival. These functions are all affected during tumor progression and their disruption parallels the loss of dystroglycan in a growing list of epithelial tumors and tumor cell lines, including breast cancer (Cross et al. 2008) and oral squamous cell carcinoma (Jing et al. 2004). Loss of dystroglycan expression or altered dystroglycan glycosylation has also been demonstrated in non-epithelial cancers, including pediatric rhabdomyosarcoma and neuroblastoma (Martin et al. 2007) and also adult glioma (Calogero et al. 2006). Exceptions to this list include pediatric liver cancers, osteosarcomas, yolk sack tumors, and Hodgkin's lymphomas (Martin et al. 2007). Thus disruption of dystroglycan function appears in many, but not all, cancer types.

Three major mechanisms have been proposed in cancer cells for disruption of dystroglycan function: proteolytic cleavage of the dystroglycan complex, altered glycosylation of α dystroglycan, and overall reduction or loss of dystroglycan expression (Losasso et al. 2000; Yamada et al. 2001; Singh et al. 2004; Martin et al. 2007; Cross et al. 2008). The first two mechanisms converge on the fact that they disrupt the ability of α dystroglycan to tether the cell to the surrounding basement membrane, either by removing carbohydrates essential for its interactions with laminin or by inducing shedding of α dystroglycan from the membrane. The latter can involve cleavage of β dystroglycan, leading to loss of the extracellular domain of β dystroglycan, which also binds α dystroglycan, from the membrane (Yamada et al. 2001; Singh et al. 2004).

Cleavage of the extracellular domain of β dystroglycan coincides with the appearance of a 31 kDa protein fragment recognized by monoclonal antibodies to the extreme C-terminus of the β dystroglycan protein (in its cytoplasmic domain) (Yamada et al. 2001; Singh et al. 2004). This protein fragment has been detected in primary oral squamous cell carcinomas (Jing et al. 2004) and cervical cancers (Sgambato et al. 2006) as well as in mammary epithelial and prostate tumor cell lines (Losasso et al. 2000; Sgambato et al. 2007b). Of note, this 31 kDa β dystroglycan protein is not unique to tumors and has been detected in normal tissues (Yamada et al. 2001; Sgambato et al. 2006). The function of this 31 kDa β dystroglycan protein is unknown (Losasso et al. 2000; Yamada et al. 2001), but it appears to be more readily targeted for degradation than intact β dystroglycan since its levels are increased following treatment with proteasome inhibitors (Singh et al. 2004). The identity of the protease mediating this cleavage of β dystroglycan remains controversial; Some studies have implicated matrix metalloproteases (MMPs) (Yamada et al. 2001; Singh et al. 2004) while others reported no (or partial) effect of MMP inhibitors (Jing et al. 2004). Adding to the confusion, MMP inhibitors can restore association of α dystroglycan with the cell membrane in cells where only full-length 43 kDa β dystroglycan is present (Singh et al. 2004). This result indicates the existence of a second proteolytic mechanism for disruption of the dystroglycan complex by MMPs that does not involve direct cleavage of either α or β dystroglycan.

Many studies have reported a specific loss of α dystroglycan in primary tumors and cancer cell lines however these often have used the IIH6 or VIA4-1 monoclonal antibodies that recognize only natively glycosylated α dystroglycan. Thus the apparent loss of α dystroglycan, as evidenced by loss of IIH6 or VIA4-1 binding, accompanied with preservation of β dystroglycan, could reflect hypoglycosylation of α dystroglycan or loss of α dystroglycan protein expression. Primary tumors where α dystroglycan appears to be affected include oral squamous cell carcinoma (Jing et al. 2004), cervical cancer (Sgambato et al. 2006), renal carcinoma (Sgambato et al. 2007a), glioma (Calogero et al. 2006), prostate cancer (Sgambato et al. 2007b), and pediatric rhabdomyosarcoma and neuroblastoma (Martin et al. 2007).

Some studies using antibodies to the core peptide of α dystroglycan have shown that this hypoglycosylated form of α dystroglycan remains associated with the cell membrane but is unable to bind laminin (Beltran-Valero de Bernabe et al. 2009),

and similar studies have also utilized antibodies to β dystroglycan, which is co-translated with α dystroglycan, to show maintained dystroglycan expression (Martin et al. 2007). Recent studies in tumor cell lines have attributed the hypoglycosylation of α dystroglycan to a selective loss of LARGE expression. Altered LARGE function is attributed to epigenetic modifications rather than mutations in the LARGE gene (Beltran-Valero de Bernabe et al. 2009). Finally, expression of β dystroglycan has been found to be reduced or absent in a relatively large set of primary cancers. These include prostate cancer (15 out of 15 tumors (Henry et al. 2001)), breast cancer (337/343 tumors (Cross et al. 2008); 6/6 tumors (Henry et al. 2001)), esophageal adenocarcinomas (10/10 tumors (Cross et al. 2008)), colorectal adenocarcinomas (102/105 tumors (Cross et al. 2008)), and ureteric transitional cell carcinomas (55/55 tumors (Cross et al. 2008)). Expression of glycosylated α dystroglycan and core protein was not assayed in most of these tumors. It is interesting that the same β dystroglycan antibody was found to strongly stain most pediatric cancers (Martin et al. 2007), suggesting adult and pediatric cancers may be fundamentally different with regard to dystroglycan expression.

Cancer progression involves loss of the differentiated phenotype as well as uncontrolled cellular proliferation and, in more advanced stages, acquisition of a metastatic potential associated with altered cellular interactions with the extracellular matrix. In normal mammary epithelial cells, the interaction of dystroglycan with laminin has been implicated in cellular differentiation, including the establishment of cell polarity and expression of β-casein, a gene expressed during the process of maturation (Weir et al. 2006). Down-regulation of dystroglycan expression by siRNA in non-tumorigenic mouse mammary epithelial cells leads to inhibition of lactogenic differentiation and apoptotic cell death. Given the known roles of dystroglycan in epithelial cell differentiation and maturation, it is important to determine whether the loss of dystroglycan is a secondary consequence of cellular transformation or a significant contributor to the malignant phenotype. Forced expression of dystroglycan in prostate and mammary epithelial tumor cell lines leads to inhibition of cell cycle progression, loss of anchorage-independent growth, and impairment in tumor formation in vivo (Sgambato et al. 2004, 2007b; Calogero et al. 2006). Interestingly, up-regulation of β dystroglycan, without restoration of glycosylated α dystroglycan, was sufficient to inhibit cell proliferation and significantly reduce tumorigenicity in these cancer cell lines. However, expression of markers of cell polarity and differentiation did depend on expression of glycosylated α dystroglycan capable of interacting with laminin (Muschler et al. 2002).

Taken together these observations suggest that dystroglycan performs a dual function in epithelial cells; the interaction of α dystroglycan with laminin is important for cellular polarization and maturation while signaling through β dystroglycan regulates cellular proliferation. These experiments are particularly relevant in light of the suggested correlation for some primary tumors between loss of dystroglycan expression and tumor grade or stage (Sgambato et al. 2003, 2006, 2007a; Martin et al. 2007). For example, a study of oral squamous cell carcinomas found that while primary tumors consistently lost expression of α dystroglycan, tumors showing metastasis lost both α and β dystroglycan expression (Jing et al. 2004). Thus, in these tumors, metastasis was accompanied by a complete loss of dystroglycan

protein. The possible correlation between dystroglycan expression and tumor grade or stage is tantalizing, however, the number of tumors used in many of these studies is often too small to support any clinical relevance. In addition, such results may be complicated by residual normal tissue within the samples and by the absence of generally available antibodies to the α dystroglycan core protein suitable for immunohistochemistry. Nonetheless, these studies support an important role for dystroglycan as a tumor suppressor and warrant further exploration as to dystroglycan's diagnostic and prognostic significance.

6.6 Dystroglycan and Signaling

Dystroglycan was first described as a core member of the dystrophin-associated glycoprotein (DAG) complex in skeletal muscle, connecting the cytoskeletal actin-binding protein dystrophin, or its synaptic orthologue utrophin, through the membrane to the extracellular matrix. The proteins liking to dystroglycan in the sarcolemmal membrane involve laminin and other ECM proteins in the basal lamina surrounding the myofiber, sarcoglycans within the membrane, and cytoplasmic structural proteins at the Z-band including dystrophin, desmin, plectin, myospryn, syncoilin and filamin C, among others (Fig. 6.2). The linkage to the sarcomere is more than just structural since myospryn also anchors protein kinase A to the sarcomere (Reynolds et al. 2008), which modulates contraction via phosphorylation of sarcomeric proteins including myosin and titin. In addition, the DAG complex is linked to proteins that control calcium dynamics, and calcium homeostasis is dysregulated in skeletal and cardiac muscles lacking dystrophin (Constantin et al. 2006; Williams et al. 2006; Williams and Allen 2007).

In non-muscle cells, where contraction is not an issue, dystroglycan has also been shown to perform a structural role in determining cell shape, such as polarity of mammary epithelial cells (Weir et al. 2006), and in the formation of cellular extensions such as microvilli (Spence et al. 2004a) (Fig. 6.2). In these processes dystroglycan also regulates the actin cytoskeleton via its interactions with laminin. It is therefore not surprising that restoration of cellular polarity in mammary tumor cell lines depends on glycosylation of α dystroglycan allowing binding to laminin. In this respect it is interesting that utrophin, the intracellular ligand of dystroglycan in epithelial cells, is mutated in some breast cancers (21/61 cancers screened) (Li et al. 2007) and its expression is lost in breast and prostate cancers in a manner similar to α dystroglycan (Henry et al. 2001).

The intracellular interactions of β dystroglycan are modulated by two processes (Fig. 6.2): engagement of α dystroglycan by extracellular ligands and phosphorylation of the cytoplasmic region of β dystroglycan on tyrosines, particularly tyrosine 892. In muscle cells, binding of laminin to α dystroglycan has been shown to induce activation of the PI3K/AKT and the Rac1/PAK1/JNK signaling pathways (Langenbach and Rando 2002; Oak et al. 2003). Experimental evidence includes biochemical studies of protein interactions via pull-downs (Oak et al. 2003; Xiong et al. 2009) as well as altered regulation of these signaling pathways in muscles of

mdx mice (Xiong et al. 2009), which lack dystrophin (Hoffman et al. 1987) and therefore have a destabilized DAG complex (Matsumura et al. 1992). The actions of dystroglycan on these signaling pathways appear to mediate muscle fiber survival and protection from atrophy (Chockalingam et al. 2002; Langenbach and Rando 2002). In non-muscle cells, dystroglycan can also act as a scaffold for the ERK/MAP kinase signaling cascade (Fig. 6.2). Here, dystroglycan can negatively modulate the ERK/MAP kinase activity by physically segregating the cellular compartments, thereby preventing their interaction (Ferletta et al. 2003; Spence et al. 2004b). Furthermore, dystroglycan can inhibit the ERK/MEK pathway in pancreatic cells to promote differentiation (Jiang et al. 2001). As the PI3K/AKT and MEK/ERK signaling cascades mediate cancer cell proliferation and survival (McCubrey et al. 2007), it is tempting to speculate that dystroglycan may have anti-proliferative and tumor suppressor activities that could be manipulated to alter tumor behavior or growth.

Phosphorylation of β dystroglycan has been reported to disengage dystroglycan from dystrophin and utrophin (James et al. 2000; Ilsley et al. 2001), rendering its cytoplasmic region available for interaction with SH2 domain containing proteins involved in signal transduction (Sotgia et al. 2001). Proteins that may bind to the cytoplasmic domain of β dystroglycan include Src, Fyn, Csk, Nck and Shc. The biological significance of these interactions is currently not known. One biological process linked to β dystroglycan phosphorylation involves the assembly of podosomes (Thompson et al. 2008). Podosomes are transient adhesion structures that mediate directional cellular migration and tissue invasion via protrusions called invadopodia. Podosomes are often formed on migrating tumor cells and their assembly is regulated by Rho family GTPases and tyrosine kinases including src. Overexpression of dystroglycan leads to inhibition of podosome formation by sequestering proteins essential for podosome assembly. Overexpression of a mutant dystroglycan lacking a src phosphorylation site is unable to interfere with podosome formation, indicating that this process relies on β dystroglycan phosphorylation.

The current knowledge on the connections of dystroglycan to major signaling pathways as well as its interplay with integrins favor the notion of different biological functions of dystroglycan depending on the cellular context (Fig. 6.2). In particular, dystroglycan appears to cooperate with integrins in muscle fibers where activation of the ERK/MEK and PI3K/AKT signaling pathways contributes to survival and prevent atrophy. In other cell types, dystroglycan appears to antagonize integrin's functions by inhibiting cell proliferation, promoting differentiation, and interfering with the formation of cell-matrix adhesion structures important for cell migration and tissue invasion.

6.7 Conclusions

The glycosylation of dystroglycan is complex and contains an unusual sialylated O-linked mannose structure not commonly found in mammals. This unique pattern of glycosylation involves a series of genes, *POMT1*, *POMT2*, *POMGnT1*, *FKTN*,

FKRP, and *LARGE*, some of which encode specialized glycosyltransferases. Mutations affecting the activity of any one of the proteins encoded by these genes leads to a group of diseases commonly referred to as dystroglycanopathies. Clinical features of these disorders include progressive and usually severe muscular dystrophy accompanied in some cases by neurological involvement and/or cardiomyopathy. While it is not clearly established whether dystroglycan is the only protein glycosylated by these specialized glycosyltransferases, tissue-specific dystroglycan knockout mice phenocopy many of the clinical pathological aspects associated with human disease. Thus, the disruption of dystroglycan function is likely a key determinant of phenotype. α dystroglycan is a receptor for a subset of extracellular matrix proteins and most of these interactions depend on or are modulated by glycosylation. The effects of hypoglycosylation of α dystroglycan in disease states such as dystroglycanopathies and cancer is providing insights on how glycosylation modulates key aspects of cellular function. Dystroglycan has also been implicated in extracellular matrix assembly, protection of the plasma membrane from mechanical stress, and intracellular signaling via a variety of pathways regulating cell survival, proliferation, differentiation and migration. Resolving the composition and structure of the different carbohydrate moieties on α dystroglycan in normal and disease states is essential to gain a clearer and broader picture of how the structural and intracellular signaling functions of the dystroglycan complex are regulated.

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