Proteomics and Genetic Studies of Dystroglycan Function in the Nervous System

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ABSTRACT

Muscular dystrophies are a group of diseases that are often caused by loss-of-function mutations affecting the dystrophin glycoprotein complex (DGC). The common feature of the diseases is muscle degeneration, which is often associated with mental retardation and various retinal defects, including ones of synaptic transmission. However, the mechanisms of the disease remain largely unknown, especially those in the central nervous system. I have focused on dystroglycan (DG), the transmembrane protein in the DGC that links the cytoskeleton to the extracellular matrix and is essential for muscle survival and brain development. I have used proteomics and *Drosophila* genetics to study DG function in the brain and retina.

Using proteomics I found that β-DG is directly associated with the GTPase dynamin 1 in the retina and in the brain together with α-DG and Grb2, and immunohistochemically β-DG was colocalized with dynamin 1 in the outer plexiform layer where photoreceptor terminals are localized. Moreover, loss of DG in differentiated DG-null embryonic stem cells significantly increases dynamin-mediated transferrin-uptake and re-expression of DG in null cells by infection with an adenovirus containing DG reduced transferrin uptake to levels seen in wild-type cells. This result implies that one of mechanisms in muscular dystrophy might be the altered synaptic vesicle endocytosis, especially in the retina where synaptic transmission defect has been known for decades.

Muscular dystrophies show not only impaired retinal synaptic transmission and several DG-related congenital muscular dystrophies also display retinal structural defects. To further understand the roles of DG in the retina, I used *Drosophila* eye as a model and

demonstrated for the first time that DG is required cell-autonomously for photoreceptor morphogenesis in the developing visual system. Deficiency of DG in the eye causes severe disruption of retinal structure, aberrant lens formation and abolition of electroretinogram in the adult fly eye. These adult defects appear derived from autonomous photoreceptor cell (PRC) defects in the early pupa including size arrest, loss of polarity and progressive degeneration. All defects in the eye, however, can be reversed by re-expression of wild type DG in DG-deficient PRCs, suggesting DG functions cell-autonomously in PRCs and non-autonomously for lens. In the 3rd instar larvae DG is present in the apical tips and the basal membranes of PRCs, two polarized locations opposing the extracellular matrix. At the pupal stage it continues to mainly distribute at the apical rhabdomere and basal membrane of PRCs. Over-expression of DG leads to larger ommatidia but the PRC number remains unchanged, suggesting that DG is both necessary for and sufficient to promote PRC expansion. By rescue experiments, I demonstrated that the extracellular DG alone could not rescue DG-deficient eye defects, whereas the intracellular DG can substantially ameliorate PRC degeneration and structural defects while some PRCs remain disorganized, a sign of disrupted PRC planar polarity in absence of the extracellular DG. Therefore, our data suggest that the degeneration and planar polarity disruption in DG-deficient PRCs are two independent processes that appear to require the respective function of intracellular and extracellular DG. In summary, our experiments demonstrated several novel findings and provided the basis for future investigations on DG function and the molecular mechanisms of nervous system defects in muscular dystrophies.

RESUME

Les dystrophies musculaires sont un groupe de maladies qui sont souvent causées par des mutations résultant en une perte de fonction du complexe associé à la dystrophine (DGC). Une caractéristique commune de ces maladies est la dégénérescence musculaire, qui est souvent accompagnée d'un retard mental et de défauts rétiniens, incluant ceux affectant la transmission synaptique. Les mécanismes pathologiques de ces maladies sont principalement inconnus, surtout ceux affectant le système nerveux central. J'ai étudié le dystroglycane (DG), une protéine transmembranaire du DGC qui lie le cytosquelette d'actine à la matrice extracellulaire et qui est essentielle à la survie musculaire et au développement du cerveau. J'ai utilisé la protéomique et la génétique de la mouche du vinaigre *Drosophila* pour étudier la fonction de DG dans le cerveau et dans la rétine.

En utilisant la protéomique, j'ai découvert que β-DG est directement associé à la GTPase dynamine 1 dans la rétine et dans le cerveau ainsi qu'à α-DG et Grb2; par immunocytochimie, β-DG co-localise avec dynamine 1 dans la couche plexiforme externe de la rétine là où se trouvent les terminaux des cellules photoréceptrices. La perte d'expression de DG dans des cellules souches embryonnaires différentiées et dépourvues de DG cause l'augmentation du recaptage de la transferrine contrôlée par la dynamine; la ré-expression de DG dans ces même cellules par infection avec un adénovirus exprimant DG réduit le recaptage de la transferrine à un niveau similaire aux cellules de type sauvage (DG +/+). Ce résultat suggère qu'un des mécanismes pathologiques des dystrophies musculaires est d'altérer l'endocytose des vésicules synaptiques, surtout dans la rétine où la transmission synaptique défectueuse a déja été décrite.

Les dystophies musculaires démontrent des défectuosités de la transmission synaptique rétinienne, et de plus, plusieurs dystrophies musculaires congénitales reliées au DG démontrent des défauts dans la structure de la rétine. Afin de mieux comprendre le rôle de DG dans la rétine, j'ai utilisé l'oeil de la mouche du vinaigre *Drosophila* comme modèle et j'ai démontré pour la première fois que DG est nécessaire par autonomie cellulaire à la morphogénèse des cellules photoréceptrices (PRC) lors du développement du système visuel. La déficience de DG dans l'oeil de mouche cause de sévères défauts dans la structure rétinienne et dans la formation du cristallin, ainsi que l'abolition de l'électrorétinogramme dans l'oeil adulte. Ces défauts chez l'adulte semblent provenir de défauts d'autonomie des PRC dans la pupe incluant un arrêt de croissance, une perte de polarité et une dégénérescence progressive. Tous les défauts de l'oeil peuvent être corrigés par la ré-expression du gène normal de DG dans les PRC dépourvues de DG, ce qui suggère que DG fonctionne par autonomie cellulaire dans les PRC et par non-autonomie cellulaire dans la formation du cristallin. Dans la larve de 3ième instar, DG est présent dans les terminaises apicales et la membrane basale des PRC qui sont deux structures polarisées adjacentes à la matrice extracellulaire. A l'étape de la pupe, DG est distribué dans le rhabdome apical et la membrane basale des PRC. La sur-expression de DG mènent à de plus grosses ommatidies, mais le nombre de PRC reste constant, ce qui suggère que DG est à la fois nécessaire et suffisant à l'expansion des PRC.

Par des expériences de restoration, j'ai démontré que la portion extracellulaire de DG ne peut pas corriger les défauts oculaires causés par la déficience de DG, alors que la portion intracellulaire de DG peut améliorer considerablement la dégénérescence et les défectuosités de structure des PRC malgré que quelques PRC restent désorganisés, ce qui

indique un défaut dans la polarité des PRC. Par conséquent, nos données suggèrent que la dégénérescence et les défauts de la polarité planaire dans les PRC dépourvues de DG représentent deux processus indépendants qui semblent requérir des fonctions respectives aux portions intracellulaire et extracellulaire de DG. En résumé, nos expériences apportent plusieurs nouvelles découvertes et établissent les fondations pour de futurs travaux sur la fonction de DG et sur les mécanismes moléculaires des défauts pathologiques du système nerveux dans les dystrophies musculaires.

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CONTRIBUTIONS OF AUTHORS

This thesis contains one published paper and a secured manuscript in preparation. The contributions of my co-authors are listed below.

Chapter 2 Evidence that dystroglycan is associated with dynamin and regulates endocytosis by Yougen Zhan, Mathieu R. Tremblay, Nadia Melian, and Salvatore Carbonetto. This paper is predominantly my work. Mathieu R. Tremblay contributed figure 2-6 and figure 2-7. Nadia Melian and Mingying Hu contributed figure 2-5c. Dr. Salvatore Carbonetto and I wrote the paper.

Chapter 3 Dystroglycan expression in photoreceptors is essential for *Drosophila* eye development by Yougen Zhan, Charles Bourque, Hannele Ruohola-Baker, Yong Rao, and Salvatore Carbonetto. This manuscript is predominantly my work. Dr. Charles Bourque did the electrophysiological recordings (Fig. 3-8), and I organized the figures and wrote the legends. The severe DG hypomorphic allele, UAS-DG and UAS-DG_{cyto} flies as well as anti-*Drosophila* DG antisera were generated by Dr. Hannele Ruohola-Baker' lab.

ABBREVIATIONS

AChR	acetylcholine receptor
α-DG	α-dystroglycan
Arm	armadillo
β-DG	β-dystroglycan
BM	basement membrane
BMD	Becker Muscular Dystrophy
CNS	central nervous system
DG	dystroglycan
DGC	dystrophin glycoprotein complex
DMD	Duchenne Muscular Dystrophy
DAPI	4'-6-Diamidino-2-phenylindole
ERG	electroretinogram
ES	embryonic stem
EGF	epidermal growth factor
FCMD	Fukuyama Congenital Muscular Dystrophy
FLP	flippase
FRT	Flp recombinase target
FGF	fibroblast growth factor
GFP	green fluorescence protein
Grb2	Growth Factor Receptor-bound Protein 2
GABA	γ-aminobutyric acid
GST	glutathione *S*-transferase
GTPas	guanosine triphosphatase
HSPG	heparin-sulfate proteoglycan
LGMD	Limb Girdle Muscular Dystrophy
LTP	long term potentiation
MEB	muscle eye brain disease
MDC1D	Congenital Muscular Dystrophy 1D
Myd	myodystrophy
mRFP	mono red fluorescence protein

MDC	Congenital Muscular Dystrophy
MuSK	muscle specific tyrosine kinase
MAPK	mitogen-activated-protein kinase
MF	morphogenetic furrow
NMJ	neuromuscular junction
nNOs	neuronal nitric oxide synthase
NMIgG	non-immune mouse IgG
NRIgG	non-immune rabbit IgG
PRC	photoreceptor cell
PSD	post synaptic density
PBS	phosphate buffered saline solution
PDZ	PSD-95, Disc-large, Z01
POMT1	protein O-mannosyltransferase-1
SH2/SH3	Src homology 2/Src homology 3
WWS	Walker-Warburg Syndrome
ZA	zonula adherens

Chapter 1

General Introduction

RATIONAL AND OBJECTIVES

Muscular dystrophy is a group of diseases that are often caused by loss-of-function mutations affecting the dystrophin glycoprotein complex (DGC). The common pathological features of the disease involve muscle degeneration, mental retardation and various retinal defects. However, mechanisms of the disease are largely unknown, especially for those in the nervous system.

The DGC is a protein complex spanning the sarcolemma in muscle (Fig. 1-1). The core members of DGC are comprised of dystrophin, syntrophin, dystrobrevin, nNOs in the cytoplasm, protein sarcoglycans (α-, β-, γ- and δ-sarcoglycans) and sarcospan in the sarcolemma, and the transmembrane protein dystroglycan (DG) (Ervasti and Campbell, 1991; Ervasti and Campbell, 1993b; Ervasti et al., 1991; Ibraghimov-Beskrovnaya et al., 1992). DG is comprised of a transmembrane β-DG and an extracellular α-DG (Ervasti and Campbell, 1991; Ibraghimov-Beskrovnaya et al., 1992). Through the carbohydrate chains of α-DG, the DGC is linked to laminin, agrin or perlecan in the extracellular matrix (Bowe et al., 1994; Gee et al., 1993; Gee et al., 1994; Ibraghimov-Beskrovnaya et al., 1992; Jacobson et al., 2001; Peng et al., 1998; Smalheiser and Schwartz, 1987), and to neurexin or the transmembrane agrin in other cells (Burgess et al., 2002; Burgess et al., 2000; Sugita et al., 2001). The composition of the DGC differs in tissues. For example, at neuromuscular junctions (NMJs), utrophin replaces dystrophin to bind to β-DG (Ohlendieck et al., 1991a); in the central nervous system (CNS), no sarcoglycans and sarcospan exist in the complex (Blake and Kroger, 2000; Imamura et al., 2000) and α-DG connects to neurexin (Sugita et al., 2001).

Therefore, it would be very interesting to identify novel protein(s) in the CNS of this complex.

Several congenital muscular dystrophies such as Fukuyama congenital muscular dystrophy (FCMD), Muscle-eye-brain (MEB) disease and Walker-Warburg Syndrome (WWS) are caused by mutations in putative glycosyltransferase genes and result in a defective glycosylation of α-DG (Yoshida et al., 2001; Michele et al., 2002; Michele and Campbell, 2003; Moore et al., 2002; Muntoni et al., 2002; Jimenez-Mallebrera et al., 2003) and display severe mental retardation, disrupted brain architecture, abnormal neuronal migration in brain (Michele et al., 2002; Moore et al., 2002) and retinal defects in human. The retina defects include severe structural abnormalities, retinal degeneration and blindness (Fahnehjelm et al., 2001; Hino et al., 2001; Vervoort et al., 2004; Zervos et al., 2002). For example, patients with FCMD have misfolded retina and impaired retinal structure (Hino et al., 2001). Some patients with MEB show retinal degeneration and early blindness (Fahnehjelm et al., 2001; Zervos et al., 2002). Mice models of FCMD show an abolished electroretinogram (ERG) and severely impaired retinal structure (Takeda et al., 2003). Mutations in *LARGE* gene, encoding a protein *LARGE* that is required for O-linked glycosylation of α-DG (Barresi et al., 2004; Kanagawa et al., 2004), lead to muscular dystrophy in humans (congenital muscular dystrophy 1D) (Longman et al., 2003) and in mice (Grewal et al., 2001; Holzfeind et al., 2002). These mice have neuronal migration defects in brain and defective synaptic transmission, and disrupted ERGs. However, eyes show no signs of neuronal migration defects and are largely functional (Grewal et al., 2001; Holzfeind et al., 2002) with only mild structural abnormalities in the outer plexiform layer (Lee et al., 2005). Although

these retinal phenotypes appear variable, they suggest that function of DG, especially the glycosylation of α-DG, is required for the normal retinal development.

It is known that DG and dystrophin isoforms are expressed in multiple layers of vertebrate retina, including photoreceptor terminals (Blake and Kroger, 2000; Blank et al., 2002; Jastrow et al., 2006; Koulen et al., 1998), and synaptic transmission defects in muscular dystrophy such as the Duchenne/Becker muscular dystrophy (Lenk et al., 1996; Pillers et al., 1993) caused by mutations in dystrophin gene (Hoffman and Kunkel, 1989; Koenig et al., 1988; Monaco et al., 1993) occur between photoreceptors and their postsynaptic target cells (Blake and Kroger, 2000). However, we do not know the mechanisms leading to the defective synaptic transmission; neither do we know those similar synaptic defects in congenital muscular dystrophies. One step toward answering the question is to look for novel DG-associated proteins in the CNS. Chapter 2 of this thesis describes experiments that show DG is associated with the GTPase dynamin 1 in brain and in the outer plexiform layer of retina and regulates dynamin-dependent endocytosis.

Furthermore, although the function of DG in the brain has been nicely studied in brain-specific DG-null mice that show similar defects as in FCMB, MEB, WWS and congenital muscular dystrophy 1D (Michele et al., 2002; Moore et al., 2002), some questions remain to be answered. First, we do not know the cell type that causes a specific phenotype in brain because DG is expressed in both neuron and glia and the gene deletion affects DG in both of them. It is critical to understand in what cell type and how the neuronal migration defects unfold and what causes the misfolding of cortex. Furthermore, as suggested from retina in the human congenital muscular

dystrophies and in *LARGE* mutant mice, the phenotypes in retina appear quite variable. Therefore, the precise roles of DG in the eye remain to be revealed in animal models. Chapter 3 of this thesis describes experiments using *Drosophila* eye as a model to study DG function in the nervous system. *Drosophila* allows the unbiased and easily manipulable genetics, and its simple anatomy can provide clear information about the requirement of DG in different developmental stages. Moreover, the highly conserved DG structural domains (discussed below in general literature review) suggest a highly conserved function in non-vertebrate and vertebrate. My genetic studies reveal that deficiency of DG in the eye causes loss of retina, a severe phenotype that is derived from autonomous photoreceptor cell (PRC) defect during early pupal development. Furthermore, with rescue experiments I demonstrate that degeneration and planar polarity disruption in DG-deficient PRCs are two independent processes, which appear to be functions of intracellular and extracellular DG respectively.

GENERAL LITERATURE REVIEW

Part I: Dystroglycan and muscular dystrophy

I: Muscular dystrophy and the dystrophin glycoprotein complex

Muscular dystrophies are a group of muscle disorders that primarily affect skeletal muscles and are marked by progressive muscle wasting and weakness. Among all the muscular dystrophies, Duchenne and Becker muscular dystrophies are the most common ones, affecting one in 3300 (Duchenne) or 30,000 (Becker) and often leading to death in late teens and early twenties (Brown, 1997; Dalkilic and Kunkel, 2003). Both diseases are the consequence of mutations in a gene called the Duchenne muscular dystrophy (DMD) gene located on the X chromosome and encoding a protein called dystrophin (Hoffman and Kunkel, 1989; Koenig et al., 1988; Monaco et al., 1986). Dystrophin is very large cytoskeletal protein that is underneath the muscle sarcolemma, and is completely absent in Duchenne muscular dystrophy (DMD) patients but partially expressed in milder patients with Becker muscular dystrophy (BMD) (Campbell, 1995). Biochemical purification of dystrophin from skeletal muscle led to identification of the dystrophin glycoprotein complex (DGC) (Campbell and Kahl, 1989; Yoshida and Ozawa, 1990; Ervasti and Campbell, 1991; Ervasti et al., 1991; Ohlendieck et al., 1991b). The identified complex contains the extracellular protein laminin, the transmembrane protein β-dystroglycan (DG) and its firmly attached extracellular protein α-DG, the intracellular proteins syntrophin, dystrobrevin as well as membrane proteins sarcoglycans and sarcospan (Chan et al., 1998; Ervasti and Campbell, 1993b). As illustrated in Figure 1-1, the DGC spans the muscle sarcolemma and links the intracellular cytoskeleton to the extracellular matrix through

multiple protein-protein interactions (Ibraghimov-Beskrovnaya et al., 1992). Intracellularly, actin binds to the N-terminus of dystrophin, whereas DG, syntrophin and dystrobrevin bind to its C-terminus. These proteins form cytoplasmic complex and are linked to the extracellular matrix through the transmembrane protein DG as its extracellular portion binds to matrix protein laminin. Within the sarcolemma four sarcoglycans including alpha, beta, gamma and delta, epsilon and zeta-sarcoglycan and one sarcospan are associated with DG (Chan et al., 1998).

It is generally believed that one important function of the DGC is to protect muscle sarcolemmal integrity against shear stresses imposed by constant muscle contraction. As is known by genetic studies, mutations in genes encoding DGC members are responsible for many muscular dystrophies (Table 1) (Campbell, 1995; Dalkilic and Kunkel, 2003; Straub and Campbell, 1997). As noted above, mutations in dystrophin gene leading to absence or reduced levels of dystrophin protein are responsible for DMD and BMD. In addition, mutations in four sarcoglycans are responsible for four variants of type 2 Limb-girdle muscular dystrophies (Bonnemann et al., 1996; Nigro et al., 1996a; Noguchi et al., 1995; Vainzof et al., 1996). Mutation in α2 laminin (merosin) is responsible for at least one form of congenital muscular dystrophy as its murine model called dy^{2j} mouse (Xu et al., 1994a; Xu et al., 1994b). There have no reported human mutations in genes encoding DG, syntrophin or dystrobrevin to cause diseases. However, recent studies have found that hypo-glycosylation of DG on its extracellular α-subunit is associated with several congenital muscular dystrophies (discuss below).

II: DG and congenital muscular dystrophies

DG was first found in the brain as a laminin receptor (Smalheiser and Schwartz, 1987; Douville et al., 1988) and biochemical studies purified it from muscle and showed that it is the core member of the DGC by binding to laminin via its carbohydrate chains and to dystrophin via its intracellular last 20 amino acids (Campbell and Kahl, 1989; Ervasti and Campbell, 1991; Ervasti and Campbell, 1993a; Ervasti et al., 1991; Gee et al., 1993; Ibraghimov-Beskrovnaya et al., 1992; Jung et al., 1995; Ohlendieck et al., 1991b). Deficiency of DG in mice leads to embryonic lethality at day 6.5 due to failure in Reichert's membrane formation (Williamson et al., 1997). Studies from *Drosophila*, which has highly conserved DG ortholog, revealed that DG is required for apical-basal polarity formation in epithelial cells (Deng et al., 2003). In mice, deletion of DG in skeletal muscles causes muscular dystrophy, similar but more severe than dystrophin-null mice (Cohn et al., 2002; Cote et al., 1999).

DG is encoded as a single polypeptide by a gene located in human chromosome 2. The polypeptide is post-translationally cleaved into α and β-subunits, and modified by glycosylation (Holt et al., 2000). β-DG is inserted into the plasma membrane as a single-pass transmembrane protein and α-DG is non-covalently, but stably, associated with the N-terminus of β-DG. Full-length DG contains 895 amino acids. The extracellular region of α-DG consists of amino acids 1-653, while the transmembrane protein β-DG consists of amino acids 654-895. There are proline-rich domains that contain PxxP or PPxY consensus sequences in the intracellular portion of β-DG, through which DG can bind to the W-W domain of dystrophin, SH3 domain of Grb2 and other proteins (discussed in part II). α-DG contains a mucin-like region from

amino acid 315 to amino acid 485 with high homology (30% identity) to intestinal human mucin-2 and other mucin-like proteins (Brancaccio et al., 1995; Ibraghimov-Beskrovnaya et al., 1992; Smalheiser and Kim, 1995). Forty percent of the amino acids in this region are proline and threonine residues, and about 50 serine and threonine residues are potential sites for O-linked glycosylation, but the particular serine and threonine that are glycosylated are unknown (Martin, 2003). Outside the mucin region are three potential N-glycosylation sites (Ibraghimov-Beskrovnaya et al., 1992) with consensus Asn-X-Ser/Thr (where X is any amino acid except proline) (Fig.1-2). Indeed β, especially α-DG is highly glycosylated. The amino acid sequence of β-DG predicts a molecular weight of 26 kilodalton (KD), but by gel electrophoresis it is 43 KD in almost all tissues. α-DG is predicted as having a molecular weight of 57 KD, but it is 156 KD in skeletal muscle, 140 KD in cardiac muscle and 120 KD in brain and peripheral nerve (Henry and Campbell, 1996; Winder et al., 2001). When purified α-DG from vertebrate skeletal muscle is treated with trifluoromethanesulfonic acid (TFMS), a chemical that removes N- and O-linked oligosaccharides, as well as glycosaminoglycan chains from proteoglycans (Soroka and Farquhar, 1991), a 57 KD core protein is shown on SDS-PAGE (Ervasti and Campbell, 1993b). Chemically deglycosylated protein is not recognized by IIH6, a monoclonal antibody raised to specific carbohydrate moieties of α-DG, nor does it bind to its ligands laminin (Ervasti and Campbell, 1993b) and agrin (Bowe et al., 1994). Removal of the N-linked glycans by treatment with N-glycosidase F, which cleaves Asn-linked high mannose and hybrid and complex oligosaccharides, alters its molecular weight by about 4 kDa but does not affect its binding to extracellular ligands (Ervasti and Campbell, 1991). Treatment with

neuraminidase, an enzyme which removes sialic acids, results in 10 KD decrease in size but does not affect laminin binding ability (Ervasti and Campbell, 1993). The lectin jacalin and peanut agglutinin bind to Galβ1-3GalNAc disaccharides of α-DG, while the binding of these lectins to α-DG is increased by neuraminidase treatment that removes the terminal sialic acid (Smalheiser and Kim, 1995), it does not affect the laminin binding to α-DG (McDearmon et al., 2003). Together, these data demonstrated that α-DG is heavily glycosylated, and the glycan chains appear critical for laminin binding.

Recent genetic and biochemical experiments have made tremendous advances in our understanding of the pathogenesis of congenital muscular dystrophies and the importance of carbohydrate moieties in these diseases. Hypo-glycosylation of α-DG secondary to mutations in enzymes required for glycosylation has been demonstrated to be responsible for Fukuyama congenital muscular dystrophy (FCMD), Muscle-eye-brain (MEB) disease, and Walker-Warburg Syndrome. In addition, muscular dystrophy patients also have severe mental retardation and neuronal migration abnormalities. FCMD is one of the most common muscular diseases in Japan (Kobayashi and Toda, 2004). The FCMD gene was defined because of a retro-transposon inserted into the 3" untranslated region, resulting in unstable transcript (Kobayashi et al., 1998). The gene encodes a protein called fukutin which is a putative fringe-like glycosyltransferase resident in the Golgi apparatus (Kobayashi et al., 1998; Manya et al., 2003). The mutant gene leads to diminished glycosylation of α-DG and inability to binds to extracellular ligands (Hayashi et al., 2001; Michele et al., 2002). A gene encoding a fukutin-related protein has also been identified to be a putative glycosyltransferase that,

when mutated, is responsible for congenital muscular dystrophy type 1C (Brockington et al., 2001a; Brockington et al., 2001b). Muscle-eye-brain (MEB) disease has similar symptoms as FCMD, which is caused by mutations in the gene that encodes O-mannose β-1,2-N-acetylglucosaminyl-transferase (POMGnT1) that is known to participate in O-mannosyl glycan synthesis (Yoshida et al., 2001). Walker-Warburg Syndrome (WWS) is another form of congenital muscular dystrophy associated with cobblestone lissencephaly (Kanoff et al., 1998). The mutant gene in this disease encodes O-mannosyltransferase 1 (POMT1) located in the endoplasmic reticulum and might involve in initial O-linked protein glycosylation (Jurado et al., 1999; Willer et al., 2003). A common feature of these muscular dystrophies appears to involve in defective glycosyltransferases, thus resulting in loss of the carbohydrate side chains of α-DG, and loss of ligand binding of α-DG to the extracellular matrix (Michele et al., 2002). Various mouse models in which these genes have been deleted by homologous recombination mimic the human diseases (Takeda et al., 2003; Willer et al., 2004; Grewal et al., 2001). Deletion of fukutin gene results in early embryonic lethality at embryonic day 6.5-7.5 prior to muscle development, and chimeric mice consisting of the mixture of fukutin-deficient and wild-type cells develop severe muscular dystrophy with loss of α-DG glycosylation and laminin-binding activity (Takeda et al., 2003). In addition, there are central nervous system (CNS) phenotypes resulting from abnormal neuronal migration and retinal disruption including loss of the retinal laminar structure and retinal detachment as well as the non-neural structural anomaly of the lens (Michele et al., 2002; Takeda et al., 2003). Targeted deletion of POMT1 gene resulted in embryonic developmental arrest at day 7.5 and death between day 7.5 and 9.5, which

appear due to failure of Reichert's membrane formation, a possible consequence of loss of α-DG glycosylation (Willer et al., 2004). In *myd* mice, a spontaneous loss-of-function mutation in the *LARGE* gene leads to deficiency in α-DG glycosylation, loss of laminin binding, muscular dystrophy, brain malformation and retinal phenotypes but mice survive to the adulthood (Grewal et al., 2001; Holzfeind et al., 2002). LARGE appears to bind to a sequence N-terminal to mucin-like domain and essential for initiating the glycosylation of α-DG (Kanagawa et al., 2004). Over-expression of LARGE in cells isolated from MEB, FYMD and WWS patients restores functional carbohydrates that can bind to laminin and circumvent the α-DG defect (Barresi et al., 2004). Mutations in the LARGE gene have also been identified in a single family with congenital muscular dystrophy 1D (MDC1D), which shows congenital onset of muscle weakness, profound mental retardation and brain malformation (Longman et al., 2003). Although no mutations in DG gene are associated with human congenital muscular dystrophies, targeted-deletion of DG in muscle and brain results in similar phenotypes of congenital muscular dystrophies (Cohn et al., 2002; Cote et al., 1999; Moore et al., 2002). These data suggested that carbohydrates of α-DG are essential for the function of DG. This is supported by recent data that viral introduction of intracellular portion of DG in DG-deficient muscles is sufficient for assembly and targeting of the DGC complex to the muscle surface, but to prevent muscular dystrophy in DG-deficient muscles, α-DG and its carbohydrates are required (Kanagawa et al., 2004).

Part II: DG and its associated proteins in muscle

Serving as the core member of the DGC and the transmembrane linker between the cytoskeleton and basement membrane, DG plays its biological roles through interactions with many extracellular and intracellular proteins.

Laminin and Basement membrane

In skeletal muscle, a layer of extracellular matrix called basement membrane (BM) coats muscle fibers providing structural and regulatory roles and involved in biological processes such as cell growth, differentiation, and migration, tissue development and repair (Erickson and Couchman, 2000; Timpl, 1996; Yurchenco et al., 2004). The BM contains glycosylated proteins but no lipid or nucleic acid (Sanes, 2003). BM proteins are classified as collagenous glycoproteins, non-collagenous glycoproteins and proteoglycans (Hall and Sanes, 1993). Collagen (collagen IV) is the major component of the BM and can self-assemble into a network. The major heparan sulfate proteoglycans (HSPG) are perlecan and agrin. The major non-collagenous proteins of BM are entactin and laminin (Carbonetto and Lindenbaum, 1995; Hall and Sanes, 1993; Jenniskens et al., 2005). The BM has two networks, the collagen type IV network and the laminin network interconnected via entactin-1 (Battaglia et al., 1992; Brown et al., 1997; Fox et al., 1991). Recent data suggest that the laminin network is prerequisite for the basement formation, which may bring the collagen, nidogen, perlecan and other components to form big BM network (Yurchenco et al., 2004).

Laminin is a glycoprotein heterotrimer consisting of a α, β and γ chains. The individual polypeptide chains are joined together to produce a molecule with one long arm and three separate short arms (Fig. 1-3) (Beck et al., 1990; Miner and Yurchenco,

2004), but laminin isoform laminin 6-9 lacks the α-chain short arm. The α-chain extends beyond the coiled-coil of the long arm and forms five β-sandwich LG modules called G-domains (Fig.1-3) (Timpl et al., 2000). Five laminin α, four β and three γ chains have been identified in mammals which can join to form 15 distinct laminin heterotrimers (Laminins 1-15) (Jenniskens et al., 2005; Timpl et al., 2000). Four out of 15 have been found in skeletal muscle. Laminin 2 (α2β1γ1) is located in the extra synaptic BM, whereas laminin 4 (α2β2γ1), laminin 9 (α4β2γ1) and laminin 11 (α5β2γ1) are located in the synaptic BM although laminin 4 is also non-synaptic (Miner et al., 1997; Miner and Yurchenco, 2004; Patton et al., 1998; Patton et al., 1997; Sanes, 2003).

Laminin binds to entactin through its γ chain at its short arms (Timpl et al., 2000) and collagen IV through the globular domains on its short arms (Charonis et al., 1985). In addition, N-terminal globular (LN) domains of short arms can interact homophilically to form laminin polymer network in the presence of calcium (Yurchenco and Cheng, 1993). Thus, laminin, entactin and collagen form an interconnected BM scaffolding (Yurchenco et al., 2004).

The five LG domains on the COOH-terminal of α chains beyond the coiled-coil region mediate interactions with the cell surface receptors (Yurchenco et al., 2004). DG and integrin are the two cell surface receptors known to interact with the G-domains of laminin. Integrins are heterodimers composed of non-covalently associated α and β subunits, acting as a mechanical linker and a signaling transducer to regulate cell proliferation, differentiation, adhesion, migration and survival (Hynes, 1992). The major forms of integrin in skeletal muscle which bind laminin are α7β1, α6β1 and

α3β1 (Fig. 1-3) (Collo et al., 1993; Kaufman et al., 1985; Mayer, 2003; Velling et al., 1996), all of which interact with LG domain 1-3 of laminin. α1 and α2 chains and α1β2 and α2β1 integrins also bind to LN domain of laminin α-chain (Miner and Yurchenco, 2004). The LG 4 of laminin α-chain interacts with carbohydrates of α-DG (Ido et al., 2004; Li et al., 2002; Talts et al., 2000; Yu and Talts, 2003) in a calcium-dependent manner (Gee et al., 1993; Ibraghimov-Beskrovnaya et al., 1992; Smalheiser and Kim, 1995). Kanagawa et al. (Kanagawa et al., 2004) have demonstrated that carbohydrates attached on the first half (amino acid 316-418) of the mucin-like domain of α-DG interact with laminin, which appears to require interaction of *LARGE* with the NH2-terminal (amino acid 29-316) of α-DG.

Laminin interacts with its receptors DG and integrin and plays roles in muscle maintenance, muscle survival, signaling and myogenesis. It is well known that laminin plays a structural role as the linker between cytoskeleton and BM to maintain the muscle integrity. Laminin could also send signals from extracellular matrix to the cell inside through DG and integrin to promote cell survival. For example, absence of the α2 chain induces high level of apoptosis (Sanes, 2003; Vachon et al., 1996) and loss of LG domain 4-5, the DG-binding domains, of laminin 1, results in early death at embryonic day 6.5 (Scheele et al., 2005), indicating active signals may come from laminin α2 and G-domain of α1 chain. Furthermore, Montanaro et al. (Montanaro et al., 1999) have found that cultured myoblasts with significantly decreased DG expression showed significant apoptosis in the myotubes. Similarly apoptosis was also found in the DG-knock-out embryoid bodies in culture (Li et al., 2002). Another interesting report comes from studies of BM and alveolar epithelial cells in the lung

(Jones et al., 2005), which shows that mechanical stretch of the lung induces activation of mitogen-activated-protein kinase (MAPK) in epithelial cells. This activation is significantly reduced when function-perturbing antibodies against α3 laminin and α-DG were used or when DG is knocked down with RNAi. No reduction in MAPK activity was observed when the tissue was treated with function-perturbing antibodies against β1 or α3 integrins, or with the RGD peptide that inhibits binding of integrin to another BM component fibronectin. Moreover, a recent report from Yurchenco and colleagues also showed that galactosyl-sulfatide (a laminin binding glcolipid)-induced laminin polymerization can activate Src kinase phosphorylation and association of c-Src with DG in culture Schwann cells, a biochemical process that requires laminin interaction with DG but not integrin (Li et al., 2005). These experiments strongly suggest that active signals could come from BM and laminin transduced through DG into the cells. Laminin is also involved in myogenesis. Myogenesis is a process including myoblast proliferation, myoblast fusion and myotube formation. Laminin has been shown to promote myoblast proliferation and myotube formation (Kroll et al., 1994; Kuhl et al., 1982; Ocalan et al., 1988; von der Mark and Ocalan, 1989), and, in the absence of laminin, formation of myotubes by myoblast fusion decreased, but is still present (Foster et al., 1987; Sanes, 2003). However, both in vitro and in vivo data suggest that the laminin receptor DG is not required for myoblast proliferation and fusion (Cohn et al., 2002; Cote et al., 1999; Montanaro et al., 1999). But, remarkably, in skeletal muscles DG appears to be required for satellite cell-mediated muscle regeneration (Cohn et al., 2002; Cote et al., 1999). When homozygous DG-null alleles were used to generate DG-deficiency in the chimeric mice, skeletal muscles showed

severe muscle degeneration with increasing age, remarkable tissue fibrosis and macrophage infiltration (Cote et al., 1999). However, in conditional knock-out mice when DG-deficiency is induced by muscle creatine kinase (MCK) promoter that does not target the satellite cells, skeletal muscle showed very mild muscle degeneration because DG-positive satellite cells make new muscle fibers (Cohn et al., 2002). The difference in muscle phenotypes in two kinds of experimental mice strongly suggests that DG is necessary for muscle satellite cell-induced regeneration.

That laminins are required for BM assembly has been well documented (Yurchenco et al., 2004; Yurchenco and Wadsworth, 2004). For example, failure to form the laminin heterotrimer leads to lack of BM assembly in γ1 and α2 chain knockout mice (Smyth et al., 1999; Xu et al., 1994a). However, whether the receptors of laminin, DG and integrin, are required for BM assembly is not conclusive. In skin β1 integrin knock-out results in disrupted epidermal basement membrane (DiPersio et al., 1997; Raghavan et al., 2000), but β1 integrin in muscle, Schwann cell, brain and cultured embryoid bodies appears not required for BM formation (Feltri et al., 2002; Schwander et al., 2003). In muscle and developing embryos, evidence does not support DG is required for BM assembly either. First, deficiency of DG does not prevent basement membrane assembly in muscle and peripheral nerve (Cohn et al., 2002; Cote et al., 1999; Moore et al., 2002; Saito et al., 2003). Secondly, genetic deletion of the DG-binding domain LG 4-5 of α chain of laminin 1 (α1β1γ1), the major BM protein in the early embryogenesis, results in lethality at embryonic day 6.5, but the BM is intact (Scheele et al., 2005). Thirdly, DG-null embryoid bodies spontaneously form basement membrane in-vitro (Li et al., 2002). These experiments suggest that for muscle and

embryoid body DG-deficiency does not result in BM disruption. Therefore, DG-deficiency-induced muscle degeneration and embryonic lethality are probably not due to disruption of BM. On the other hand, several observations appear to support that DG is required for BM assembly in some instances. First, DG-null embryos at day 6.5 appeared to result in failure in Reichert's membrane formation (Williamson et al., 1997). Second, in brain DG is required for integrity of the glial limitans that separates the marginal zone from the subarachnoid space, which is disrupted in DG-null mice (Moore et al., 2002). Third, in the neuromuscular junctions, DG appears to regulate BM molecules such as laminin, achetylcholinesterase, perlecan to the synaptic sites (Jacobson et al., 2001). Therefore, laminin and its receptor DG are required for normal cell function, but BM assembly doesn't appear to require the laminin receptor in some tissues, at least in muscle and developing embryos.

Different isoforms of laminins are also found in *Drosophila* basement membranes with similar trimeric subunit composition as that in the vertebrate (Fessler et al., 1987). α3,5; β1; γ1 was first identified in almost all organs (Fessler et al., 1987; Montell and Goodman, 1988; Montell and Goodman, 1989). Null mutations in α3,5, also called Laminin A (LamA), lead to embryonic lethality with visible defects in mesoderm-derived tissues such as heart with cell migration defect, and muscle with distorted myotube shape during its extension and failure to attach to epidermis (Prokop et al., 1998; Yarnitzky and Volk, 1995). Pathfinding defects are also observed in ocellar neurons (Garcia-Alonso et al., 1996). At the neuromuscular junctions, the functional synapses form properly without α3,5, but the extent of contact between neuronal and muscle surface is reduced significantly (Prokop et al., 1998), and in contrast embryos

lacking PS integrin have increased nerve-muscle contact surface (Prokop et al., 1998). Hypomorphic mutants of LamA also give rise to rough eyes with fused photoreceptor cells, disorganized cell clusters and sometimes few photoreceptor cells in pupa and adult retina (Henchcliffe et al., 1993). Another *Drosophila* laminin isoform called *wing blister* was also found to cause similar phenotypes as LamA (Martin et al., 1999). *Wing blister* encodes the *Drosophila* counterpart of vertebrate α2 chain and homozygous *wing blister* clones show disorganized photoreceptor cells in the retina (Martin et al., 1999).

Perlecan and Biglycan

In addition to laminin in the BM as the ligand for DG, perlecan and biglycan are also ligands of DG. Perlecan was first identified from murine tumor cell line as a heparan sulfate proteoglycan in BMs (Hassell et al., 1980). Subsequent biochemical studies showed it contains a ~395 kDa single polypeptide core with three glycosaminoglycan side chains on its N-terminus and one chondroitin sulfate chain at the C-terminus (Ledbetter et al., 1985). The amino acid sequence of perlecan contains five distinct domains (Noonan et al., 1991). Domain I is the N-terminus containing sites for heparan sulfate attachment; domain II is similar to cholesterol binding region of the low density lipoprotein; domains III is the EGF-like repeats; domain IV contains 21 Ig-like repeats. At the C-terminus is domain V characterized by three laminin-like globular G-domains. A homologue of perlecan has also been found in C. *elegans* (Rogalski et al., 1993) and *Drosophila* (Friedrich et al., 2000). Perlecan binds to BM proteins including laminin, collagen IV, entactin and fibronectin through domain IV

(Hopf et al., 1999). Fibroblast growth factor (FGF) binds to the core proteins and glycosaminoglycan side chains of perlecan (Aviezer et al., 1994; Mongiat et al., 2001), thus perlecan modulates growth factor signaling for cell proliferation and differentiation (Aviezer et al., 1994; Iozzo et al., 1994). Through the G-domains in its C-terminus, perlecan binds to DG and integrin (Brown et al., 1997; Talts et al., 1999). At the neuromuscular junctions (NMJ), perlecan interacts with acetylcholinesterase and clusters it within the synaptic BM through interaction with DG (Peng et al., 1999). Perlecan-null mice have no acetylcholinesterase clusters at the NMJ (Arikawa-Hirasawa et al., 2002) and DG-null mice have disrupted localization of acetylcholinesterase at the NMJ (Jacobson et al., 2001). In general, perlecan-null mice often die at embryonic day 10.5 with defective cephalic and cardiac development, and some survive until birth but have skeletal dysplasia characterized by shortened long bones and craniofacial abnormalities (Arikawa-Hirasawa et al., 1999; Costell et al., 1999) indicating perlecan is essential for cartilage development and BM integrity.

Biglycan belongs to a small leucine-rich proteoglycans (SLRP) family that is abundantly expressed in tissue such as bone and skin (Ameye and Young, 2002; Young et al., 2002). Biglycan has two chondroitin sulfate glycosaminoglycan chains attached to the N-terminus, and is an extracellular matrix protein. Biglycan binds to collagen and biglycan knockout mice develop various phenotypes which are primarily resulted from abnormal collagen fibrillogenesis (Ameye and Young, 2002). The 125 kDa biglycan was first identified to interact with DG in *Torpedo* electric organs by a ligand blot overlay assay (Bowe et al., 2000). Biglycan binds with its chondroitin sulfate side chains to the core protein of the α-DG at the muscle membrane and the NMJs (Bowe et

al., 2000). Moreover, in *mdx* mice biglycan expression is increased in response to changes in the DGC.

Dystrophin and Utrophin

In addition to above extracellular proteins that bind to α-DG, several other intracellular proteins are known to bind β-DG to form the core members of the DGC. Positional cloning of the gene altered in Duchenne or Becker muscular dystrophy led to identification of dystrophin (Monaco et al., 1986). Biochemical characterization identified dystrophin as part of a protein complex, the DGC, at the plasma membrane of all muscle fibers. The dystrophin gene consists of 79 exons spanning 2.4 million base pairs of the human short arm of the X chromosome (Koenig et al., 1988). Full-length dystrophin is translated from a 14 Kb transcript and has 3685 amino acids with a molecular weight of 427 kDa. Alternative splicing generates several C-terminal dystrophin isoforms that include Dp260, Dp140, Dp116 and Dp70. These dystrophin isoforms are expressed tissue-specifically. Full-length dystrophin is expressed in skeletal muscle, epithelial cells and in brain (Blake and Kroger, 2000; Mehler, 2000; Nguyen et al., 1991). In brain, it is expressed in retina and in the postsynaptic density of excitatory synapses (Blake and Kroger, 2000; Lidov et al., 1990). Dp71 is expressed in a variety of tissues including brain, retina and muscle (Austin et al., 1995). Dp140 is brain specific (Lidov et al., 1995) and Dp116 is expressed in the Schwann cells (Byers et al., 1993; Imamura et al., 2000). Dp260 is expressed specifically in the outer plexiform layer of the retina (D'Souza et al., 1995). The different expression pattern suggests different DGC complexes and distinct functions. Full-length dystrophin

contains multiple domains with overall similarity to the cytoskeletal proteins β-spectrin and α-actinin (Koenig et al., 1988). The N-terminus from amino acid 1-260 of dystrophin is the actin-binding domain similar to the actin-binding region of actinin and has been shown to bind to actin (Corrado et al., 1994; Levine et al., 1990; Root, 1997). Next to the actin-binding region is 24 spectrin-like repeats called the rod region that spans from amino acids 300-3000 (Koenig et al., 1988). C-terminal to the rod region lies the cysteine-rich region that includes a WW region, two EF-hand-like regions and one ZZ domain. The WW domain is a ~30 amino acid region containing two conserved tryptophans (hence WW). The EF-hand like region is usually a motif involved in calcium binding, but the consensus sites for calcium-binding in this region are not conserved (Koenig et al., 1988). Instead, calcium-calmodulin has been shown to bind to the ZZ domain (Anderson et al., 1996), a putative zinc finger motif found in many proteins. The most important domains of dystrophin are its WW domain and the coiled-coil domain, C-terminal to the cysteine-rich domain. Through the WW domain dystrophin binds to the PPXY motif on the last 15 amino acids of DG (Jung et al., 1995; Yoshida and Ozawa, 1990), which can be blocked by tyrosine phosphorylation on PPXY motif (Sotgia et al., 2001). Through the coiled-coil domain, dystrophin binds to syntrophin and dystrobrevin (Ahn et al., 1996; Peters et al., 1997b; Piluso et al., 2000; Suzuki et al., 1995). Mutations in dystrophin gene that leads to truncated proteins containing no N-terminal actin-binding domain, no cysteine-rich domain, or no C-terminal coiled-coil domain result in severe Duchenne muscular dystrophy in human (Campbell, 1995).

Utrophin is a 395 kDa dystrophin-related protein encoded by an autosomal gene. Utrophin is quite similar to dystrophin in its amino acid sequence and contains all the secondary domain structures of dystrophin (Blake et al., 1996b). Unlike dystrophin that is expressed uniformly in the muscle sub-sarcolemma and concentrated at neuromuscular junctions (NMJs), utrophin is only expressed at the NMJs, where it replaces dystrophin to associate with other DGC members (Matsumura et al., 1992; Nguyen et al., 1991). In the regenerating muscle fibers of *mdx* mice, a line of mice which lacks dystrophin (Sicinski et al., 1989), utrophin is expressed in the extra-synaptic membranes to compensate for dystrophin (Khurana et al., 1991; Nguyen et al., 1991; Ohlendieck et al., 1991a; Pons et al., 1991), thus, no obvious muscle phenotypes are found in these mutant mice (Carnwath and Shotton, 1987; Coulton et al., 1988; Torres and Duchen, 1987). Unlike single mutants, mice deficient in both dystrophin and utrophin develop severe phenotypes resembling those of DMD (Deconinck et al., 1997; Grady et al., 1997). However, these double mice survive to adulthood, which are different from DG-null mutant mice that die at embryonic day 6.5. This difference between DG-null and dystrophin and utrophin-double null mice demonstrates that DG plays much broader biological roles and may have other molecule(s) associated. In addition to utrophin, there is another dystrophin-related protein called dystrophin related protein 2 (DRP2), which is expressed in brain and enriched in postsynaptic density (Dixon et al., 1997; Roberts and Sheng, 2000). DRP2 is also expressed in Schwann cells of periphery nerve and is associated with L-periaxin, suggesting a role in regulating myelination (Sherman et al., 2001).

Syntrophin and dystrobrevin

Syntrophin and dystrobrevin are two intracellular core members of the DGC, which interact with DG indirectly through dystrophin. Syntrophin was originally identified as a 59 kDa protein in the *Torpedo* electroplaques and skeletal muscles (Froehner et al., 1987). When dystrophin was purified from the skeletal muscle by lectin chromatography, a 59 kDa protein was co-purified with other DGC members, which is now named as syntrophin (Butler et al., 1992; Ervasti and Campbell, 1991; Ervasti et al., 1991). Studies have identified five syntrophins encoded by five different genes, and subsequently named as α1, β1, β2, γ1 and γ2 (Adams et al., 1993; Ahn et al., 1996; Piluso et al., 2000; Yamamoto et al., 1993). All syntrophins can bind to the coiled-coil region of dystrophin or DRPs (Piluso et al., 2000; Suzuki et al., 1995). α1-syntrophin, like the full length dystrophin, is expressed primarily in skeletal muscle sarcolemma and enriched in the neuromuscular junctions but also in cardiac muscle and brain (Adams et al., 1993; Peters et al., 1997a). β1-syntrophin has a similar distribution pattern to α1-syntrophin in brain, skeletal muscle and the neuromuscular junctions, but is highly expressed in liver (Ahn et al., 1996). β2-syntrophin, however, is expressed only in the neuromuscular junctions of skeletal muscles, but not the extra-synaptic membrane (Adams et al., 1993; Peters et al., 1997a). γ1 and γ2-syntrophin are exclusively expressed in the brain and associated with Dp71 and Dp140 (Piluso et al., 2000). Syntrophin is believed to serve as an adapter protein to recruit signaling molecules to the DGC. All syntrophins contain several distinct domains: a pleckstrin homology (PH) domain, a PDZ domain (so named to indicate its presence in postsynaptic density protein 95 (PSD-95), *Drosophila* disc large, and the zonula

occludens-1 (ZO-1) (Sealock and Froehner, 1994), and a highly conserved-carboxyl syntrophin-unique (SU) domain (Adams et al., 1995) through which it binds to dystrophin and DRPs (Adams et al., 1995; Piluso et al., 2000). The PDZ domain is a protein motif involved in protein-protein interaction. PDZ domains can interact homophillically or bind to the S/T XV (where X stands for any amino acid) motif at the C-terminus of other proteins (Brenman et al., 1996; Kornau et al., 1995). The PDZ domain of α1-syntrophin has been known to bind to PDZ protein neuronal nitric oxide synthase (nNOs) in brain and in muscle (Brenman et al., 1996; Hashida-Okumura et al., 1999). nNOs is a required enzyme for production of nitric oxide that regulates myogenesis (Lee et al., 1994), metabolism (Roberts et al., 1997), contraction (Kobzik et al., 1994) and blood flow (Sander et al., 2000; Thomas et al., 1998) in skeletal muscle. β2-syntrophin binds to a family of PDZ domain-containing proteins called microtubule-associated serine/threonine kinase 205 kDa (MAST205) and syntrophin associated serine/threonine kinase (SAST) at the neuromuscular junctions (Lumeng et al., 1999). Syntrophin also interacts with sodium channels whose C-terminus contains the PDZ-binding motif S/TXV in the skeletal muscles (Gee et al., 1998). At the endfeet of astrocytes which attach to blood vessels in the brain, syntrophin interacts with the aquaporin-4 water channel through the consensus motif Ser-Ser-Val (Adams et al., 2001). In α1-syntrophin-deficient mice astrocytic endfeet membrane and skeletal muscle show significant loss of localization of aquaporin-4 (Neely et al., 2001), indicating a role of DGC in stabilizing the water channel. Consistent with this, a recent study demonstrated that laminin could induce aquaporin-4 aggregation via the DGC in the cultured astrocytes (Guadagno and Moukhles, 2004). Brain γ1-syntrophin interacts

with diacylglycerol kinase-z (DGK-z), an enzyme containing PDZ binding-consensus sequence and could functionally convert diacylglycerol into phosphatidic acid (Hogan et al., 2001). DGK-z, syntrophin and GTPase Rac appear to regulate the neurite outgrowth in the cultured cells (Yakubchyk et al., 2005). In muscle, localization of DGK-z to the plasma membrane and enrichment at the neuromuscular junction require the interaction with syntrophin (Abramovici et al., 2003).

When α1-syntrophin gene is deleted, mice have no muscular dystrophy, but loss of nNOs and utrophin in the sarcolemma and disrupted neuromuscular junctions. As well there is loss of aquaporin-4 in the sarcolemma and astrocyte (Adams et al., 2000; Kameya et al., 1999; Neely et al., 2001). Loss of nNOs may contribute to the pathogenesis of muscular dystrophy by an aberrant regulation of adrenergic vasoconstriction and the resulting defective control of muscle blood flow in lack of nNOs (Crosbie et al., 2001). An interesting study showed that, in the perivascular membranes of astrocyte endfeet (a part of blood-brain-barrier), removal of α1-syntrophin which causes loss of aquaporin-4 results in a delayed buildup of brain edema (Amiry-Moghaddam et al., 2004) in experimental model of acute hyponatremia. This data indicates a therapeutic potential that could benefit from interfering with the binding of syntrophin to aquaporin-4 in acute brain edema. A recent study showed that PDZ-domain of α1-syntrophin binds to potassium channel Kiv4.1 in brain, and α1-syntrophin null mice show disrupted submembrane localization of Kiv4.1 (Connors et al., 2004). An interesting report shows that patients with mesial temporal lobe epilepsy (MTLE) and hippocampal sclerosis lose perivascular dystrophin (Dp71) in the hippocampus, and have disrupted subcellular localization of aquaporin 4 in astrocytes

and abnormal accumulation of extracellular K^+ (Eid et al., 2005). Despite their highly conserved sequences and PDZ domain, β1 and β2-syntrophin have function distinct from α1-syntrophin. For example, neither β1 nor β2-sytrophin binds to nNOs in skeletal muscle (Adams et al., 2000). β2-syntrophin null mice show no overall phenotypes or muscular dystrophy, and form morphologically normal sarcolemma and neuromuscular junctions (Adams et al., 2004). Mice null for both α1 and β2–syntrophin show normal muscle histology but more severe disrupted neuromuscular junctions than those of α1-syntrophin null mice (Adams et al., 2004), indicating α and β2-syntrophin play distinct roles and each can compensate for the loss of the other.

Dystrobrevin was identified from co-purifications of DGC in skeletal muscle (Ervasti et al., 1991) and was initially named A0 (Yoshida and Ozawa, 1990), or an 87 kDa postsynaptic protein (Butler et al., 1992; Wagner et al., 1993). The amino acid sequence of dystrobrevin reveals three major domains: the cysteine-rich and coiled-coil domain, which is homologous to dystrophin, and a C-terminal dystrobrevin-unique domain (Wagner et al., 1993). Dystrobrevin binds directly to syntrophin through amino acids 427-480, upstream of the coiled-coil domain (Ahn and Kunkel, 1995; Sadoulet-Puccio et al., 1997a; Sadoulet-Puccio et al., 1997b). It also interacts directly with dystrophin through its C-terminal coiled-coil region (Sadoulet-Puccio et al., 1997b). Two dystrobrevin genes, α and β, are broadly expressed in mice, and give rise to at least 5 splicing variants (Blake et al., 1998; Blake et al., 1996a). α-dystrobrevin, the first identified 87 kDa protein in *Torpedo* electric organ, has two major isoforms in muscle, which are full-length α-dystrobrevin-1 and the C-terminal truncated α-dystrobrevin-2 (Blake et al., 1996a). α-dystrobrevin-1 is highly restricted to the NMJs,

whereas α-dystrobrevin-2 is expressed both on the sarcolemma and at the NMJs (Peters et al., 1998). β-dystrobrevin is not expressed in muscle, but in brain, kidney, and other organs where it is directly associated with Dp71 and Dp140 (Blake et al., 1998; Peters et al., 1997b). When the α-dystrobrevin gene is deleted in mice, neither α-dystrobrevin-1 nor α-dystrobrevin-2 is expressed but normal muscle integrity and localization of other DGC members are observed (Grady et al., 1999). Muscles of these mice appear histologically normal during the first two weeks after birth but dystrophic phenotypes such as degenerating and regenerating muscle fibers, necrosis and centralized nuclei appear by one-month of age (Grady et al., 1999). These mice also show significant loss of nNOs in the sarcolemma and fail to produce the second messenger cyclic GMP with electrical stimulation (Grady et al., 1999), consistent with previous findings that nitric oxide appears to stimulate cGMP production in contracting muscle (Lau et al., 1998). At the neuromuscular junctions of α-dystrobrevin deficient mice, clusters of acetylcholine receptors are distributed more diffusely and sometimes fragmented (Grady et al., 2000). These experiments indicate the importance of dystrobrevin in cell signaling, structure and maintenance and neuromuscular junction formation. Furthermore, a new coiled-coil protein dysbindin has been identified that interacts with α-dystrobrevin in muscle and with β-dystrobrevin in brain (Benson et al., 2001). Mutations in dysbindin have been shown to be associated with schizophrenia (Schwab et al., 2003; Straub et al., 2002; Van Den Bogaert et al., 2003) and with an abnormal vesicle trafficking disease called Hermansky-Pudlak syndrome (Li et al., 2003). In *mdx* mouse brain, dysbindin expression was increased, but disrupted in synaptic regions

(Sillitoe et al., 2003). However, direct biochemical or molecular evidence regarding schizophrenia and dysbindin/dystrobrevin or other DGC members remains unclear.

Sarcoglycan and Sarcospan

Sarcoglycan and sarcospan are a group of single pass transmembrane proteins that are part of the DGC. Several sarcoglycans, α, β, γ, δ, ε and ξ-sarcoglycans have been cloned. Among them, α-, β-, γ- and δ-sarcoglycans were first characterized as a heterotetramer complex that is tightly associated with sarcospan and β-DG. Like most other DGC members, α-sarcoglycan/adhalin (50 kDa), β-sarcoglycan (43 kDa) and γ-sarcoglycan (35 kDa) were co-purified from muscle sarcolemma with dystrophin by lectin affinity purification (Campbell and Kahl, 1989; Ervasti et al., 1991; Ohlendieck and Campbell, 1991; Yoshida and Ozawa, 1990). They could be biochemically separated from the rest of the DGC by the detergent n-octyl β-D-glucoside (Yoshida et al., 1994). The sarcoglycan genes were cloned following genetic studies in which mutations in these loci have been linked to limb-girdle muscular dystrophies (LGMD) (Table 1). The α-sarcoglycan gene was the first found, and is located on chromosome 17 and associated with mutations leading to LGMD 2D (Roberds et al., 1994). Subsequent discoveries showed a total of 39 mutations in α-sarcoglycan in unrelated families and sporadic cases with LGMD 2D (Carrie et al., 1997; Piccolo et al., 1995). Homozygous missense mutations in β-sarcoglycan gene were found in a large Amish family with LGMD 2E (Bonnemann et al., 1995; Lim et al., 1995). The disease gene of severe childhood autosomal recessive muscular dystrophy (now LGMD 2C) was identified as γ-sarcoglycan and is located on chromosome 13 (Noguchi et al., 1995). δ-

sarcoglycan (35 kDa) was identified and characterized as the mutant gene on chromosome 5 with a single nucleotide deletion affecting the reading frame, from two Brazilian families with LGMD 2F (Jung et al., 1996; Nigro et al., 1996a; Nigro et al., 1996b). ε-sarcoglycan, a homologue of α-sarcoglycan, was discovered to replace α-sarcoglycan in smooth muscle to form a new type of sarcoglycan complex (Ettinger et al., 1997; Straub et al., 1999), and mutations in ε-sarcoglycan have been implicated in myoclonus dystonia (Muller et al., 2002; Zimprich et al., 2001), an autosomal dominant disorder characterized by bilateral, alcohol-sensitive myoclonic jerks involving mainly the arms and axial muscles (Zimprich et al., 2001). ξ-sarcoglycan has not found to associate with any diseases. The amino acids of ξ-sarcoglycan show a high homology to γ-and δ-sarcoglycan (Wheeler et al., 2002), and ξ-sarcoglycan forms a stable complex with α-, β-, γ- and δ-sarcoglycans in the skeletal muscle and is reduced in δ and γ-sarcoglycan deficient muscle and in *mdx* mice (Wheeler et al., 2002). How ξ-sarcoglycan binds to the sarcoglycan complex is unknown, but, on the sarcolemma β, γ and δ-sarcoglycan associate with one another very tightly by forming intramolecular disulfide bonds whereas α-sarcoglycan binds less tightly (Chan et al., 1998). Moreover, they tightly connect to β-DG through δ-sarcoglycan (Chan et al., 1998). Sarcospan is a 25 kDa core member of the DGC and forms a complex with sarcoglycans and its localization on the muscle sarcolemma is dependant on sarcoglycans (Crosbie et al., 1997; Crosbie et al., 1999). It is known that sarcoglycans, DG and sarcospan form a large complex in the Golgi network (Noguchi et al., 2000). In fact, loss of any one of sarcoglycans (α-, β-, γ-, δ-) in LGMD patients leads to failure of the sarcoglycan

complex to assemble (Ozawa et al., 2005; Vainzof et al., 1996), which results in a damaged integrity of muscle sarcolemma.

Animal models have been established to further investigate the mechanisms of type 2 LGMD caused by mutations in α, β, γ, δ-sarcoglycan (Araishi et al., 1999; Coral-Vazquez et al., 1999; Duclos et al., 1998b; Durbeej et al., 2000; Hack et al., 1998; Okazaki et al., 1996). For each type, skeletal muscles begin to degenerate around 2 weeks after birth. Cell infiltration, fibrosis and other typical muscular dystrophic phenotypes such as sarcolemmal injury and creatine kinase efflux are observed (Ozawa et al., 2005). In γ-sarcoglycan deficient mice, the other three sarcoglycans and sarcospan are present on the sarcolemma in trace amount and high level of programmed cell death in the muscle fibers was observed (Hack et al., 1998). In α-, β- or δ–sarcoglycan deficient mice, the remaining sarcoglycans and sarcospan are not present (Crosbie et al., 1999). This indicates loss of γ-sarcoglycan alone may be sufficient to cause the disease and activate apoptotic signaling pathways. Moreover, loss of sarcoglycan complex causes reduction of α-DG and an unstable DGC in the sarcolemma (Duclos et al., 1998a; Duclos et al., 1998b; Iwata et al., 1993; Roberds et al., 1993; Straub et al., 1998), although β-DG and dystrophin are present in the sarcolemma in sarcoglycan-null muscles (Araishi et al., 1999; Coral-Vazquez et al., 1999; Duclos et al., 1998b; Durbeej et al., 2000; Hack et al., 1998; Okazaki et al., 1996). Therefore, one role of sarcoglycan complex appears to preserve the whole DGC and the linkage between extracellular matrix and cytoskeleton. On the other hand, when DG is absent from the skeletal muscle, α, β, γ and δ-sarcoglycan as well as sarcospan are all lost from the sarcolemma (Cote et al., 1999; Cohn et al., 2002). Re-introduction

of β-DG in DG-deficient skeletal muscle restores the sarcoglycans and sarcospan complex in the sarcolemma (Kanagawa et al., 2004). Reduction of sarcoglycans is also found in *mdx:utrophin-/-* mice (Grady et al., 1997). These data establish mutual functional connections between DG and sarcoglycan-sarcospan complex as well as between dystrophin-utrophin and sarcoglycan-sarcospan. However, we may not assume that DG-deficiency-induced phenotypes come from loss of sarcoglycans. Several known results support this. 1), DG-deficiency causes lethality but sarcoglycans-deficiency doesn't. 2), In the skeletal muscle, restoration of sarcoglycan-sarcospan complex on the sarcolemma by introduction of β-DG in DG-null muscles does not prevent the muscular dystrophy (Kanagawa et al., 2004). This strongly suggests that sarcoglycan and sarcospan doesn't contribute significantly to DG-deficiency-induced phenotypes. To understand if loss of sarcoglycans contributes to the muscle dystrophic phenotype in DMD/BMD, similar rescue experiment with re-introduction of sarcoglycan in *mdx:utrophin-/-* mice would provide interesting information.

Like the skeletal muscle, the cardiac muscle contains same sarcoglycans (α-, β-, γ- and δ-sarcoglycan) and sarcospan. Interestingly, a cardiomyopathy including a dilated ventricle, areas of necrosis and fibrosis was reported from β, γ and δ-sarcoglycan knock-out mice, but not from α-sarcoglycan knock-out mice (Coral-Vazquez et al., 1999; Okazaki et al., 1996). Just as in skeletal muscle, β-DG is not altered in cardiac muscle even in the loss of sarcoglycan-sarcospan complex (Coral-Vazquez et al., 1999). In the smooth muscle of coronary arteries, ε, β, δ-sarcoglycan and sarcospan form a complex (Straub et al., 1999), and mice deficient in δ-sarcoglycan develop an irregular coronary artery that was shown to underlie the pathogenesis of

cardiomyopathy in sarcoglycan knock-out mice (Coral-Vazquez et al., 1999). Unlike the skeletal and smooth muscles, Schwann cells of the peripheral nerve contain only δ, β- and ε-sarcoglycan with no sarcospan (Imamura et al., 2000). In neurons, however, neither sarcoglycan nor sarcospan is expressed (Blake and Kroger, 2000; Imamura et al., 2000).

Grb2

In addition to core members of the DGC, there are several other proteins considered to be members of the DGC. Grb2 (growth factor receptor-bound protein 2) is a small SH2/SH3 (Src homology 2/Src homology 3) domain-containing signaling molecule that binds to the C-terminus of β-DG through its SH3 domains (Yang et al., 1995). Grb2 is well known as an adapter protein that participates in receptor-tyrosine kinase, PI3 kinase (phosphoinositide 3-kinase) and MAPK (mitogen-activated protein kinase) signaling cascades. Grb2 is comprised of a central SH2 domain and an SH3 domain at its NH2- and COOH-terminus. Through its SH2 domain, Grb2 typically binds to the tyrosine-phosphorylated receptor after ligands activate the receptor and induce tyrosine residue autophosphorylation (Mayer et al., 1988; Olivier et al., 1993; Schlessinger, 2000). The SH3 domains of Grb2 bind to proline-rich proteins such as Sos (Son of sevenless) (Schlessinger, 1994), and brings Sos in close proximity to the plasma membrane protein Ras (Mayer et al., 1988; Olivier et al., 1993; Schlessinger, 2000). Many experiments have demonstrated that Sos then transforms inactive GDP-Ras to active GTP-Ras, which can then activate several effector proteins such as Raf and PI3 kinase (McCormick, 1993; Schlessinger, 1994; Schlessinger, 2000). Activated

Raf stimulates MAP-kinase-kinase (MAPKK), and the activated MAPKK phosphorylates MAP-kinase (MAPK), which in turn phosphorylates many cytoplasmic and membrane-linked substrates as well as some transcription factors after translocation into nucleus (McCormick, 1993; Schlessinger, 1994; Schlessinger, 2000). Thus, Grb2 couples receptor tyrosine kinase activation to a variety of cellular processes.

DG contains the consensus motif PXXP (P, proline; X, any amino acid) for SH3 domain binding on its C-terminus. DG binds to both SH3 domains of Grb2 in muscle and in brain, but with higher affinity for the N-terminal SH3 domain (Russo et al., 2000; Yang et al., 1995). Therefore, Grb2 may link DG to the receptor tyrosine kinase signaling by affecting Ras activation and MAPK signaling pathway. There have no direct evidence for this, however, individual experiments showed that tyrosine residue (Y892) of β-DG can be phosphorylated by Src kinase and recruit SH2-domain containing proteins in co-transfected cells (Sotgia et al., 2003; Sotgia et al., 2001), and extracellular stress in alveolar epithelial cells induces MAPK activation which is dependent on DG (Jones et al., 2005). More interestingly, perturbation of DG-laminin binding in muscle cells appears to cause decreased phosphorylation of AKT and glycogen synthase kinase (GSK)-3β and an increased apoptosis resulting from disrupted the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway (Langenbach and Rando, 2002). These experiments suggest that DG play roles in intracellular signaling pathways suck as MAPK and PI3K pathways.

Part III: DG in the nervous systems

I Agrin, MuSK and rapsyn in formation of the Neuromuscular Junctions (NMJs)

NMJs are the sites where motor nerves make contact with postsynaptic muscle fibers to establish chemical synapses, in which nicotinic acetylcholine receptors (AChRs) in muscle receive signals from the nerve. In the newly formed myotubes, AChRs are inserted into the plasma membrane and reach a density of ~1000/μm^2 prior to the nerve innervation (Sanes and Litchtman, 2001; Burden, 2002). In adult muscles, however, AChRs are considerably concentrated and reach a density of >10,000/μm^2 at the muscle membrane opposing the nerve terminal, whereas other muscle membrane has very little (~10/μm^2) AChRs (Sanes and Lichtman, 2001). The clustering of AChRs at the synaptic site is a hallmark of synaptogenesis. The mechanisms of AChR clustering have been intensively studied. Agrin, MuSK and rapsyn have been demonstrated as the key molecules required in this process (Sanes and Lichtman, 2001; Burden, 2002; Kummer et al., 2006).

Agrin

In the late seventies, scientists found that plaques of basal lamina heparan sulfate proteoglycan are associated with aggregates of acetylcholine receptors in the neuromuscular junction (Anderson and Fambrough, 1983; Burden et al., 1979; Godfrey et al., 1984; Nitkin et al., 1983; Sanes et al., 1978). In the 1980s, McMahan and colleagues isolated agrin as the bioactive component of the basal lamina that organizes post-synaptic proteins in muscle membranes (McMahan, 1990; Nitkin et al., 1987). Agrin is a large (400-600 kDa) heparan sulfate proteoglycan with a 200 kDa core

(Gesemann et al., 1995; Tsen et al., 1995), secreted, most notably, by neurons (Smith et al., 1987) and muscle (Lieth et al., 1992) and deposited in the BM of the NMJs by binding to γ1 chain of laminin (Denzer et al., 1998; Kammerer et al., 1999). Alternative splicing generates a non-BM associated transmembrane agrin containing a short N-terminus and specifically expressed in neurons (Burgess et al., 2002; Burgess et al., 2000). Like laminin, agrin contains three LG domains at it C-terminus: the first G-domain (G1), the second G-domain (G2) which contains 0 or 4 amino acid insert due to alternative splicing; and the third G-domain (G3) which contains a 0, 8, 11 or 19 amino acid insert (Carbonetto and Lindenbaum, 1995; Ferns et al., 1992). The biological activity of agrin to promote AChR clustering is located in its G-domains. Isoforms with 4 amino acid insert in G2 and 8, 11 or 19 (8+11) amino acid insert in G3 at a site called Z site in mammals, which is secreted by motor neurons but not by myotubes, are the most active forms of agrin to aggregate the receptors in cultured myotubes (Ferns et al., 1992; Ferns et al., 1993; Hoch et al., 1993; Ruegg et al., 1992). The G-domains of agrin bind directly to carbohydrate chains of α-DG and the binding could be blocked by heparin and monoclonal antibody IIH6 (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994). Both nerve and muscle-derived agrins bind α-DG with high affinity, but nerve-derived agrin is much more active in promoting AChR clustering (Ferns et al., 1992; Ferns et al., 1993; Gesemann et al., 1995; Sugiyama et al., 1994) and is indispensable for the NMJ synaptogenesis whereas muscle-derived agrin appears not required in synaptogenesis (Burgess et al., 1999). When the agrin gene was deleted, the size, number and density of AChR clusters were dramatically reduced at the NMJs of diaphragm and muscles from agrin-deficient mice fail to make mature NMJs, which

results in death at birth due to failure of synaptic transmission (Gautam et al., 1996). This experiment firmly established an essential role of agrin in the NMJ formation.

MuSK

Similar to failure in formation of mature synapses in agrin-deficient mice, when the MuSK gene is deleted the NMJs completely lack AChR clusters and lack synaptic differentiation and animals die at birth because they have difficulty breathing (DeChiara et al., 1996; Sanes and Lichtman, 2001). Early experiments have suggested MuSK is the agrin receptor. Agrin induces rapid tyrosine phosphorylation in the juxta-membrane domain of MuSK which appears to be required for agrin-induced AChR clustering (Glass et al., 1997; Glass et al., 1996; Herbst and Burden, 2000; Meier et al., 1996; Zhou et al., 1999). Since agrin does not bind directly to MuSK, a hypothetical co-receptor is required to activate MuSK (Glass et al., 1997; Glass et al., 1996). Interestingly, the ectodomain of MuSK was shown to be essential to cluster AChRs (Apel et al., 1997) in which the first immunoglobulin (Ig)-like domain is required for agrin response whereas the fourth Ig-like domain for interaction with rapsyn, the effector of AChRs (Zhou et al., 1999). Thus, a hypothetical protein is proposed to link the fourth Ig-domain and rapsyn (Apel et al., 1997; Zhou et al., 1999). Moreover, the juxtamembrane and kinase domains of MuSK interact with dishevelled, a downstream molecule in Wnt/Frizzled signaling pathway (Luo et al., 2002). This interaction appears to mediate agrin-induced activation of a small GTPase, p21-activated kinase (PAK) that is required for AChR clustering by regulating the cytoskeleton (Luo et al., 2002). In cultured myotubes agrin induces phosphorylation of β- and δ-subunits of AChRs that precedes the receptor clustering events and MuSK is required for this phosphorylation

process (Ferns et al., 1996; Glass et al., 1997; Qu and Huganir, 1994; Wallace, 1994). Phosphorylation of AChR stabilizes its association with the cytoskeleton (Borges and Ferns, 2001).

However, recent genetic studies suggest that agrin is not required for initiating AChR clusters and for MuSK activation, even more, these data challenge the central role of nerve innervation in the postsynaptic differentiation (Ferns and Carbonetto, 2001). On embryonic day 14, AChR clusters do form in the central band of muscle in the agrin-deficient mice, but do not form in MuSK-deficient muscle (Lin et al., 2001). In addition, overexpression of mutant MuSK without its ectodomain is sufficient to induce AChR clustering without ligand activation (Sander et al., 2001). Furthermore, Burden and colleagues (2001) have demonstrated that the embryonic patterning of AChR clustering does form in the absence of nerve innervation, but not in the absence of MuSK (Yang et al., 2001), thus, they propose that nerve-derived signals such as agrin refine the endogenous patterning (Yang et al., 2001). It has been shown later by an independent group that muscle intrinsic factors but not nerve released-factors or agrin assemble the AChR clusters (Pun et al., 2002). Time-lapse imagining has revealed the sequential events for synaptogenesis at the zebrafish NMJs in which postsynaptic AChRs clusters form in advance of growing axons, even before the axons leave the spinal cord, and these pre-innervation clusters later incorporate into the synapse (Flanagan-Street et al., 2005). More recent experiments showed that nerve-released acetylcholine in fact serves as a negative factor to disperse the pre-existing AChR clusters, whereas neural agrin counters the effect of transmitter to refine the clusters of AChRs at the late stage of differentiation (Lin et al., 2001; Lin et al., 2005;

Misgeld et al., 2002; Misgeld et al., 2005). These experiments suggested that it is muscle-intrinsic factors including MuSK is required for initiating AChR clustering and MuSK can be activated independently of agrin. Moreover, they suggest that nerve innervation and nerve-released agrin are required to refine the clusters in the late developmental stage, a process that is also essential for normal synaptogenesis because agrin-null mice have severe NMJ defects (Gautman et al., 1996).

In addition, agrin also works with other molecules to promote AChR clustering. For example, with DG and the DGC (discuss below), and Rho family of the small GTPase such as Rac1 and Cdc42 (Weston et al., 2003; Weston et al., 2000). Rac1 and Cdc42 can be activated by agrin and the activation appears required for agrin-refined AChR clustering (Weston et al., 2003; Weston et al., 2000).

Rapsyn

Rapsyn, a 43 kDa cytoplasmic protein, has long been known to tightly associate with AChRs in the *Torpedo* electric organ and mammalian postsynaptic muscle. Rapsyn co-distributes perfectly with AChRs from the earliest stage of synaptogenesis and is present at 1:1 stoichiometry with AChRs (Burden et al., 1983; Froehner et al., 1990; Noakes et al., 1993; Phillips et al., 1993; Sobel et al., 1977). Rapsyn-deficient mice die at birth because animals fail to move and breathe due to defective NMJs (Gautam et al., 1995), a similar phenotype as agrin or MuSK-deficient mice. Histological studies in these mice revealed loss of AChR clusters and loss of utrophin and DG in the postsynaptic muscle membrane, but normal synaptic localization of MuSK, acetylcholinesterase and laminin (Apel et al., 1997; Gautam et al., 1995), demonstrating that synaptic localization of MuSK is independent of rapsyn. Co-

expression of AChRs and rapsyn in heterologous cells leads to formation of AChR-rapsyn clusters whereas AChR is diffusely distributed when expressed on their own (Froehner et al., 1990; Phillips et al., 1991). Interestingly, treatment of agrin does not promote AChR clustering in rapsyn-deficient muscle (Fuhrer et al., 1999) and forced expression of rapsyn in heterologous cells clusters MuSK and recruits AChRs and other synaptic components to the MuSK-containing scaffold (Apel et al., 1997; Gillespie et al., 1996). These and many other experiments have firmly established that rapsyn is the downstream effector of agrin or MuSK (Sanes and Lichtman, 2001). Rapsyn contains several domains: a myristoylated amino terminus (Frail et al., 1988), eight tetratricopeptide repeats (TPRs) involved in self-association and a coiled-coil domain that binds to the AChR β-subunit and is necessary for AChR clustering (Burden et al., 1983; Ramarao et al., 2001; Ramarao and Cohen, 1998). In addition there is a RING-H2 domain that is involved in interacting with the intracellular juxtamembrane portion (from amino acid 784 to 819) of β-DG (Bartoli et al., 2001; Cartaud et al., 1998; Ponting and Phillips, 1996). Over-expression and deletion of this DG region disrupted agrin-induced AChR clustering in cultured myotubes (Kahl and Campanelli, 2003). AChRs, utrophin and DG form membrane-associated clusters in the non-muscle cells only when they were co-transfected with rapsyn (Apel et al., 1995). Therefore, rapsyn serves as the link between AChRs and the DGC.

II Roles of DG in the NMJs

In addition to MuSK, agrin and rapsyn, many other proteins are also involved in the NMJ formation, which include the extracellular protein laminin (Vogel et al., 1983),

acetylcholinesterase and the DGC. The DGC members are concentrated at the NMJs and match with AChRs in addition to its peripheral sarcolemma expression (Matsumura et al., 1992; Ohlendieck et al., 1991b). DG colocalizes with AChRs at very early stages before and after nerves make contact with muscles (Cohen et al., 1995). At the muscle membrane of the NMJ, DG serves as the receptor of agrin and laminin (Gee et al., 1993; Gee et al., 1994; Campanelli et al., 1994; Bowe et al., 1994; Montanaro et al., 1998) and many experiments have demonstrated DG is involved in the NMJ formation. When the DG gene is deleted, the NMJs and AChR clusters are formed, but the clusters are dispersed and fragmented both in vivo and in vitro (Cote et al., 1999; Grady et al., 2000; Jacobson et al., 2001), which is similar to phenotypes observed in α-dystrobrevin- and utrophin/dystrophin-deficient NMJs (Grady et al., 2000; Kong and Anderson, 1999). The synaptic localization of acetylcholinesterase is also disrupted in DG-deficient myotubes because the DG-perlecan complex anchors acetylcholinesterase (Jacobson et al., 2001; Peng et al., 1999). In addition, other BM proteins such as laminin and perlecan are no longer concentrated at the AChR aggregates but agrin and rapsyn are not affected (Jacobson et al., 2001). These genetic and cell biology data demonstrated DG is required for the stabilization and condensation of AChR clusters and formation of synaptic BM. There are several possible mechanisms to explain the involvement of DG in the formation of AChR clusters. First: laminin 1 was demonstrated to induce AChR clustering twenty years ago (Vogel et al., 1983), independent and additive to the effect of agrin (Sugiyama et al., 1997). The laminin-induced effect was shown to function through DG (Montanaro et al., 1998; Sugiyama et al., 1997), and experiments have demonstrated that antibody inhibition of α-DG blocks

its ability to induce AChR clustering (Gee et al., 1994) and in DG-deficient cells laminin is no longer able to induce AChR aggregates (Jacobson et al., 2001; Montanaro et al., 1998). Recent data have suggested that the sialylated core 1 oligosaccharides of α-DG that differ from the laminin-binding oligosaccharides mediate laminin-induced AChR clustering (McDearmon et al., 2003). Intracellularly DG binds to rapsyn (Bartoli et al., 2001; Cartaud et al., 1998) and rapsyn is also essential for laminin-induced AChR clustering (Marangi et al., 2002). Therefore, a pathway through laminin-DG-rapsyn-AChRs effectively participates in postsynaptic differentiation. Second, DG also interacts with agrin and functional blocking of DG and agrin interaction has negative effect on AChR clustering (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994). A recent study indicated that over-expression of the intracellular β-DG can serve as a dominant negative molecule and inhibited agrin-induced AChR clustering because the intracellular β-DG sequesters its binding partners (Kahl and Campanelli, 2003). Especially noteworthy for agrin's effect on AChR clustering, over-expression of a construct containing the rapsyn-binding juxtamembrane domain of DG is sufficient to inhibit agrin-induced AChRs clustering (Kahl and Campanelli, 2003). Very interestingly, a recent experiment suggested that non-neural muscle agrin can induce AChR clustering in cooperation with phosphorylations of MuSK and AChR β-subunit, a process that is laminin-dependent and requires laminin polymerization (Smirnov et al., 2005). This provides a likely mechanism for pre-existing AChR clusters in the aneral muscles, and future experiments would be to determine the role of DG in this laminin-dependent and non-neural (muscle) agrin-induced AChR clustering.

III DG in the Schwann cells

DG is located at the outermost wrapping of myelin in peripheral nerves opposing the basal lamina (Masaki et al., 2000; Saito et al., 1999; Yamada et al., 1994). There, it interacts with agrin and laminin 2 (Occhi et al., 2005; Yamada et al., 1996), and is complexed intracellularly with Dp116, δ, β- and ε-sarcoglycan (Imamura et al., 2000). In addition, DG is also complexed with DRP2 and L-periaxin (Sherman et al., 2001). Mutations of L-periaxin cause demyelination of peripheral nerves in mice and human (Boerkoel et al., 2001; Gillespie et al., 2000). α- and β-DG, Dp116, utrophin are also enriched at the microvilli of Schwann cells at nodes of Ranvier and extracellularly connect to α2 chain of laminin, whereas DRP2 and periaxin are not present at the nodes of ranvier (Occhi et al., 2005; Saito et al., 2003). Mutation in α2 laminin causes muscular dystrophy and non-myelinated nerves (Shorer et al., 1995). In DG-deficient Schwann cells, other members of the DG complex are also lost or dramatically reduced, and mice display extensively folded and long myelin sheaths, reduced sodium channel density in nodes of Ranvier and disorganized Schwann cell microvilli, which leads to reduced conduction velocity and reduced sensitivity to pain in aged animals (Saito et al., 2003).

Interestingly DG is also the Schwann cell receptor for *Mycobacterium leprae*, a pathogen that causes leprosy (Rambukkana, 2001; Rambukkana, 2004). By binding to carbohydrate chains of α-DG in the presence of LG-domains of α2-laminin (Rambukkana et al., 1997; Rambukkana et al., 1998), *Mycobacterium leprae* enters the Schwann cell and cause demyelination in vivo and in vitro (Rambukkana et al., 2002). In dy/dy dystrophic mice that do not express α2 chain of laminin 2, *Mycobacterium*

leprae fails to attach to the Schwann cell basal lamina (Rambukkana et al., 1997) and fail to infect the Schwann cells (Rambukkana et al., 1998).

IV DG in the brain

As opposed to muscle, the extracellular space among neurons in the CNS has no basement membranes but the extracellular matrix is abundant and contains hyaluronan, the main structural component of brain extracellular matrix, and proteolgycans such as agrin, perlecan, syndecan, aggrecan, versican, neurocan and more (Holt and Dickson, 2005; Rauch, 2004; Rutka et al., 1988) with little laminin and collagen (Jucker et al., 1996; Ruoslahti, 1996). However, DG, dystrophin and other DGC members are abundantly expressed in neurons and glia of many brain regions (Blake and Kroger, 2000; Ibraghimov-Beskrovnaya et al., 1993; Kim et al., 1992; Lidov et al., 1990; Moukhles and Carbonetto, 2001; Tian et al., 1996). Unlike in muscle where the function and composition of the DGC is better understood, the composition of the DGC and its subcellular localization are just beginning to be studied in brain. However, it has been known for decades that one third of DMD and BMD patients have cognitive defects and mental retardation (Emery, 1998), and recent *mdx* mice display retention-related behavioral abnormalities (Vaillend et al., 1995).

Dystrophin isoforms including Dp427, Dp140 and Dp71 are expressed in cerebral cortex, hippocampus, cerebellum, striatum and thalamus (Blake and Kroger, 2000; Gorecki and Barnard, 1995; Lidov et al., 1995). Full length Dp427 is neuronal specific and localized at central synapses and enriched at the postsynaptic densities of excitatory synapses (Blake et al., 1999; Kim et al., 1992; Lidov et al., 1990), where it

forms a complex with syntrophin and β-dystrobrevin (Blake et al., 1999). In differentiated astrocytes, Dp71 replaces Dp427 and utrophin to associate with syntrophin and α-dystrobrevin (Blake et al., 1999; Imamura and Ozawa, 1998). Together with DG and the aquaporin4 water channel, they form a complex at astrocyte endfeet surrounding the basement membrane of blood vesicles and regulate brain fluid volume (Moukhles and Carbonetto, 2001; Neely et al., 2001; Tian et al., 1996; Zaccaria et al., 2001). Like Dp427, DG is also expressed in the neurons and glia of similar brain regions (Moukhles and Carbonetto, 2001; Tian et al., 1996; Zaccaria et al., 2001). However, unlike Dp427 DG is not enriched biochemically in the postsynaptic density of excitatory synapses (Moukhles and Carbonetto, 2001; Mummery et al., 1996). Instead, in cultured hippocampal neurons, DG was found at the postsynaptic sites of inhibitory GABAergic synapses together with syntrophin and Dp427 (Brunig et al., 2002; Levi et al., 2002). But it appears that formation of postsynaptic GABA receptors and GABAergic synaptogenesis is independent of the complex of DG, dystrophin and syntrophin (Brunig et al., 2002; Levi et al., 2002). Therefore, these data suggest different complexes exist in the excitatory, inhibitatory synapses and in astrocyte endfeet with unrevealed functions. Future experiments should address whether DG is localized at the presynaptic terminals of excitatory synapses and whether it is localized at the CNS cholinergic synapses.

Recently, neuronal specific cell surface protein neurexins are shown to form a physical complex with α-DG (Sugita et al., 2001). Neurexins are encoded by three genes and several promoters, which generates longer α-neurexins and shorter β-neurexins (Missler and Sudhof, 1998). Both types of neurexins are transmembrane

proteins and contain laminin-like G-domains within their extracellular domain structures (Rudenko et al., 1999). β-neurexin interacts with the post-synaptic protein neuroligin to initiate the excitatory synapses (Graf et al., 2004; Ichtchenko et al., 1995; Song et al., 1999). The neurexin-neuroligin complex is linked to intracellular scaffolding protein postsynaptic density 95 (PSD95) via neuroligin (Irie et al., 1997), and to the presynaptic scaffolding protein CASK at the neurexin side (Butz et al., 1998). The synaptic proteins, neurexin and neuroligin, have been shown to be able to induce both pre- and post-synaptic differentiation (Graf et al., 2004; Scheiffele et al., 2000). Similar to laminin, single G-domains of α- and β-neurexin bind to carbohydrate chains of α-DG, which is tissue-specific and dependent on presence of calcium (Sugita et al., 2001). However, at inhibitatory synapses, neurexin seems unable to cluster DG together with other postsynaptic proteins in cultured hippocampal neurons (Graf et al., 2004), which certainly raises the question about the role of neurexin-DG complex in brain.

A decisive illustration of DG functions in central nervous system came from the brain-specific DG-null mice (Moore et al., 2002). These DG-null mice have fusion of cerebral interhemisphere and cerebellar folia, disorganized cerebral layers, aberrant migration of granule cells, and discontinuous glia limitans that normally separates the marginal zone of the cortex from the subarachnoid space (Moore et al., 2002). These mutant mice also show severely blunted long-term potentiation (LTP), suggesting that DG may have a role in learning and memory (Moore et al., 2002). Importantly, these mutant mice provided suggestions for brain pathogenesis of several congenital muscular dystrophies in human. Walker-warburg syndrome, muscle-eye-brain disease

and Fukuyama congenital muscular dystrophy display 'cobblestone lissencephaly' in their brains, in which overmigrated neurons result in a brain with a bumpy, cobblestone appearance and loss of normal brain folding pattern (Muntoni et al., 2004; Ross, 2002; Montanaro and Carbonetto, 2003). These diseases are caused by putative or confirmed glycosyltransferase mutant genes (described in the page 10-12), which leads to a greatly reduced glycosylation of α-DG and loss of laminin binding in the extracellular matrix (Michele et al., 2002). As suggested from the DG-null mice that DG-deficiency causes disrupted glia limitans and aberrant neuronal migration (Michele et al., 2002; Moore et al., 2002), thus 'Cobblestone lissencephaly' in human may form due to neuron over-migration into the subarachnoid space (Ross, 2002). DG mutant brain phenotypes were further demonstrated in *myd* mice which bear a mutant *LARGE* protein. *LARGE* has been confirmed to participate directly in the glycosylation of α-DG (Kanagawa et al., 2004; Barresi et al., 2004) and *myd* mice show loss of glycosylation of α-DG and display similar brain phenotypes including neuronal migration defects in cerebral cortex, hippocampus, and cerebellum and discontinued glia limitans (Grewal et al., 2001; Holzfeind et al., 2002; Michele et al., 2002). Therefore, α-DG subunit appears essential for the function of DG protein in brain and loss of glycosylation of α-DG, similar as loss of DG, causes abnormal brain structure and organization. Moreover, it suggests that glycosylation defect of α-DG might be the common pathway leading to those congenital muscular dystrophies, although this remains to be precisely determined.

However, these CNS data do not prove that carbohydrates of α-DG are the only functional units required in the CNS development. Very simply, loss of ligand-binding

could affect the intracellular DG function, such as its associated proteins or signaling molecules or the structure of β-DG. For example, DG-null muscles lose the β-DG binding partners including dystrophin, sarcoglycans and sarcospan from the muscle sarcolemma (Cote et al., 1999; Cohn et al., 2002), thus, loss of β-DG alone could disrupt the muscle membrane integrity because the sarcolemma loses some of its internal proteins which are indeed known to cause the muscle dystrophy. Nevertheless, open questions remain for clarifying roles of DG in the CNS. For example, since DG is expressed in both neuron and glia, we don't know in which cell type loss of DG gives rise to the respective brain phenotype, and how they happen? Moreover, we don't know if any intracellular signalings that are associated with β-DG contribute to the brain phenotype.

Similar to the phenotypes in DG-null brain, deletions of extracellular matrix components such as α5, γ1 laminin or their receptors such as α6, β1 integrin in brain often result in cortical disorganization, abnormal lamininination and disrupted glia limitans (Georges-Labouesse et al., 1998; Graus-Porta et al., 2001; Miner et al., 1998; De Arcangelis et al., 1999). Interestingly, loss of the intracellular focal adhesion kinase (fak), an effector of integrin activation, and integrin-linked kinase in non-neuronal cells lead to 'cobblestone lissencephaly', aberrant neuronal migration and defective cortical lamination similar to the congenital muscular dystrophies (Beggs et al., 2003; Niewmierzycka et al., 2005). These data suggest that disruption of the intracellular signaling components is sufficient to cause similar phenotypes as disrupted binding between the receptor and extracellular ligands

V DG in the retina

In addition to muscle and brain, DG together with dystrophin and its other associated proteins functions in the retina. The first evidence for retinal involvement came from the electroretinogram (ERG) defects in some of the DMD/BMD patients and the *mdx*^{3cv} mice which lacks all dystrophin isoforms and results in disrupted DG location in the retina (Blank et al., 1999; Dalloz et al., 2001; Moukhles and Carbonetto, 2001). The ERGs in these patients and mice are reduced and delayed in the amplitude of the b-wave under dark-adapted (scotopic) condition, but the ultrastructure of the retina is well preserved (Pillers et al., 1990; Pillers et al., 1993);(Cibis et al., 1993; Pillers et al., 1995). The ERG is a summed electrical signal evoked by a flash of light (Weleber and Eisner, 1988). The b-wave of the ERG is a positive summed potential which is thought to originate from the depolarization of ON-bipolar cells that receive and are activated by synaptic transmitter glutamate released from photoreceptor cells (Schmitz and Drenckhahn, 1997a; Stockton and Slaughter, 1989). Cone photoreceptor cells make synapses on both ON- and Off-bipolar cells, whereas rod photoreceptor cells are only connected to ON-bipolar cells (Schiller, 1982; Wassle et al., 1991). Some experiments have demonstrated that b-wave defects in DMD/BMD patients result from depolarizing ON-bipolar cells that receive transmission from both cone and rod photoreceptor cells (Cibis et al., 1993; Fitzgerald et al., 1994; Pillers et al., 1993; Schmitz and Drenckhahn, 1997a). In addition, Muller cells appear also involved in the generation and modification of the b-wave in which the synaptic current (K^+) flows into Muller cells to contribute to b-wave (Wen and Oakley, 1990). However, more evidence

suggests that b-wave defects in DMD/BMD are originated from impaired synaptic transmission from rods and cones to postsynaptic cells (Blake and Kroger, 2000).

In the vertebrate retina, full length Dp427 and isoform Dp260 are expressed exclusively in the photoreceptor cell membrane of the outer plexiform layer (D'Souza et al., 1995; Claudepierre et al., 1999), where photoreceptor cells form the invaginating ribbon synapses with bipolar and horizontal cells (Muntoni et al., 2003). The ribbon is a structurally and functionally specialized presynaptic organelle that constitutes an electron-dense band extending from the site of transmitter release into the cytoplasm (Sterling and Matthews, 2005). An invagination is created because the presynaptic photoreceptor cell membrane forms a cavity that contains the postsynaptic dendrites of bipolar and horizontal cells. All bipolar cells that have dendrites within the invagination belong to ON-pathway and respond to illumination with depolarization whereas bipolar cells that do not have dendrites within the invagination belong to OFF-pathway and hyperpolarize in response to light (Schmitz and Drenckhahn, 1997a). The altered b-wave of the ERG in DMD/BMD patients appears to be produced by the ON-pathway mediated by the bipolar cells within the invagination (Gurevich and Slaughter, 1993). DG is also expressed in the outer plexiform layer (Drenckhahn et al., 1996; Montanaro et al., 1995). Electron microscopy studies together with genetic and biochemical studies have revealed that DG and dystrophin immunoreactivities are found in the photoreceptor membranes that form the invagination and retrieve vesicles, but little is found at the site where the ribbon attaches and vesicles are released (Dalloz et al., 2001; Claudepierre et al., 1999; Koulen et al., 1998; Schmitz and Drenckhahn, 1997b; Sterling and Matthews, 2005; Ueda et al., 1997; Ueda et al., 1995). Therefore, DG and

Dp427/Dp260 form a presynaptic membrane complex in the outer plexiform layer, together with β-dystrobrevin, syntrophin and extracellular matrix protein agrin (Jastrow et al., 2006; Blank et al., 2002; Kroger and Mann, 1996) at the sites of endocytosis (Sterling and Matthews, 2005). *mdx* mice that lack Dp427 do not show b-wave abnormalities (Cibis et al., 1993), but deletion of Dp260 results in reduced level of DG at the outer plexiform layer and an abnormal scotopic b-wave in both mice and human (D'Souza et al., 1995; Kameya et al., 1997; Pillers, 1999; Pillers et al., 1999), which is quite similar to what is observed in mdx^{3cv} mice, which lack all dystrophin isforms, and DMD/BMD patients. Therefore it is reasonable to assume that Dp260 contribute to normal ERG production at the presynaptic membranes of photoreceptor cells. Indeed, other dystrophin isoforms such as Dp427 and Dp71 are not sufficient to replace the function of Dp260 (Fitzgerald et al., 1999). Isoform Dp71 and DG are also expressed and form a complex with utrophin, δ-sarcoglycan, α-dystrobrevin and α1-syntrophin in the endfeet of Muller glial cells, which make contact with agrin in the inner limiting membranes, as well as in the perivascular astrocyte endfeet (Claudepierre et al., 2000; Dalloz et al., 2003; Kroger and Mann, 1996; Mann and Kroger, 1996). Deficiency of Dp71 in this two places leads to reduced level of DG, impaired clustering of the rectifying potassium channel Kir4.1 and aquaporin 4, but does not lead to abnormal b-wave (Dalloz et al., 2003). Because loss of Dp71 and reduced level of DG in Muller cells and astrocyte endfeet lead to no disrupted b-wave, it suggests that defective synaptic transmission in DMD/BMD patients mainly result from the disrupted complex of Dp260 and DG located at the presynaptic membranes of the photoreceptor cells. However, how this disrupted complex in the presynaptic photoreceptor cells leads to b-

wave defects remains unknown. Although the DGC could play a structural role by linking the presynaptic photoreceptor cells to the postsynaptic cells, the normal ultrastructure in the *mdx*^{3cv} mice and DMD/BMD patients (Cibis et al., 1993; Pillers et al., 1995), which show b-wave defects, suggests that the synaptic defects in the retina are unlikely due to structural defects.

DG has an altered expression and localization at the photoreceptor membranes in Dp260-deficient animals, in *mdx*^{3cv} mice as well as in DMD/BMD patients (Blake and Kroger, 2000; Blank et al., 1999; D'Souza et al., 1995; Kameya et al., 1997). Mutations in the DG gene are not found so far in human and mouse models with eye-specific DG gene deletion are not available for studying the function of DG in retina. However, recent findings from the several human mutations in genes that alter glycosylation of DG have indicated important functions of DG in the retina. In particular with mutation in *LARGE*, that has resultant hypo-glycosylation of α-DG, the amplitude of the b-wave is significantly reduced and profound mental retardation is displayed in human (Longman et al., 2003) but vision is largely normal. Reduced amplitude and delay in the b-wave are also observed in *myd* mice bearing LARGE mutations, but retinal layering appears normal and no sign of neuronal migration defects (Holzfeind et al., 2002). In fukutin-deficient chimeric mice with hypoglycosylation of α-DG, there is extinction of the b-wave, anomalous formation of eye ball and lens, loss of laminar structure of the retina and retinal detachment (Takeda et al., 2003). Similar to mice, humans suffering from FCMD also have eye abnormalities including extensive detachment and mis-folding and fusion of the retina (Hino et al., 2001). Furthermore, some reported MEB patients have severely impaired

ocular system including retinal degeneration, bilateral glaucoma, corneal clouding and are blind at an early age (Fahnehjelm et al., 2001; Vervoort et al., 2004; Zervos et al., 2002). Because the severity and histological abnormalities in the retina of individual mutant genes seem not to phenocopy one another, this raises some doubts whether hypo-glycosylation of α-DG is the cause of these retinal defects, rather it might be the consequence or only partially contribute to the pathologies observed in the eye. A decisive dissection of DG function in the ocular system will need an animal model with eye-specific deletion of DG, or an animal model with a mutated DG having defective glycosylation of α-DG, or a rescue experiment with α-DG re-introducing into the DG-null eye. These experiments not only can clarify the function of DG in the ocular system, they can also help understand the mechanisms of retinal impairments in several congenital muscular dystrophies including MEB, FCMD, WWS and congenital muscular dystrophy type 1D..

In chapter 3, I will describe experiments using *Drosophila melanogaster* as a model to answer some of the questions mentioned above, especially the roles of DG in the visual system and distinct function of DG domains in the *Drosophila* visual system. Below, I would like introduce some background in the *Drosophila* and its visual system, especially focusing on those relevant to DG.

Part IV: DG in *Drosophila melanogaster*

I *Drosophila* DGC

Drosophila melanogaster is a powerful model organism being used to understand a particular gene function by "reverse" genetics and to look for genes

involved in a biological process by "forward" genetic screen, because of its completed genomic sequence, sophisticated genetics and evolutionary conservation of most cellular events. DG and many of its associated proteins in vertebrates are also found in *Drosophila* (Dekkers et al., 2004; Greener and Roberts, 2000; Roberts and Bobrow, 1998). The *Drosophila* ortholog of dystrophin gene encodes at least four isoforms, three of which are expressed during embryogenesis (Neuman et al., 2001). The mRNAs of longer dystrophin isoforms dmDLP1 and dmDLP2 are mainly located in the mesoderm and gut, whereas mRNA of small isoform dmDLP186 is predominantly located in the ventral nerve cord and brain (Dekkers et al., 2004). The *Drosophila* dystrobrevin ortholog is expressed in the ventral nerve cord, the brain and the muscle during late embryonic stage (Dekkers et al., 2004). The *Drosophila* syntrophin 1 is mainly expressed in the ventral nerve cord and brain, but syntrophin 2 is expressed in the visceral mesoderm, the dorsal pharyngeal muscle as well as midgut (Dekkers et al., 2004). α, β and δ-sarcoglycan *Drosophila* orthologs are expressed in the ventral nerve cord and midgut as well as muscle (Dekkers et al., 2004). These data indicate that like in vertebrate *Drosophila* dystrophin, syntrophin, dystrobrevin and sarcoglycan are predominantly expressed in the muscle and some of them are also in the central nervous system.

Drosophila DG

Drosophila DG is encoded by a single gene located on the second chromosome and alternative splicing generates three isoforms, and several bands appear in the SDS-PAGE ranging from 75 kDa to 200 kDa (Deng et al., 2003), probably due to different

glycosylation. The *Drosophila* DG ortholog is highly conserved comparing with its human counterpart. The entire amino acid sequence is about 27% identical, while the intracellular portion is about 31% identical and the last 20 amino acids in the C-terminus is 51% identical. In vertebrates, residues from 653-657 (GSIVV) of the DG polypeptide are conserved among several vertebrate species and a S654A point mutation at the cleavage site can prevent the cleavage of DG polypeptide into α- and β-subunits and leads to muscular dystrophy in vivo, but normal localization of DG on the cell surface (Esapa et al., 2003; Jayasinha et al., 2003). In the *Drosophila* DG, although the attachment site of two subunits are conserved from amino acid 934-969 (Sciandra et al., 2001), the cleveage site appears mutated. In the N-terminus, the two longer isoforms of *Drosophila* DG (type A and C) (*CG1825-PA* and *CG1825-PC*) (Deng et al., 2003) contain the mucin-like domain that is enriched with serine and threonine. In the mammals, the mucin region of α-DG contains 19 consensus sequences {S(T)P or PXXS(T)} for O-linked glycosylation (Martin and Freeze, 2003). By sequence comparison, the mucin-like region of *Drosophila* DG type C has 30% identity in amino acid sequence to human mucin-1 and 21% identity to human mucin 2 and contains 15 consensus sites for O-linked glycosylation (Fig. 1-2). *Drosophila* DG (type A) has 17.3% identity to human mucin-1 and 20% identity to mucin-2 and contains 5 putative O-linked glycosylation sites whereas the shortest DG (type B) has 6 putative sites (Fig.1-2). However, so far no reported data show that the *Drosophila* ortholog, like the vertebrate one, is glycosylated. Interestingly, the *Drosophila* orthologs of human protein O-mannosyltransferase-1 (hPOMT1) known to participate in glycosylation of α-DG and lead to WWS, when mutated, have been identified as dPOMT1 and dPOMT2, which

seem to function together in transferring a mannose to the vertebrate α-DG (Ichimiya et al., 2004). RNAi of dPOMT2 knock-down causes a "twisted abdomen" phenotype (Ichimiya et al., 2004) similar to *rotated abdomen (rt)* as seen in mutant dPOMT1 flies (Bridges and Morgan, 1923). Both dPOMT1 mutant and dPOMT2 RNAi knock-down flies have reduced viability and escapers show a "rotated abdomen" phenotype due to defects in muscle development (Martin-Blanco and Garcia-Bellido, 1996; Ichimiya et al., 2003). However, as in vertebrates, homozygous DG-null alleles are embryonic lethal in *Drosophila* (Deng et al., 2003), no escaper can live to adulthood. This viability difference simply suggests a functional difference between dPOMT and *Drosophila* DG.

During the embryogenesis DG mRNA is located in many places including the brain, visceral mesoderm, the ventral nerve cord and midgut (Dekkers et al., 2004). Indeed, in the follicle cell epithelium, DG-null cells lose their apical-basal polarity. In the DG-null epithelial cells, apical proteins such as crumbs (*crb*), β-Heavy-Spectrin, discs lost (*dlt*) are mislocalized to both basolateral and basal sides, whereas expression of basal protein such as Disc large (*Dlg*) is reduced (Deng et al., 2003). In oocytes, DG is also required for the anteroposterior polarity in that the microtubule organization center (MTOC) fails to localize to the anterior part of the oocytes in DG-deficiency (Deng et al., 2003). These data suggest that in the early embryonic development DG serves as a cell polarity determinant.

Drosophila Eye Development

Drosophila has a typical insect compound eye. Each eye is composed of about 800 functional units, the light-sensitive ommatidia. Each ommatidium is a ~20-cell assembly containing eight photoreceptor neurons (R1-R8), and 12 accessory cells including four non-neuronal cone cells, three classes of pigment cells and a bristle complex (Longley and Ready, 1995; Ready et al., 1976). In adult ommatidia each photoreceptor cell (PRC) extends a photosensitive organelle, called rhabdomere, toward the center of the ommatidium that is separated by a space called interrhabdomeral space. The rhabdomeres of R7 lie in the center position surrounded by R1-R6 whereas rhabdomere of R8 lie underneath the rhabdomeres of R7. The rhabdomere is a dense stack of microvilli of photoreceptor apical membranes, whereas the basal membranes of the photoreceptor cells are connected to the surrounding pigment cells mainly by cell adhesion molecules (Cagan and Ready, 1989; Longley and Ready, 1995).

The eye develops from a sheet of epithelial tissue called the eye imaginal disc. Beginning at the 3rd instar larval stage, an indentation called morphogenetic furrow (MF) in the disc, caused by the apical shift of a monolayer epithelial cells, initiates the cell differentiation at the posterior tip and move anteriorly in a wave across the disc (Hsiung and Moses, 2002). This process appears dependent on specific signals mediated by Hedgehog, Decapentaplegic (Dpp), the epidermal growth factor (EGF) receptor homolog (Egfr) and Notch (Borod and Heberlein, 1998; Curtiss and Mlodzik, 2000; Dominguez and Hafen, 1997; Kumar and Moses, 2001b). Photoreceptors are the first cells to differentiate followed by non-neuronal lens-secreting cone cells and primary pigment cells (Nagaraj and Banerjee, 2004; Tomlinson and Ready, 1987).

Among all photoreceptor cells, R8 is the first to be specified followed by R5 and R2, then R4 and R3 to form five cell clusters (Fig. 1-4), posterior to morphogenetic furrow. Around this five cell stage, PRCs begin to rotate as a group. In dorsal part of disc these PRCs rotate clockwise whereas in the ventral part they rotate counterclockwise (Zheng et al., 1995; Strutt and Strutt, 2003)(Fig. 1-4). The boundary that separates the two parts is called "equator" (Fig. 1-4). Following the five cell stage, R7, R1 and R6 are finally recruited to form an eight cell trapezoid (Hsiung and Moses, 2002; Nagaraj and Banerjee, 2004). These processes generate mirror-symmetrically patterned PRC clusters so that they have a uniform pattern at one side of equator and another part has an opposite pattern (Fig.1-4). This polarity is formed within a plane perpendicular to the apical-basal direction and is referred as planar cell polarity of PRC.

Cell-cell communications play a major role in PRC differentiation and fate determination. Best known is the notch-delta signaling pathway. Activation of the notch ligand, delta requires high level of EGFR signaling, and delta in PRC activates notch in the undifferentiated neighboring cells and initiate the signaling (Baker and Rubin, 1989; Baker and Rubin, 1992; Nagaraj and Banerjee, 2004; Tsuda et al., 2002). This process is required specification of all cells in the eye (Freeman, 1996). In addition, *bride of sevenless* (Boss), a seven-pass transmembrane protein in R8, binds to and activates *sevenless,* a receptor tyrosine kinase expressed in R7, R3/R4 and R1/R6 (Banerjee et al., 1987; Reinke and Zipursky, 1988). This binding results in activation of Ras by Grb2/Sos complex, which in turn activates the MAPK signaling cascades (Biggs et al., 1994; Biggs and Zipursky, 1992; Bonfini et al., 1992; Rogge et al., 1991; Simon et al., 1991; Simon et al., 1993). Through combination of complex signaling

pathways, the specification of PRCs, cone cells and pigment cells are completed when the pupation begins.

Starting with the pupal stage, following the 90° rotation the apical surfaces of PRC become toward the center of ommatidia and apical-basal axis is perpendicular to entering light (Longley and Ready, 1995), and cells within the ommatidia begin their final differentiation, a process involving in massive cell shape change, cell movement, and controlled cell death. After approximately 33% of pupal development the differentiation and patterning of the retina is nearly complete, showing already the same number of ommatidia and the highly regular hexagonal array observed in the adult (Ready et al., 1976; Tomlinson, 1988). Between 37 and 55% of pupal life, PRCs dramatically expand their cell volume, and its apical membranes elongate to form long, densely-packed arrays of light-sensitive microvilli called rhabdomeres. And, rhabdomeres reach and anchor to the retina floor that is formed by meeting of four cone cell feet (Longley and Ready, 1995; Pichaud and Desplan, 2002). Cone cells are recruited apically into the PRCs cluster in the 3rd instar larvae and lie entirely to the sides of the PRCs until 14% pupa life and form the cone cell plate at 37% pupa life (Longley and Ready, 1995) and begin to secrete lens at 45% pupal development (Cagan and Ready, 1989). The primary pigment cells begin to enwrap the cone cell at 20% of pupal development and form a membrane above the retinal floor called fenestrated membrane at ~67% of pupal development (Cagan and Ready, 1989). The cell-cell contacts mediated mainly by adherens junctions between PRCs and between PRCs and cone cells are maintained and extended during this morphogenesis of the eye (Longley and Ready, 1995). The adherens junctions between PRCs are localized sub-apically and

are connected to the rhabdomeres by the stalk membranes (Longley and Ready, 1995; Pellikka et al., 2002). When the adherens junctions are disrupted in the pupal stage by mutant polarity determinants such as bazooka and crumbs, the final differentiation of PRC is impaired (Hong et al., 2003; Izaddoost et al., 2002; Pellikka et al., 2002). The mutant PRC rhabdomeres fail to reach the retinal floor and are mis-positioned in the ommatidia, but the PRCs are normally polarized and there is no degeneration in pupal PRCs (Hong et al., 2003; Izaddoost et al., 2002; Pellikka et al., 2002). Many more genes involved in polarity formation, transcription regulation, cytoskeleton modification, protein trafficking and cell adhesion have been shown to be required for eye morphogenesis.

To understand if DG is required for eye morphogenesis, chapter 3 of this thesis describes evidence that DG plays essential roles for autonomous PRC differentiation and for eye morphogenesis by loss-of-function, gain-of function and other methods. Moreover it shows distinct roles of intracellular and extracellular DG in PRC development in *Drosophila* eye. In addition, chapter 2 of the thesis describes identification of a new DG interacting protein, the GTPase dynamin 1, in the brain and in the outer plexiform layer of vertebrate retina and its functional implication in endocytosis is reported. Together, by experiments describing in *Drosophila* visual system (chapter 3) and in vertebrate (chapter 2), I show several novel functions of DG in the nervous system and its implications will be discussed.

Table 1. Muscular dystrophies and their gene products

Disease	Disease subtype	Inheritance pattern	Gene product
DMD/BMD		XR	Dystrophin
LGMD	1C	AR	Caveolin3
	2B	AR	Dysferlin
	2C	AR	γ-sarcoglycan
	2D	AR	α-sarcoglycan
	2E	AR	β-sarcoglycan
	2F	AR	δ-sarcoglycan
MDC	1A	AR	Laminin α2 chain
	1C *	AR	Fukutin related protein
	1D *	AR	LARGE
	Fukuyama CMD *	AR	Fukutin
	MEB *	AR	O-mannoseβ-1,2-N-acetyl-glucosaminyl transferase
	WWS *	AR	O-mannoslytransferase 1

This table lists mainly the muscular dystrophies and their gene products that are related to the dystrophin glycoprotein complex (DGC). For a complete list, please see Dalkilic and Kunkel (2003). DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; LGMD, limb-girdle muscular dystrophy; MDC, congenital muscular dystrophy; CMD, congenital muscular dystrophy; MEB, muscle eye brain disease; WWS, Walker-Warburg Syndrome; XR, X-linked recessive; AR, autosomal recessive. *, disease having a defective glycosylation of α-DG.

Dystroglycan and its associated proteins

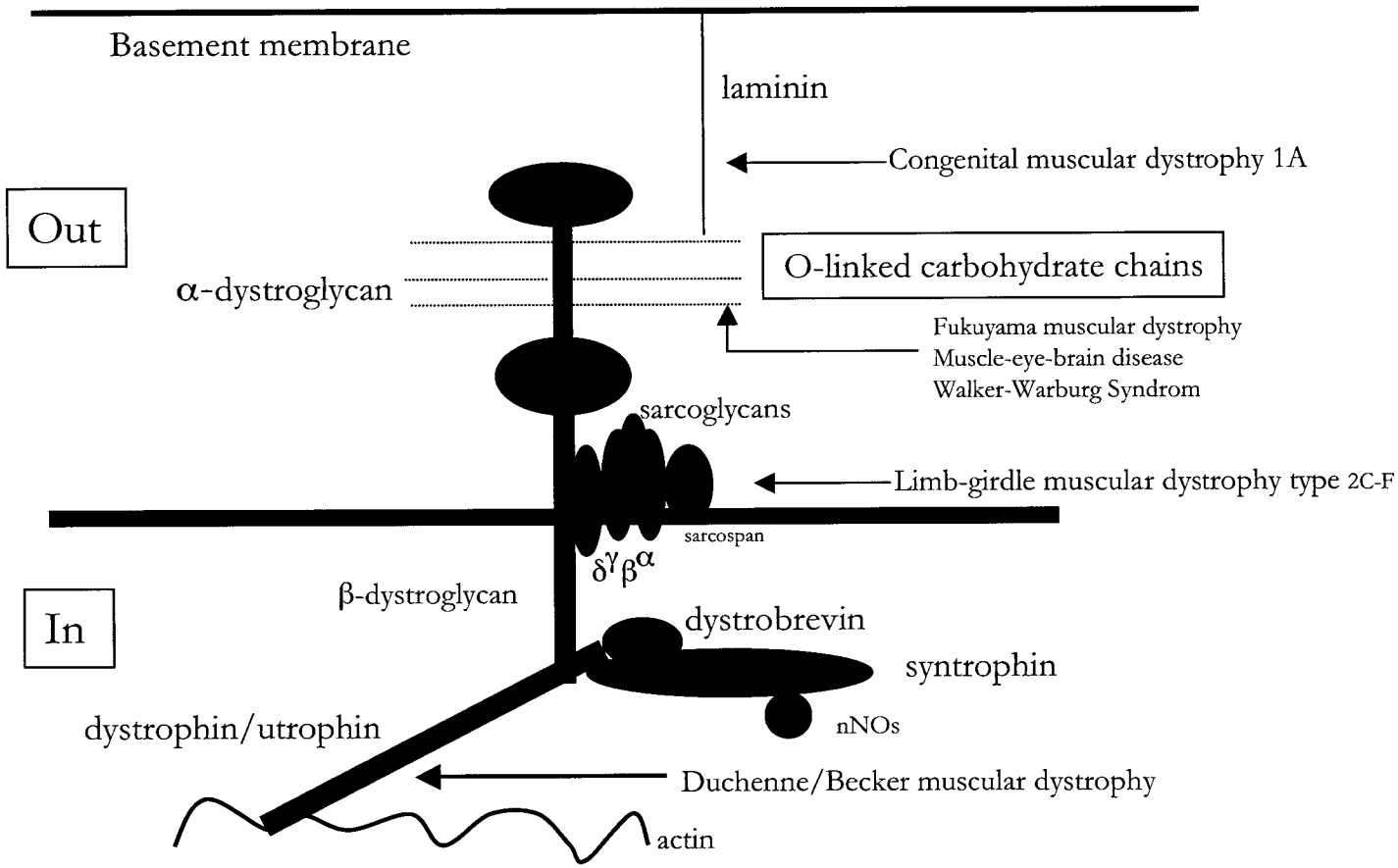


Figure 1-1: Structure of the dystrophin glycoprotein complex (DGC) in muscle. The DGC links the cell from intracellular actin to the extracellular laminin, agrin or perlecan in the basement membrane. The DGC can be divided into three subcomplexes: the cytoplasmic complex containing dystrophin/utrophin, syntrophin, dystrobrevin and syntrophin-binding protein nNOs; the sarcoglycan and sarcospan complex containing transmembrane protein sarcoglycan (α, β, γ, δ) and sarcospan (SS); the dystroglycan complex containing β and α-dystroglycan and its extracellular ligands laminin and agrin. Disruption of the DGC causes various muscular dystrophies: Mutations in dystrophin causes Duchenne/Becker muscular dystrophy; Mutations in sarcoglycans causes limb girdle muscular dystrophy type 2C-F; Mutations that affect glycosylation of α-DG are found in Fukuyama muscular dystrophy, Muscle-Eye-Brain disease and Warker-Warberg Syndrome. Mutations in α2 chain of laminin cause congenital muscular dystrophy type 1A. No mutations in human have been found in agrin and perlecan genes.

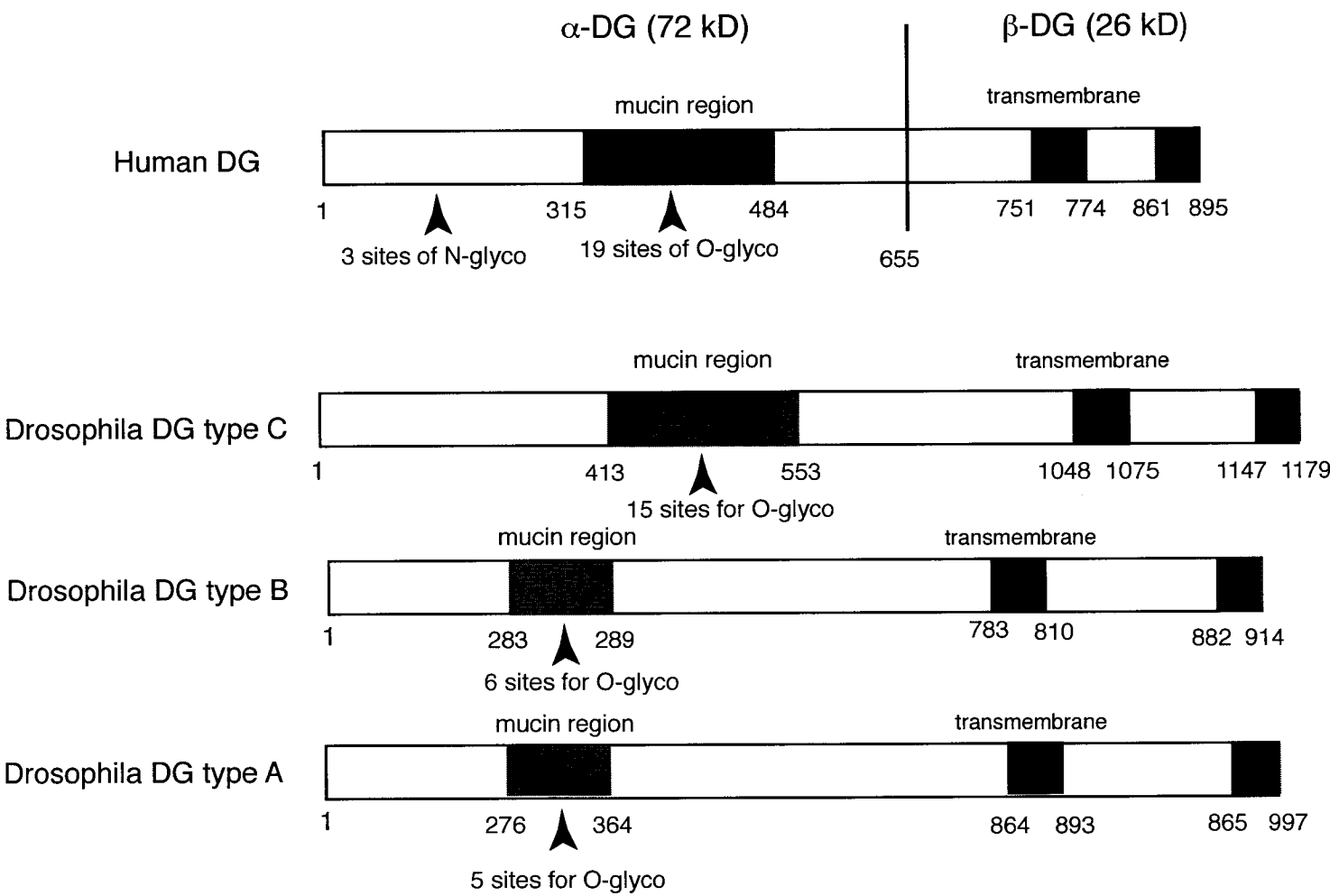
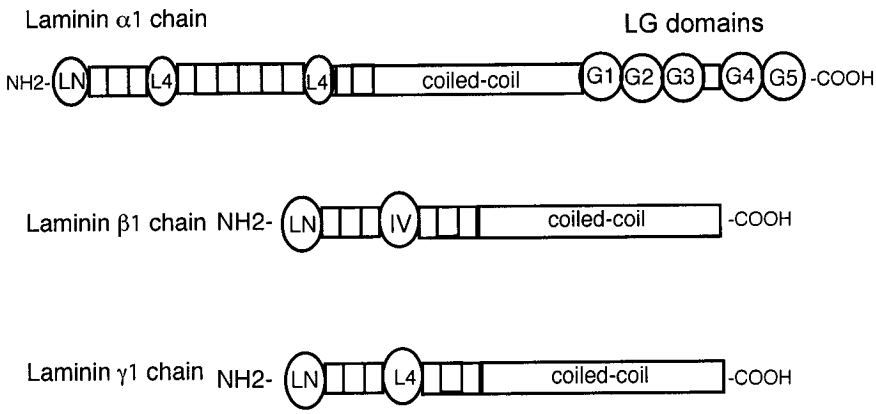
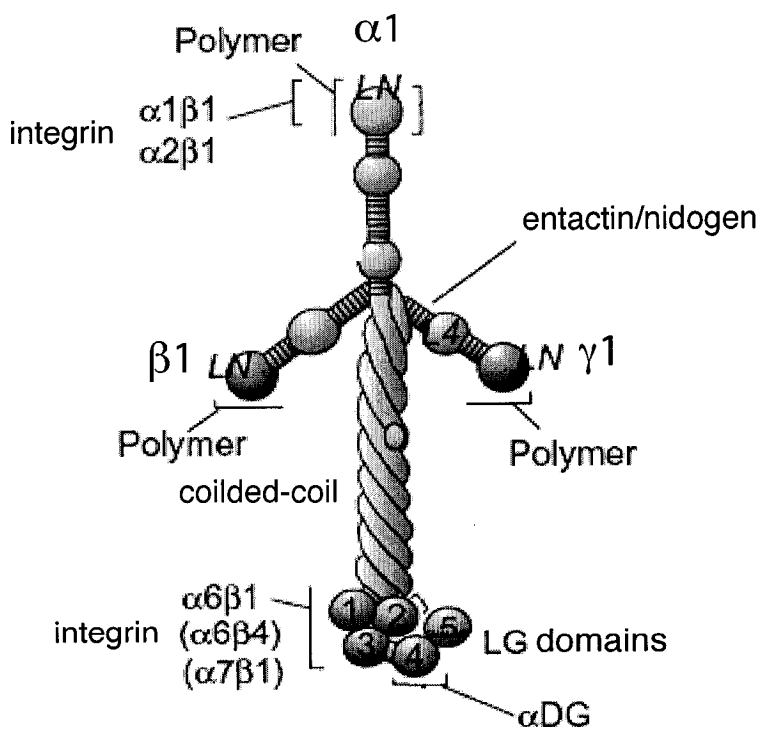

α-DG (72 kD)
β-DG (26 kD)
mucin region
transmembrane
Human DG
1
315
484
751
774
861
895
3 sites of N-glyco
19 sites of O-glyco
655
mucin region
transmembrane
Drosophila DG type C
1
413
553
1048
1075
1147
1179
15 sites for O-glyco
mucin region
transmembrane
Drosophila DG type B
1
283
289
783
810
882
914
6 sites for O-glyco
mucin region
transmembrane
Drosophila DG type A
1
276
364
864
893
865
997
5 sites for O-glyco

Figure 1-2: Illustration of human and *Drosophila* dystroglycan (DG) proteins. The human DG is comprised of α and β-DG with a cleavage site at serine 655. α-DG contains 653 amino acids within which a mucin region starting from amino acid 315 to 484 has 19 consensus sites for O-linked glycosylation (Martin, 2003). Outside the mucin region, there are three putative N-linked glycosylation sites. Glycosylation changes the molecule weight of α-DG protein (72 kDa) to 100-200 kDa that is found in tissues. Glycosylation of the transmembrane β-DG increases its molecular weight from 26 to 43 kDa. Alternative splicing generates three isoforms of DG in *Drosophila* (Deng et al., 2003). The longest one is type C DG which contains a highly conserved mucin region in its extracellular domain (from amino acid 413 to 553) and a C-terminal region (from amino acid 1147 to 1179). By sequence comparison, fifteen serines or threonines are putative O-linked glycosylation sites in the mucin region of the *Drosophila* DG type C. In the type B and A the consensus sites for O-linked glycosylation are six and five respectively. The cleavage site is not conserved and it is not unkown if *Drosophila* DG is cleaved into α and β-subunits or not. So it is not marked. Nor do we know the precise molecule weights following the glycosylation. The transmembrane domains are marked in *Drosophila* DG and the conserved C-terminal domains in type A and B are labeled too.

Figure 1-3





(Bottom: modified from Figure 1 in Yurchenco and Wadsworth, 2004)

Figure 1-3: Structure of laminin 1 comprised of α1, β1 and γ1 chains. The three chains of laminin are shown individually (top) and as a heterotrimer (bottom). The domains in individual chains are marked. The N-terminal and L4 globular domains are shown in LN (α1, β1, γ1 chain) and L4 (α1, γ1 chain) as circles which are interrupted by EGF repeats shown as rectangles (α1, β1, γ1 chain). The coiled-coil regions in α1 chain are joined together to form the heterotrimer with the C-terminus lie beyond the coiled-coil region and three independent short arms (bottom). Five LG domains (LG modules) are marked as circles in laminin trimer (α1 chain and bottom), in which LG 1-3 interact with integrin whereas LG 4-5 interact with α-DG. Integrins also interact with LN domain of α chain and Entactin/nidogen interacts with the EGF repeat in γ chain (pointed in bottom). The self-assembly of laminin requires the N-terminal LN domains of α, β, γ-chain (pointed as polymer).

Figure 1-4

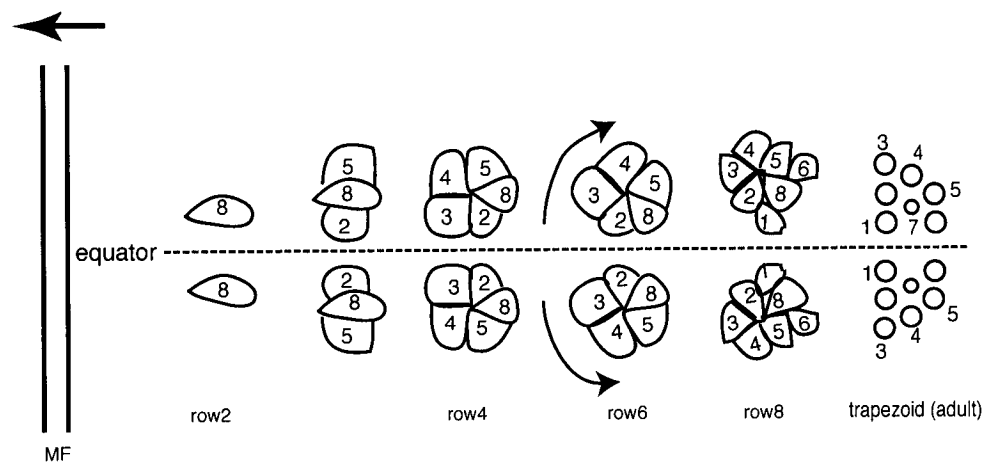


Figure 1-4: Photoreceptor patterning and rotation in the 3rd instar larvae disc.

A wave of differentiation initiated by morphogenetic furrow (MF) moves from posterior to anterior and photoreceptor cells are sequentially recruited as the disc matures. R8 photoreceptor cell is first to be specified row 2 behind the MF followed by R2 and R5, then R3 and R4 are added to form a five-cell cluster. PRCs around this stage begin to rotate clockwisely dorsal to the equator, and counterclockwisely ventral to the equator. As disc matures, R1, R6 and R7 are recruited to form a trapezoid and adult PRCs have rotated 90° to form a mirror-symmetric pattern. All R7s in the trapezoid on both sides point to the equator whereas R3 and R4 face the pole of disc. This polarized pattern is referred as PRC planar polarity. R8 is below R7 and not shown in the adult trapezoid. Anterior is toward left. Posterior is right. Dorsal is up and ventral is down.

Chapter 2

Evidence that dystroglycan is associated with dynamin and regulates endocytosis

by

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ABSTRACT

Disruption of the dystroglycan gene in humans and mice leads to muscular dystrophies and nervous system defects including malformation of the brain and defective synaptic transmission. To identify proteins that interact with dystroglycan in the brain we have used immunoaffinity purification followed by mass spectrometry (LC/MS-MS) and found that the GTPase dynamin 1 is a novel dystroglycan-associated protein. The β-dystroglycan-dynamin 1 complex also included α-dystroglycan and Grb2. Overlay assays indicated that dynamin interacts directly with dystroglycan and immunodepletion showed that only a pool of dynamin is associated with dystroglycan. Dystroglycan was associated and co-localized immunohistochemically with dynamin 1 in the central nervous system in the outer plexiform layer of retina where photoreceptor terminals are found. Endocytosis in neurons is both constitutive, as in non-neural cells, and regulated by neural activity. To assess the function of dystroglycan in former we have assayed transferrin uptake in fibroblastic cells differentiated from embryonic stem cells null for both dystroglycan alleles. In wild-type cells dystroglycan formed a complex with dynamin and codistributed with cortactin at membrane ruffles which are organelles implicated in endocytosis. Dystroglycan-null cells had a significantly greater transferrin uptake, a process well known to require dynamin. Expression of dystroglycan in null cells by infection with an adenovirus containing dystroglycan reduced transferrin uptake to levels seen in wild type ES cells. These data suggest that dystroglycan regulates endocytosis possibly as a result of its interaction with dynamin.

INTRODUCTION

Dystroglycan (DG) is encoded by a single gene (*dag1*), and is synthesized as a precursor protein that is cleaved into α and β subunits. α-DG is a peripheral membrane protein that binds, via its carbohydrate side chains (Ervasti and Campbell, 1993b; Michele et al., 2002), to globular motifs in the α chain of several laminins (Gee et al., 1993; Ido et al., 2004; Talts et al., 2000), agrin (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), perlecan (Jacobson et al., 2001; Peng et al., 1998) and α-neurexin (Sugita et al., 2001). β-DG is a transmembrane protein bound non-covalently to α-DG that interacts intracellularly with dystrophin, utrophin, rapsyn, caveolin-3 and growth factor receptor-bound protein 2 (Grb2) (Winder, 2001). In skeletal muscle α- and β-DG form the functional core of a larger complex that links the extracellular matrix to the cytoskeleton and serves to maintain the integrity of myofibers faced with the stress of repeated contractions (Durbeej and Campbell, 2002). Hence, mice with skeletal muscle deficient in DG develop a severe muscular dystrophy (Cohn et al., 2002; Cote et al., 1999).

DG is expressed in the nervous system and in many tissues other than skeletal muscle (Durbeej et al., 1998) and mice null for *dag1* die at E6.5-7.0, long before muscle differentiation (Williamson et al., 1997). DG has been implicated in the formation of several (Jacobson et al., 2001; Michele et al., 2002; Moore et al., 2002), but not all basement membranes (Cohn et al., 2002; Cote et al., 1999; Holzfeind et al., 2002; Li et al., 2002) and in *Drosophila* DG is necessary for epithelial polarization (Deng et al., 2003). In the nervous system DG is localized at glial endfeet in contact with basement membranes around blood vessels (Tian et al., 1996) and beneath the

meninges of the brain (Michele et al., 2002). DG is also concentrated at peripheral (Cohen et al., 1995; Gingras and Ferns, 2001; Leschziner et al., 2000) and central synapses (Brunig et al., 2002; Koulen et al., 1998; Levi et al., 2002; Moukhles and Carbonetto, 2001). Recent work has shown that myodystrophy (*myd*) mice, which have a mutation in *LARGE*, a gene that encodes an enzyme involved in O-glycosylation of α-DG (Grewal et al., 2001), have decreased affinity for laminin and disrupted meningeal basement membranes (Michele et al., 2002). As well, these mice have defects in neuronal migration in the cerebral cortex and cerebellum (Michele et al., 2002). In humans similar defects, called lissencephalies, are associated with profound mental retardation and are manifested in Walker Warburg Syndrome, Muscle Eye Brain Disease and Fukuyama Muscular Dystrophy. All of these are congenital muscular dystrophies that result from mutations in genes known or suspected to regulate glycosylation of α-DG (Montanaro and Carbonetto, 2003). Interestingly, mice with a targeted deletion of *dag1* in the brain have a similar phenotype (Moore et al., 2002) suggesting that many functions of DG are mediated by carbohydrate side chains which are responsible for most ligand binding (Ervasti and Campbell, 1993b; Michele et al., 2002) (Kanagawa et al., 2004). In addition, mice lacking DG in the brain have defects in long-term potentiation in the hippocampus (Moore et al., 2002) and *myd* mice have an abnormal electroretinogram with no signs of disrupted neuronal migration in the retina (Holzfeind et al., 2002). DG is also necessary for stabilization of the postsynaptic density of acetylcholine receptors on skeletal myotubes in culture (Grady et al., 2000; Jacobson et al., 2001) and at nerve-muscle synapses in vivo (Cote et al., 1999; Jacobson et al., 2001).

Emerging evidence indicates that the proteins that interact with DG in non-muscle tissues differ from those in muscle. For example, α-neurexin is complexed with α-DG in the central nervous system (CNS) (Sugita et al., 2001) while periaxin is associated with DG in Schwann cells (Sherman et al., 2001). To identify novel DG interactors we have used immunoaffinity chromatography to purify DG and its associated proteins from rat brains. We report that β-DG forms a complex with dynamin 1, a GTPase essential for several modes of endocytosis including regulated endocytosis during synaptic vesicle release and constitutive endocytosis which occurs in neuronal and non-neuronal cells alike (D'Hondt et al., 2000; Jarousse and Kelly, 2001; Praefcke and McMahon, 2004).The β-DG-dynamin complex in the CNS also contains α-DG and the SH2 (src homology 2)/SH3 (src homology 3) adapter protein Grb2 and has the potential to anchor dynamin at discrete sites within the cell. In an overlay assay, β-DG interacts directly with brain dynamin. In the retina DG is associated and immunohistochemically colocalized with dynamin at the outer plexiform layer where previous ultrastructural studies (Blank et al., 1999; Koulen et al., 1998) have found DG to be distributed presynaptically in synapses formed by photoreceptors onto bipolar and horizontal cells. Finally, in fibroblasts differentiated from embryonic stem (ES) cells DG associates with dynamin and codistributes with cortactin at membrane ruffles, which are organelles implicated in endocytosis. Cells null for DG have an increased uptake of transferrin, and re-introduction of DG into these null cells reduces this increase to normal levels.

EXPERIMENTAL PROCEDURES

Antibodies

Antisera to β-DG and monoclonal antibody (mAb 1B7) to α-DG were characterized previously (Jacobson et al., 2001; Moukhles et al., 2000). MAbs to the following proteins were used for western blotting: β-DG (1:250) (*Novocastra Laboratories Ltd.*); α-DG (IIH6) (1:1000) (*Upstate Biotechnology*); Grb2 (1:500); GST (1:1000) (*Santa Cruz Biotechnology*). MAb Hudy-1 (*Upstate Biotechnology*) that recognizes dynamin 1 found in the brain as well as the more ubiquitous dynamin 2 (Damke et al., 1994) was used at 1:40,000 for western blotting and 1:200 for immunocytochemistry. MAbs to cortactin (*Upstate Biotechnology*) were used at 1:250. Antisera to amphiphysin (1874) were a gift from Peter McPherson (McGill). Horseradish peroxidase–conjugated goat anti-mouse (1:2000) and goat anti-rabbit antisera (1:4000) (*Jackson ImmunoResearch Laboratories*) were used as secondary antibodies in western blots. Rhodamine (TRITC) conjugated donkey anti-rabbit (1:200) and fluorescein (FITC) conjugated donkey anti-mouse antisera (1:200) (*Jackson ImmunoResearch Laboratories*) were used for immunofluorescence staining.

Tissue extraction

Rat brains and hippocampi were dissected from adult rats euthanized according to the Guidelines of McGill University Health Center Animal Facility. Tissues were homogenized on ice four times (10 sec each) with a Polytron tissue homogenizer in phosphate-buffered saline (PBS; pH 9.0) containing 1% sodium deoxycholate, 0.5mM $CaCl_2$, and a protease inhibitor cocktail (*Boehringer Mannheim*). The homogenates

were incubated at 37° for 30 min followed by addition of Triton X-100 (TX-100) to a final concentration of 0.5% in a total volume of 6 ml. After 2 hrs of incubation at 4° with occasional vortexing, the homogenates were centrifuged at 37,000x g for 30 min. The supernatants were collected and protein concentrations were determined (DC Protein Assay Kit; *Bio-Rad*).

Synaptosomes were prepared from rat brains as described by Sugita et al. (2001) with minor modification. Briefly, rat brains were removed and homogenized with a Teflon glass homogenizer in a buffer containing 20mM Tris-HCL (pH7.2), 0.32M sucrose, 1mM $CaCl_2$, and protease inhibitor cocktail. The homogenates were centrifuged for 10 min at 700x g to remove nuclei. The post-nuclear supernatants were collected and centrifuged for 30 min at 9,800x g, and the resulting pellets were collected as crude synaptosomes.

Immunoaffinity chromatography

Antisera to β-DG (11), bovine serum albumin (control) or rabbit immunoglobulin (control) were covalently coupled to Affigel 10 following the manufacturer's instructions (*Bio-Rad*). Five mgs of hippocampal extract (2-3ml) were incubated overnight with 1 ml of each Affigel-protein conjugate. The slurry was packed into a column, washed first with 10 ml of PBS (pH 9.0) containing 1% sodium deoxycholate, 0.5% TX-100 and 0.15M sodium chloride (buffer A), followed by a wash with 10 ml of 0.5M NaCl in buffer A, and finally 10ml of 5mM EDTA in buffer A. Proteins still bound to the column were eluted at low pH with 0.1M glycine (pH2.5) and neutralized

with 0.3ml of 1M Tris-HCl (pH8.0). The columns then were equilibrated each with 10 ml of PBS and any remaining proteins were eluted with 3ml of 0.1M triethylamine (pH11.5) and neutralized with 0.3ml of 1M Tris-HCl (pH8.0). The glycine and triethylamine eluates were pooled, dialyzed against PBS overnight and then concentrated to equal volumes (*Millipore Centricon* filters), and equal aliquots of the eluates were subjected to SDS-PAGE. The SDS-PAGE and western blotting were carried out as described previously (Gee et al., 1993).

Immunoprecipitation

Crude synaptosomes or retinas were solubilized in PBS (pH 9.0) containing 1% sodium deoxycholate, 0.5% TX-100, 1mM $CaCl_2$ and protease inhibitor cocktail (buffer B) and centrifuged for 37,000x g for 30 min. 60-100 µl of supernatant was pre-cleared with 20 µl of protein G agarose (*GibcoBRL*) for 1 hr. After preclearing 20 µl of protein G beads conjugated to relevant antibodies or pre-immune sera were added to the pre-cleared supernatants, and incubated overnight at 4° with constant shaking. The agarose beads were then pelleted by centrifugation at 4,000x g for 2 min. The supernatants were collected and the beads were washed twice with 1 ml of buffer C, followed by 0.5 ml of 0.5M NaCl in buffer B, and finally with 0.5 ml of buffer B containing 5mM EDTA. The washed beads were then boiled in sample buffer for 5 min and subjected to SDS-PAGE and western blotting.

Overlay Assay

The cytoplasmic tail of β-DG was amplified by PCR from a plasmid containing the full-length DG cDNA sequence using sense 5'-GAAGATCTTCTATCGCAAGAA

GAGG-3', and anti-sense primers 5'-GCTCTAGATTAAGGGGGAACATACGG-3'. The amplified β-DG_{cyto} was digested with Bgl II and gel purified. Plasmid pGEX-3x (*Amersham Biosciences*) was digested with EcoR I, blunt-ended, digested with Bam HI and gel-purified. The purified pGEX-3X vector and β-DG_{cyto} fragment were ligated to produce pGST-βDG_{cyto} plasmid. The DH5α *E. coli* strain was transformed with plasmids pGST-3X and pGST-βDG_{cyto}, and proteins induced with 1mM IPTG for 2-3 hrs were purified according to the procedures described by Frangioni and Neel (Frangioni and Neel, 1993) using glutathione Sepharose (*Amersham Biosicence*).

To assay the interaction of dynamin with β-DG, dynamin was immunoprecipitated from 0.4 mg of synaptosomal extracts with 6μg of mAb Hudy-1 (Warnock et al., 1995). The immunoprecipitated dynamin was electrophoresed in a 4-15% acrylamide gel and electroblotted onto a PVDF membrane (*Bio-Rad*). The membrane was blocked for 1 hour with 5% powdered skim in TBST (10mM Tris-HCL, 150mM NaCL, 0.1%Tween-20) and incubated overnight with GST (20 μg/ml) or GST-βDG_{cyto} (20 μg/ml) in TBST containing 5% skim milk. The membranes were washed three times with TBST (10 min each) and incubated with HRP-conjugated mouse anti-GST antibody (*Bio-Rad*) for 1 hr. After three washes with TBST (15 min each), the signals were visualized by chemiluminescence (*PerkinElmer Life Sciences*).

Cell culture, immunocytochemistry and transferrin uptake assay

Wild type and DG null embryonic stem cells were engineered as previously described (Cote et al., 1999). Cells were differentiated into embryoid bodies that were grown on 0.1% gelatin-coated tissue culture dishes (Jacobson et al., 2001). After 3 d in culture,

cells from the embryoid bodies spread and fibroblast-like flat cells appear at the periphery. At this point, cultures were treated with 200 μg/ml transferrin (*SIGMA*) at 4° for 15 min, after which cells were washed extensively with PBS and incubated with 20 μg/ml of rhodamine-conjugated transferrin in fresh growth medium at 37° for 15 or 120 mins. After washing in PBS, cells were fixed in 4% paraformaldehyde in PBS, and permeablized in 0.5% TX-100 in PBS for 10 min. Nuclei were stained with DAPI (*Sigma*) for 10 min at room temperature. Immunocytochemistry followed previously described methods (Jacobson et al., 2001). Briefly, cells were washed with PBS, fixed in 4% paraformaldehyde, permeablized in 0.5% TX-100, incubated with primary antibodies for 1 hr, then washed with PBS 3 times and incubated with fluorescently-labeled secondary antibodies for 1 hour. Cells were washed prior to mounting on slides in Immunofloure mounting medium (*ICN Biomedicals, Inc.*) and observed under a 63X objective using a Zeiss Axioskop fluorescent microscope. Images were captured with a Retiga 1300 Monochrome 10 bit digital camera (*Qimaging Corp.*) and analyzed with Northern Eclipse software (*Empix*). Quantifications were done on 8-bit images using threshold values of 86-255 pixels (Jacobson et al., 2001). The sum of the areas that were fluorescent were obtained for each field and normalized to the number of cells per field. Measurements were tabulated with Microsoft Excel and analyzed statistically using StatView (*Abacus Concepts, Inc.*).

To isolate and expand fibroblastic cells, wild type and DG null embryoid bodies were grown for 3 weeks. At this point embryoid bodies were surrounded by a halo of fibroblastic cells. The central cell mass was aspirated and the remaining adherent cells on the periphery were grown for 2d after which cells that did not appear fibroblastic

were carefully aspired. The fibroblastic cells were then harvested and grown on 175 cm^2 dishes for 4d. Some cells were passaged onto dishes for transferrin uptake assays with same procedures as before except that cells are incubated with transferrin at 37° for 15 min. Others were harvested, solubilized in 1% TX-100 containing 20mM Tris-HCl, 150mM NaCl, 1mM $CaCl_2$ and protease inhibitor cocktail, centrifuged at 16,000x g for 20 min and supernatants were used for immunochemical studies.

Adenoviral Constructs and Infection

To express full-length DG in DG-null fibroblastic cells, we generated a replication-defective adenovirus using the AdEasy system (*Qbiogene*) as described by the manufacturer. In brief, DG cDNA was subcloned into the multiple cloning site of pAdTrack-CMV(GFP) (GFP and DG are under separate CMV promoters producing two separate proteins), linearized using PmeI, and cotransfected with pAdEasy-1 into BJ5183, an electrocompetent E.coli strain. Recombinants (AdDG/GFP) were screened by colony size and confirmed by restriction digests. They were then linearized using PacI, purified, and transfected into HEK293 cells using Lipofectamine reagent (*Invitrogen*). Adenovirus production was observed by plaque formation and by GFP fluorescence. Viral particles were isolated from cellular lysates and amplified through several rounds of infections in HEK293 cells. Titers were determined by $TCID_{50}$ infection test as described by the manufacturer (*Qbiogene*). For DG expression, ES cells were grown at low confluence, infected with 10^3 plaque forming units/mL (MOI 5) of control AdGFP or of AdDG/GFP for 18 hrs in growth medium. Infection

efficiency was monitored by GFP reporter gene expression and was maximal 2 days post-infection. At this point, transferrin uptake was assayed.

Immunohistochemistry

Three-month-old male mice (strain C57BL/6) were anaesthetized using xylazine (2mg/ml, *Bayer Inc.*) and ketamine hydrochloride (15 mg/ml, *Ayerst veterinary Lab.*). After anesthesia mice were perfused intraventricularly with PBS followed by 4% paraformaldehyde. Whole eyes were dissected from the animals and post-fixed overnight in 4% paraformaldehyde followed by cryopreservation in 30% sucrose. Eyes were then frozen in Tissue-Tek O.C.T. compound (*Sakura Finetek U.S.A.*) on crushed dried ice and stored at -80°. Eight μm horizontal sections were cut using a Leica CM3050S cryostat and stored at -20°. Sections were thawed to room temperature, washed with PBS and blocked with 10% horse serum and 0.3% TX-100 in PBS for 1 hr prior to immunostaining. The retinal sections were incubated 1.5 hrs in blocking buffer containing both Hudy-1 and β-DG antiserum. Incubation with secondary antibodies was done in blocking buffer containing both RITC-conjugated donkey anti-rabbit antiserum and FITC-conjugated donkey anti-mouse immunoglobulins. Sections were washed 3 times with PBS after both primary and secondary antibody incubations, mounted in immunofloure mounting medium (*ICN*) and visualized using a Leica TCSNTSP confocal microscope.

RESULTS

Identification of dynamin 1 as a β-DG associated protein in brain

Hippocampal homogenates were extracted directly with 1% sodium deoxycholate (pH9.0) and 0.5% TX-100. This effectively solubilizes DG as well as most pre- and post-synaptic proteins (Huang et al., 2000). Immunoaffinity columns conjugated with antisera to β-DG, non-immune rabbit IgGs (control), or BSA (control) were equilibrated with the extracts, washed extensively and eluted first at low and then at high pH (Methods). Following SDS-PAGE analysis of the eluates and Coomassie blue staining, several protein bands were evident at 43 kDa and ~100 kDa (Fig, 2-1A) that bound specifically to β-DG antibody columns. The 43 kDa band was confirmed to contain β-DG in western blots (Fig. 2-1B). The ~100 kDa band was cut out, digested with trypsin and subjected to LC/MS-MS analysis (Montreal Proteomics Network). This yielded 21 tryptic peptides whose sequences matched that of rat dynamin 1 (gi: 118966; 97 kDa) with the peptides covering 25% of the sequence of dynamin 1. The 55 kDa band yielded peptides matching to the IgG heavy chain. Peptides from the ~66 kDa band matched to Hsc70 (not shown). Consistent with the MS results, western blotting indicated that dynamin 1 was enriched along, with β-DG, in brain extracts subjected to immunoaffinity chromatography (Fig. 2-1B). Since dynamin is a relatively abundant cytosolic protein and β-DG is a transmembrane protein, we sought to determine the amount of dynamin 1 bound to β-DG. β-DG could be immunodepleted from brain extracts, although a significant amount of dynamin 1 remained in the supernatant (Fig. 2-2A, B). No immunoprecipitation of either protein with control IgG

(Fig. 2-2B) was observed. These results suggest that there is a pool of dynamin 1 tightly associated with β-DG, and a pool of unbound dynamin 1.

Detection of other DG-associated proteins in brain

To further characterize the β-DG-dynamin complex in the CNS we used antibodies to several DG-associated proteins in muscle. Immunoaffinity-purified fractions of β-DG from brain were western blotted with two monoclonal antibodies (mAb IIH6 and 1B7), that detect differentially glycosylated forms of α-DG (Leschziner et al., 2000; Moukhles et al., 2000). MAb IIH6, that recognizes larger and more heavily glycosylated forms, detected a band of α-DG enriched in the affinity-purified fraction (lane 2 of Fig. 2-3A), consistent with our previous data (Moukhles and Carbonetto, 2001). MAb 1B7 that recognizes smaller and, hence, hypoglycosylated forms of α-DG (Moukhles and Carbonetto, 2001) also detected an enriched band with lower molecular weight (lane 3 of Fig. 2-3A). Thus the brain contains complexes of β-DG and α-DG with different levels of glycosylation that may affect ligand binding of the α subunit (Leschziner et al., 2000; Michele et al., 2002).

Grb2 is an SH2-SH3 containing adapter protein that is associated with dynamin in brain through its SH3 domains (Gout et al., 1993; McPherson et al., 1994). In muscle Grb2 has also been shown to associate with β-DG through its SH3 domain (Russo et al., 2000; Yang et al., 1995). We therefore asked whether Grb2 could be part of a complex with DG and dynamin 1 in brain. All three proteins (DG, dynamin and Grb2) were immunoprecipitated from the synaptosomes using antibodies against β-DG (Fig. 2-3B),

and in the immunoaffinity-purified fractions from brain (data not shown). Taken together these data indicate that the brain contains a complex of α-/β-DG with dynamin 1 and Grb2.

Although Grb2 has the potential to mediate binding to dynamin, other data indicate that SH2-SH3 domain-containing proteins such as amphiphysin (David et al., 1996; Ramjaun et al., 1997) and endophilin (de Heuvel et al., 1997; Ringstad et al., 1997) preferentially bind to proline-rich domains of dynamin (McPherson, 1999). This suggested that dynamin might bind directly to β-DG. To test this, brain dynamin was immunoprecipitated with Mab Hudy-1, fractionated electrophoretically and transferred to PVDF membranes. The PVDF membranes were overlaid with a recombinant protein encompassing the entire cytoplasmic domain of β-DG fused to GST (GST-βDGcyto). We found that GST-βDGcyto but not GST alone bound to bands containing dynamin (Fig. 2-4A). Thus there appears to be a direct interaction of dynamin with the intracellular region of DG. Interestingly, β-DG immunoaffinity purification (Fig. 2-3, lanes 6, 7) revealed no detectable association of DG with amphiphysin suggesting that binding of Grb2 directly or via its SH3 domain occludes binding of amphiphysin in dynamin associated with DG.

Association and colocalization of DG and dynamin in the outer plexiform layer of the retina

DG has been localized to synaptic regions in the brain (Blake and Kroger, 2000; Moukhles et al., 2000; Zaccaria et al., 2001) as well as at GABAergic synapses in

culture by immunofluorescence microscopy (Brunig et al., 2002; Levi et al., 2002). It is challenging to localize antigens within most regions of the CNS so as to ascertain whether they are synaptically localized without resorting to EM. In the retina, however, rod and cone photoreceptors synapse on bipolar and horizontal cells and form so-called "ribbon" synapses where the presynaptic ribbon is readily detectable by light microscopy as plaques (cones) or large puncta (rods) running through the outer plexiform layer (Blake and Kroger, 2000). Both α- and β-DG have been shown to be expressed in the outer plexiform layer of retina (Dalloz et al., 2001; Koulen et al., 1998; Montanaro et al., 1995), where they are thought to form a complex with dystrophin and β-dystrobrevin (Blank et al., 2002). To extend these studies we determined whether β-DG is associated with dynamin in the retina. As in the brain, both dynamin and β-DG were co-precipitated (Fig.2-5A) with antisera to β-DG. Conversely immunoprecipitation with an antibody to dynamin (Fig. 2-5B) precipitated β-DG and Grb2 together with dynamin. The amount of β-DG precipitated was less than that in Fig. 2-5A, consistent with previous data (Fig. 2-2A) that there is a pool of dynamin unbound to β-DG. Immunohistochemically β-DG immunoreactivity (Fig.2-5C, arrowheads) was found in puncta and bands in the outer plexiform layer of retina characteristic of rod and cone photoreceptor terminals (Blank et al., 1999; Koulen et al., 1998). Dynamin immunoreactivity (Fig. 2-5C, arrowhead) was also restricted largely to the outer plexiform layer of the retina and, as expected, its distribution is more diffuse than that of β-DG. Double labeling immunofluorescence showed, however, that essentially all photoreceptor synapses labeled with antisera to β-DG were also positive for dynamin (Fig. 2-5C, merge). Grb2 was expressed diffusely in virtually all cells of

the retina, and, while it overlapped with the distribution of β-DG and dynamin, it is not obvious that this is meaningful (data not shown). Taken together, these data suggest that in the retina β-DG, dynamin and possibly Grb2 form a complex in the outer plexiform layer and this complex is presynaptically localized.

DG in endocytosis

Previously, we have studied DG function in differentiated ES cells wherein both *dag1* alleles had been disrupted by homologous recombination (Cote et al., 1999; Jacobson et al., 2001; Li et al., 2002). Here we first generated mixed cultures of differentiated cells to monitor by immunofluorescence the effect of deletion of *dag1* on dynamin-mediated endocytosis. Differentiated ES cells yield a variety of cell types, including fibroblastic cells that quickly migrate out of the embryoid body and are easily visualized as well-spread cells on the periphery of the cell mass. In addition, we have established lines of these fibroblastic cells that permit biochemical analysis. Unfortunately, we have not been able to isolate neural stem cells for similar studies nor do we have mice null for *dag1* in the CNS from which we could make primary neuronal cultures (Moore et al., 2002). Nevertheless, constitutive endocytosis of transferrin is essentially the same in neuronal and non-neuronal cells and shares many features with regulated vesicular endocytosis in nerve terminals, including a requirement for dynamin (Jarousse and Kelly, 2001).

Fibroblastic cells differentiated from wild-type ES cells express both α-DG (data not shown) and β-DG (Fig. 2-6C and G) on the surface of the plasma membrane,

preferentially in regions called membrane ruffles (Fig. 2-6A). Ruffles also contain dynamin, β-DG and cortactin (Fig. 2-6). Thus DG in these cells might be involved in endocytosis, which frequently occurs at membrane ruffles, and/or could regulate the actin cytoskeleton, a function also attributed to dynamin (McNiven et al., 2000). To assess this further, wild-type or DG-null cells were differentiated from embryoid bodies, and incubated with RITC-labeled transferrin. In mixed cultures both wild-type and DG-null fibroblastic cells take up transferrin, as demonstrated by the presence of many small, fluorescent puncta visible throughout the thickness of the cells (Fig. 2-7). Wild-type cells showed both submembranous and cytoplasmic localization of labeled transferrin, indicative of uptake into early and late endosomes (Fig. 2-7A and C). Few cells had perinuclear staining. In contrast, in DG-null cells transferrin labeling was less frequently concentrated in the region of plasma membrane and most, if not all cells had intense labeling in the cytoplasm especially around the nucleus (Fig. 2-7D). We quantified the area occupied by transferrin labeling in wild-type and DG-null cells (methods). DG-null cells show a significant, 2.25-fold increase ($P<0.05$), in transferrin uptake (Fig. 2-7E) when compared with wild-type cells. To confirm that DG is associated with dynamin we isolated and expanded fibroblastic cells that appeared essentially identical to those which migrate from embryoid bodies (Fig. 2-6,7). Transferrin uptake by these cell lines was the same as that of fibroblastic cells in mixed cultures (Fig. 2-8A and B). Furthermore, immunoprecipitation from wild type cells showed that a portion of dynamin is associated with β-DG (Fig. 2-8C, lane wt of upper panel under IP). There was no DG detectable in DG-null cells (Fig. 2-8C, Pre-IP) and no dynamin was precipitated from these cells (Fig. 2-8C, IP). Taken together, these

observations indicate that β-DG interacts with dynamin at membrane ruffles, and suggests a role for DG in regulating endocytosis of transferrin.

To confirm that the difference in endocytosis between wild-type and DG-null cells was due to DG, we re-introduced DG into the DG-null fibroblastic cells by infection with an adenovirus containing DG/GFP. In these experiments, GFP expression driven by a separate CMV promoter served as a reporter of successful infection. In DG-null cells infected with the DG/GFP virus DG is expressed in the plasma membrane and in ruffles (arrowhead, Fig. 2-9A, c') similar to the pattern in wild-type cells (Fig. 2-6). There was no DG expression seen in cells infected with the control adenovirus (GFP) (Fig. 2-9A, b'). To determine whether transferrin uptake was restored to wild-type levels in infected cells, we incubated wild-type and DG-null cells with rhodamine conjugated transferrin for 15 min 24 hrs after adenovirus infection (Fig. 2-9B). In uninfected cells transferrin uptake was significantly greater in DG null cells than in wild-type cells, as noted previously (Figs. 2-7 and 8). In DG null cells infected with DG/GFP there was a decrease in transferrin uptake when compared with DG null cells infected with GFP alone. The decrease was equivalent to that seen in wild-type cells uninfected or infected with GFP. DG-null cells infected with virus containing DG/GFP (DG-null:DG/GFP) had about a 2-fold reduction in transferrin uptake compared to DG-null cells or null cells infected with GFP ($P<0.05$), but no difference compared to wild-type cells or wild-type cells infected with GFP ($P>0.05$). Wild-type cells infected with GFP alone have the same transferrin uptake as non-infected wild-type cells ($P>0.05$).

DISCUSSION

Our results reveal that a complex of β-DG, α-DG, dynamin 1, and Grb2, is found in presynaptic terminals of CNS neurons. This complex is also present in non-neuronal cells where it appears to regulate endocytosis. Several lines of evidence support this claim. First, immunopurification of β-DG-associated proteins followed by SDS-PAGE yielded a 97 kDa band, whose sequence by LC/MS-MS resulted in 21 peptides that covered 25% of the sequence of rat dynamin 1. Sixteen of these peptides were unique for dynamin 1 and five were shared with dynamin 2 but not dynamin 3, both of which are also expressed in the CNS (Urrutia et al., 1997). This suggests that dynamin 1 is the major dynamin isoform associated with DG. Second, the association of dynamin was confirmed by immunoprecipitation and extended to show interaction of β-DG with α-DG and Grb2. Other immunoprecipitation data indicate that only a subset of dynamin is associated with β-DG in brain and retina. In an overlay assay, dynamin purified from brain bound directly to the intracellular portion of recombinant β-DG. Third, β-DG is associated and immunohistochemically colocalized with dynamin at ribbon synapses within the outer plexiform layer of the retina. Fourth, in fibroblast-like cells in culture DG is associated with dynamin at cortactin-rich membrane ruffles that are sites of endocytosis (Cao et al., 2003; Lynch et al., 2003). Finally, ES cells null for DG had a greater uptake of transferrin, a dynamin mediated process, than that of wild type cells. Expression of full length DG in DG-null ES cells restored transferrin endocytosis to levels seen in wild-type cells.

Dystroglycan is found in nerve terminals in the CNS

DG is concentrated at some central (Levi et al., 2002; Moukhles and Carbonetto, 2001) and peripheral synapses (Gee et al., 1994; Gingras and Ferns, 2001; Leschziner et al., 2000). At neuromuscular junctions DG binds to perlecan (Peng et al., 1998; Jacobson et al., 2001) and contributes to the assembly of acetylcholinesterase (Jacobson et al., 2001) in the synaptic basement membrane. In addition, DG participates in the aggregation of acetylcholine receptors (Grady et al., 2000; Jacobson et al., 2001) into stable plaques within the myotube membrane. In the hippocampus DG is involved in LTP (Moore et al., 2002) where it has also been reported to function postsynaptically. In our studies immunoaffinity chromatography identified dynamin 1, a neuron-specific form of dynamin (Grady et al., 2000), as the dominant dynamin isoform associated with DG. Dynamin 2 is also expressed in the CNS and is recognized by mAb Hudy-1 (Damke et al., 1994). As a result immunoprecipitations with this mAb from brain homogenates may contain DG that is associated with dynamin 2. However, in synaptosomes DG is likely bound to dynamin 1, the dominant isoform in neurons. Kroger and colleagues (Blank et al., 1999; Koulen et al., 1998) have localized DG in the vicinity of synaptic ribbons in the nerve terminals of photoreceptors in the outer plexiform layer of the retina (Blank et al., 1999; Koulen et al., 1998) and we have found that DG and dynamin colocalize in ribbon synapses in the outer plexiform layer of the retina (Fig. 2-5) that are readily detectable by light microscopy (Koulen et al., 1998; Montanaro et al., 1995). We conclude that DG is localized presynaptically where it is associated with dynamin 1.

Interactions of DG-dynamin in the CNS

Blank et al (2002) have suggested that β-dystrobrevin and dystrophin are bound to β-DG based on their localization in the retina. Our biochemical and other data indicate that Grb2 and dynamin also interact with the cytoplasmic tail of β-DG. Thus in the CNS α and β-DG appear to be complexed with conventional components found in muscle, such as dystrobrevin, as well as a novel one, dynamin. Grb2 is an SH2-SH3 domain protein that binds to β-DG via either of its SH3 domains (Oak et al., 2001; Yang et al., 1995) but with different affinities (Russo et al., 2000). Grb2 also binds to dynamin 1 in brain, again via SH3 domain binding to proline-rich motifs (Gout et al., 1993; McPherson et al., 1994). The N-terminal SH3 domain of Grb2 is essential for binding to dynamin (Vidal et al., 1999). So it is reasonable to think that Grb2 may bind to dynamin with its N-terminal SH3 domain and to DG with C-terminal SH3 domain, to form DG-Grb2-dynamin complex. Other studies indicated that amphiphysin and endophillin rather than Grb2 are the major SH3-domain containing protein that interacts with dynamin at nerve terminals (David et al., 1996; Micheva et al., 1997). We have not, however, detected amphiphysin in immunoprecipitates of DG that contain dynamin (Fig. 2-3A). Moreover, our overlay data (Fig. 2-4) indicated that DG can interact directly with dynamin and may not require the intercession of Grb2 in-situ. Taken together it appears that DG, Grb2 and dynamin form a complex distinct from dynamin and amphiphysin.

In addition to its intracellular interactions, β-DG also binds to α-DG conferring on the complex the ability to interact with its ligands in the extracellular matrix (Montanaro and Carbonetto, 2003) as well as on other cells (Sugita et al., 2001). For many of its ligands of α-DG binds via its carbohydrate side chain(s) (Ervasti and

Campbell, 1993b) to laminin-like globular motifs (Gee et al., 1993; Michele and Campbell, 2003). Recent data show that aberrant glycosylation of DG in several muscular dystrophies and in the *myd* mouse leads to greatly reduced ligand binding and electroretinograms with altered b-waves indicative of defective synaptic transmission in the outer plexiform layer (Grewal et al., 2001; Holzfeind et al., 2002). Agrin, which contains several G domains and binds to DG (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1994; Sugiyama et al., 1994), is found within the synaptic cleft in the outer plexiform layer of the retina (Koulen et al., 1999). Moreover, the ability of α-DG to bind ligand appears necessary for normal synaptic function (Grewal et al., 2001; Michele et al., 2002). Thus linkage of dynamin to a transmembrane receptor complex and possibly to the matrix or cell adhesion molecules (Gee et al., 1993; Gee et al., 1994; Peng et al., 1998; Smalheiser and Kim, 1995; Sugita et al., 2001) may be important in vesicle recycling during synaptic transmission where spatially regulated subdomains of endocytosis and exocytosis are thought to be critical for evoked release of neurotransmitter (Estes et al., 1996).

Dystroglycan in endocytosis

To assess the biochemical interactions of DG in cells we have resorted to cultured cells that are null for DG. Unfortunately, we do not have lines of neuronal precursors null for DG, but we do have fibroblastic and epitheliod cells. As a result, we have assayed in fibroblasts dynamin-mediated uptake of transferrin, a constitutive mode of endocytosis which employs many of the same molecules, including dynamin and clathrin, as the tightly regulated release during synaptic transmission (Jarousse and Kelly, 2001). We

have found that transferrin uptake is significantly greater in cell lines null for DG when compared with wild-type cells (Fig. 2-7-9). This holds for cells in mixed cultures differentiated from embryoid bodies as well as fibroblastic cell lines established from differentiated ES cells. In the latter instance, dynamin, most likely dynamin 2, is associated with DG (Fig. 2-8). This complex appears to be concentrated at membrane ruffles which have a high density of transferrin receptors (Bretscher and Aguado-Velasco, 1998) and are also prominent sites of endocytosis (Cao et al., 2003; Lynch et al., 2003). Furthermore, Grb 2, that is associated with DG, has been implicated in endocytosis, most notably of the EGF receptor (Huang et al., 2004; Vieira et al., 1996; Wang and Moran, 1996; Yamazaki et al., 2002), but also in the internalization of β-adrenergic receptors in response to insulin (Karoor et al., 1998).

In principle the inhibition of endocytosis by DG could be achieved by a number of mechanisms. Most obviously, DG may decrease the GTPase activity of dynamin, which is essential for its function in endocytosis. This seems unlikely to occur via Grb2 bound to DG since SH3-domain containing proteins are reported to increase the enzymatic activity of dynamin and endocytosis (Shupliakov et al., 1997; Simpson et al., 1999; Wigge et al., 1997). It is possible, however, that direct binding of DG to dynamin is inhibitory or that indirect binding via its SH3 domain displaces amphiphysin from a subset of dynamin (Fig. 2-3A) associated with DG at the cell surface. The SH3 domain protein amphiphysin 1 appears to function in the assembly of dynamin 1 into the ring-like structures around coated pits (McPherson, 1999; Schmid et al., 1998; Takei et al., 1999). Conceivably Grb2 localized to the cell surface through DG could compete with amphiphysin or other SH3 domain proteins to inhibit their function in endocytosis and

regulate the activity of membrane-associated dynamin (Simpson et al., 1999). Second, anchorage of a pool of dynamin at the membrane via DG may restrict it from movement into active sites of vesicles turnover. This may be the case in photoreceptors where DG is distant from the synaptic ribbon (Koulen et al., 1998). Third, in addition to its mechanochemical role in pinching off vesicles during endocytosis, dynamin can reorganize the cytoskeleton (McNiven et al., 2000; Orth and McNiven, 2003) by interacting with several actin-binding proteins including cortactin (McNiven et al., 2000) at membrane ruffles (Orth and McNiven, 2003). DG itself may then contribute to actin organization. In fact β-DG has been reported to bind directly to actin (Chen et al., 2003) and to reorganize the actin cytoskeleton (Chen et al., 2003; Spence et al., 2004). This may be related to observations that the cortical actin cytoskeleton inhibits endocytosis (Qualmann et al., 2000). Thus, it is conceivable that the differences seen in endocytosis of wild-type and DG null cells are an indirect manifestation of altered cytoskeletal organization. We have not, however, detected any obvious abnormalities in cell attachment, actin or tubulin distribution in these cell types. Future experiments will be aimed at determining the mechanism of inhibition of endocytosis and whether DG directly inhibits dynamin function. At this point, however, our findings indicate that DG is associated with the GTPase dynamin, and provide evidence that this complex regulates endocytosis. In view of recent work implicating DG in synapse formation/function and in neural development, these observations may contribute to our understanding of the mental retardation associated with Duchenne and several other muscular dystrophies (Montanaro and Carbonetto, 2003).

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FOOTNOTES

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The abbreviations used are: Dystroglycan, DG; ES cell, embryonic stem cell; Monoclonal antibody, mAb; CNS, central nervous system; EM, electronic microscopy; SH2/SH3, src homology 2/src homology 3. NRIgG, non-immune rabbit IgG; NMIgG: non-immune mouse IgG; pAb, polyclonal antibody; Grb2, Growth Factor Receptor-bound Protein 2; Myd, myodystrophy.

Figure 2-1

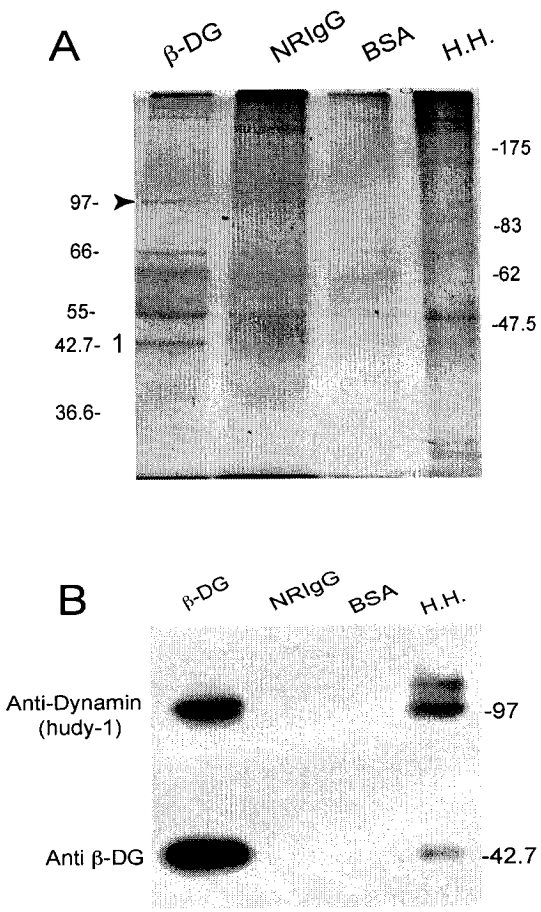

A
β-DG
NRIgG
BSA
H.H.
97-
66-
55-
42.7-
36.6-
1
-175
-83
-62
-47.5
B
β-DG
NRIgG
BSA
H.H.
Anti-Dynamin
(hudy-1)
Anti β-DG
-97
-42.7

Figure 2-1: Immunoaffinity purification of dystroglycan-associated proteins from brain. A: Equivalent volumes of eluate (Methods) from anti β-DG antibody affinity column (β-DG), non-immune rabbit IgG column (NRIgG), BSA column (BSA), and hippocampal homogenates (H.H., 2μg.) were loaded, and subjected to SDS-PAGE and stained with Coomassie blue. Two sets of molecular weight markers (36.6-175 kDa) are shown. The arrowhead points to an ~100 kDa band seen only in eluates from the β-DG antibody affinity column. Band 1 is β-DG (43 kDa). The band at 55 kDa is the IgG heavy chain and the band at ~70 kDa is Hsc70 (data not shown). The band at ~100 kDa was identified as dynamin 1 (97 kDa) by LC/MS-MS. B: Column eluates (5%) from 5 mg of hippocampal homogenates were collected, electrophoresed and western blotted with antibodies to dynamin and β-DG to confirm the data in A. Equivalent amounts of eluate from the NRIgG and BSA columns served as controls. The antibodies used as probes are noted on the left and molecular weight markers (kDa) are on the right. Image A or B is from a single gel.

Figure 2-2

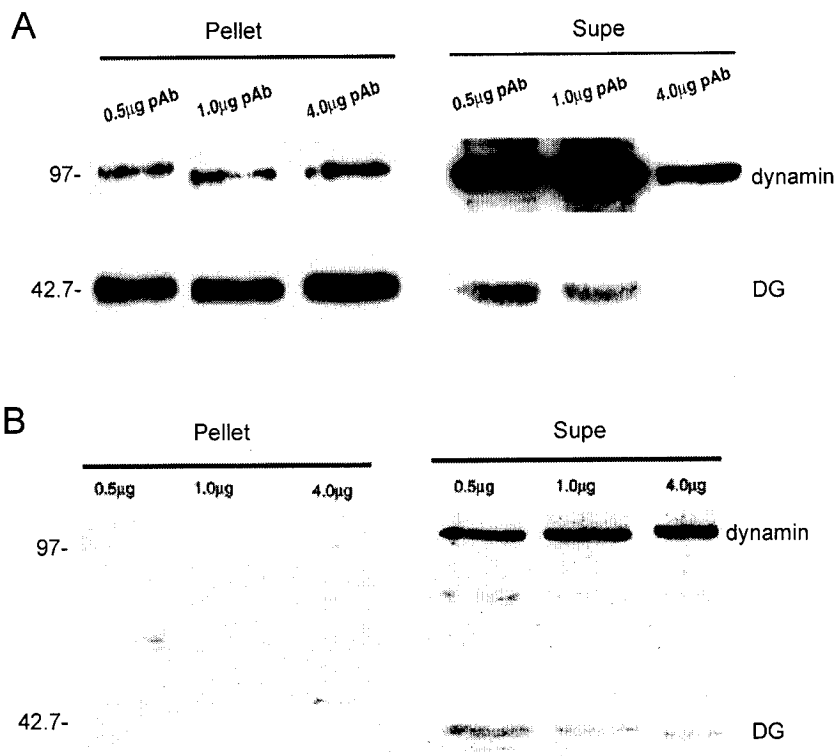
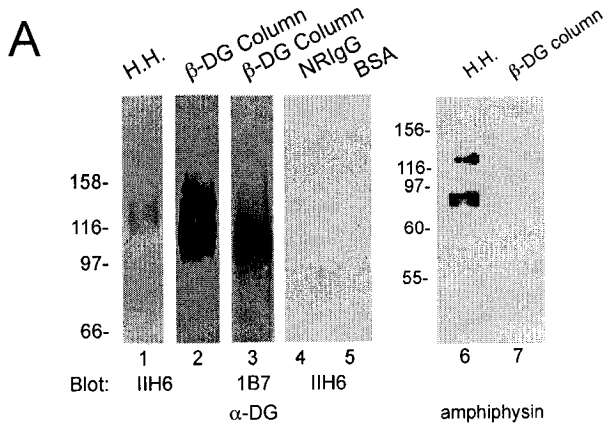

A
Pellet
Supe
0.5µg pAb
1.0µg pAb
4.0µg pAb
0.5µg pAb
1.0µg pAb
4.0µg pAb
97-
dynamin
42.7-
DG
B
Pellet
Supe
0.5µg
1.0µg
4.0µg
0.5µg
1.0µg
4.0µg
97-
dynamin
42.7-
DG

Figure 2-2: Immunodepletion of β-DG from rat brain extracts. 60 μg of rat brain homogenate was immunoprecipitated with 0.5 μg, 1.0 μg or 4.0 μg of anti β-DG antiserum (pAb) coupled to agarose beads (A) or with non-immune rabbit IgG coupled to agarose beads (B). The beads were washed extensively (Methods) with solubilization buffer containing 5 mM EDTA and 0.5M NaCl, and the precipitated proteins (Pellet) as well as the supernatants (Supe) were analyzed by western blotting with antibodies to dynamin and β-DG. With increasing amounts of β-DG antiserum (A) there is an increase in the amount of both β-DG and dynamin in the pellet and corresponding reduction in the Supe. At 4.0 μg of antiserum β-DG is entirely depleted from the Supe but a prominent band of dynamin remains. (B) Immunoprecipitations with agarose beads coupled to non-immune rabbit IgG produced no precipitation of β-DG or dynamin (Pellet) which remained in the Supe. The lanes in B contain 30% of the supernatants. Lanes in A or B are grouped from a single western blot that was scanned and computer reconstructed under the same conditions.

Figure 2-3


A
H.H.
β-DG Column
β-DG Column
NRIgG
BSA
H.H.
β-DG column
158-
116-
97-
66-
156-
116-
97-
60-
55-
1 2 3 4 5
6 7
Blot: IIH6 1B7 IIH6
α-DG
amphiphysin

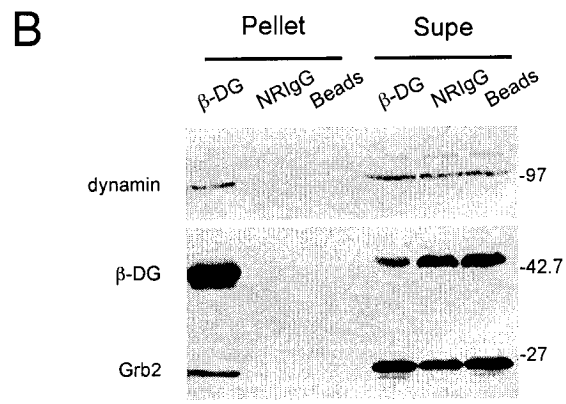

B
Pellet
Supe
β-DG
NRIgG
Beads
β-DG
NRIgG
Beads
dynamin
β-DG
Grb2
-97
-42.7
-27

Figure 2-3: α-DG, Grb2 and β-DG co-purification from brain. A: Five mgs of hippocampal homogenate were subjected to immunoaffinity purification and 10% of the eluate volume was electrophoresed and blotted with α-DG antibodies IIH6 (lane 2) and 1B7 (lane 3). An equivalent volume of eluate from columns with normal rabbit IgG (lane 4) or BSA (lane 5) served as controls. 35 μg of hippocampal homogenate (H.H.) (lane 1) was loaded as positive control. Note that α-DG is recognized by both mAbs IIH6 and 1B7, moreover, the forms recognized by mAb 1B7 are smaller reflecting hypoglycosylation (Leschziner et al., 2000). Antiserum (1874) detected amphiphysin I and II (Ramjaun et al., 1997) in the homogenates (lane 6), but no amphiphysin in the β-DG column eluates (lane 7). B: β-DG, Grb2 and dynamin are associated in synaptosomes. Solubilized synaptosomes (Method) were immunoprecipitated with an antibody to β-DG, and the precipitated proteins (Pellet) and the supernatant (Supe) were subjected to SDS-PAGE and probed with antibodies to β-DG, Grb2 and dynamin. Both Grb2 and dynamin are co-immunoprecipitated with β-DG (lane β-DG, Pellet). The non-immune rabbit IgG (NRIgG) and agarose beads alone were used as controls. Antibodies used for western blots are indicated (left) and the position of molecular markers are shown (right). Lanes 1-5, lanes 6-7 and lanes in B are grouped from a single western blot that was scanned and computer reconstructed under the same conditions.

Figure 2-4

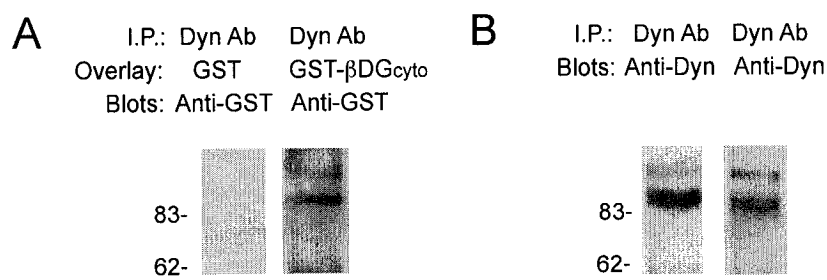

A
I.P.: Dyn Ab Dyn Ab
Overlay: GST GST-βDGcyto
Blots: Anti-GST Anti-GST
83-
62-
B
I.P.: Dyn Ab Dyn Ab
Blots: Anti-Dyn Anti-Dyn
83-
62-

Figure 2-4: The intracellular portion of β-DG interacts with dynamin. A: Dynamin was immunoprecipitated (I.P.) from 300 μg of brain synaptosomes with 6 μg of dynamin antibodies, subjected to SDS-PAGE in 4-20% acrylamide gradient gel (*Bio-Rad*) and transferred to a PVDF membrane. The membranes were incubated with 20 μg/ml of recombinant GST-β-DG_{cyto} (right lane) or GST alone (left lane) overnight, washed and blotted with a HRP-conjugated anti-GST antibody. GST-β-DG binds to two bands that react with antibodies to dynamin (right lane, B). The two bands either represent a dynamin doublet (Cook et al., 1994; Robinson et al., 1993) or possibly a proteolytic product of dynamin. Neither of these bands reacted with antibodies to GST (left lane, A). B: Membranes were subsequently stripped and re-probed with anti-dynamin antibodies (Hudy-1). Lanes in A or B are from one single gel and membrane.

Figure 2-5

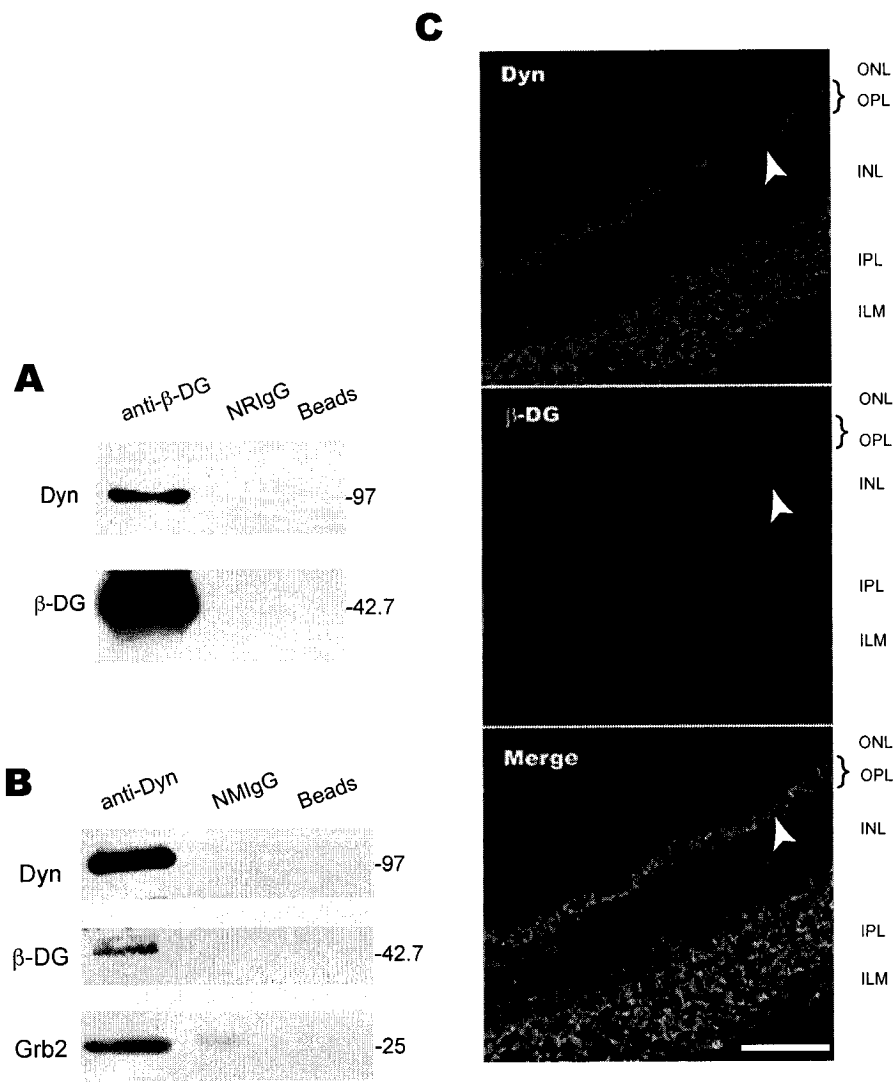

A
anti-β-DG
NRIgG
Beads
Dyn
-97
β-DG
-42.7
B
anti-Dyn
NMIgG
Beads
Dyn
-97
β-DG
-42.7
Grb2
-25
C
Dyn
β-DG
Merge
ONL
OPL
INL
IPL
ILM

Figure 2-5: β-DG, dynamin 1 and Grb2 are associated in the retina. β-DG was immunoprecipitated from rat retinal homogenates with antisera to β-DG (anti-β-DG) (A), or antibody to dynamin (Hudy-1, anti-Dyn) (B). In A the controls included normal rabbit IgG (NRIgG), and agarose beads alone (Beads), and in B, normal mouse IgG (NMIgG) and agarose beads alone (Beads). The precipitated proteins were subjected to SDS-PAGE and probed with antibodies (panel A and B, left) to dynamin (Dyn), β-DG (β-DG) or Grb2. The positions of molecular weight markers are shown on the right. C: Immunohistochemistry in the mouse retina shows that dynamin 1 (Top) is localized in the outer plexiform layer (OPL) and inner plexiform layer (IPL). β-DG (C, middle) is localized in outer plexiform layer (OPL), inner limiting membrane (ILM) and blood vessels. In the outer plexiform layer (OPL) puncta of β-DG and dynamin 1 are often colocalized as shown by the merged image (C, yellow). Arrowheads point to one example of puncta or ribbon synapse. ONL: Outer nuclear layer. OPL: Outer plexiform layer. INL: Inner nuclear layer. IPL: Inner plexiform layer. ILM: Inner limiting membrane. Lanes in A or B were grouped from single gel and under same exposure. Bar, 50 microns.

Figure 2-6

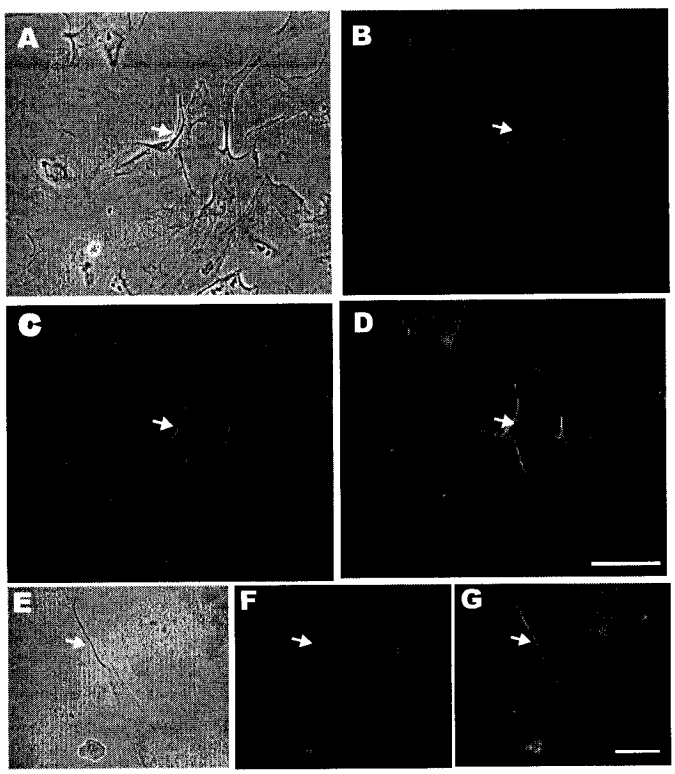


Figure 2-6: DG, dynamin and cortactin are co-localized at membrane ruffles of fibroblastic cells. A and E: Phase images of fibroblastic cells derived from wild type ES cells. The arrow shows a typical membrane ruffle. B, C, F and G: Immunolocalization of dynamin (B, Hudy-1), β-DG (C, G) and cortactin (F) show that all three proteins are concentrated in ruffles. D: The merged image of B and C shows DG and dynamin in ruffles. Bars, 50 microns.

Figure 2-7

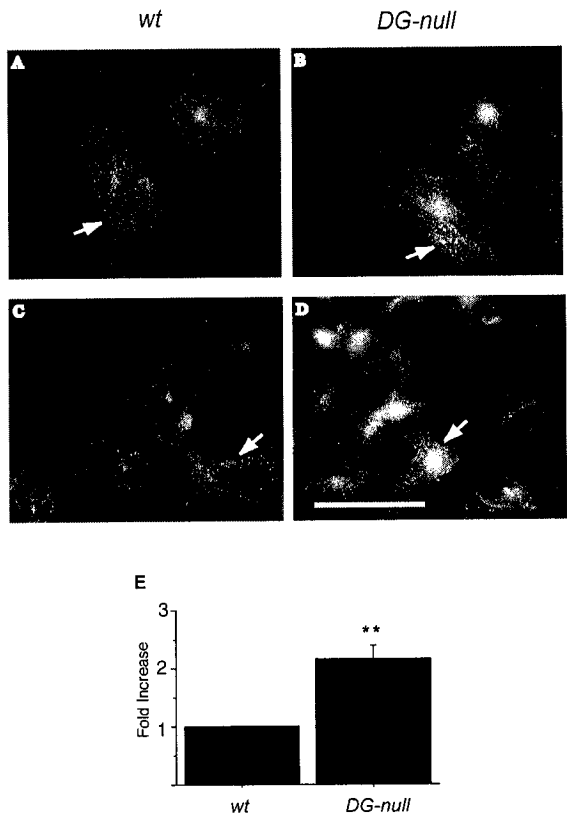


Figure 2-7: DG regulates endocytosis in differentiated ES cells in culture. Uptake of rhodamine-labeled transferrin was studied in fibroblastic cells on the periphery of adherent embryoid bodies derived from wild-type (wt) (A and C) and DG-null (B and D) ES cells. Arrowheads point to typical labeled cells. Fluorescent puncta were observed in the cytoplasm and around the nuclei of DG-null cells (B, D), but this was substantially sparser and less intense than in the wild type cells (A, C). E shows quantification of the area occupied by transferrin labeling (Methods) in (A, C and B, D). Error bars show the S.E.M. Bar, 50 microns.

Figure 2-8

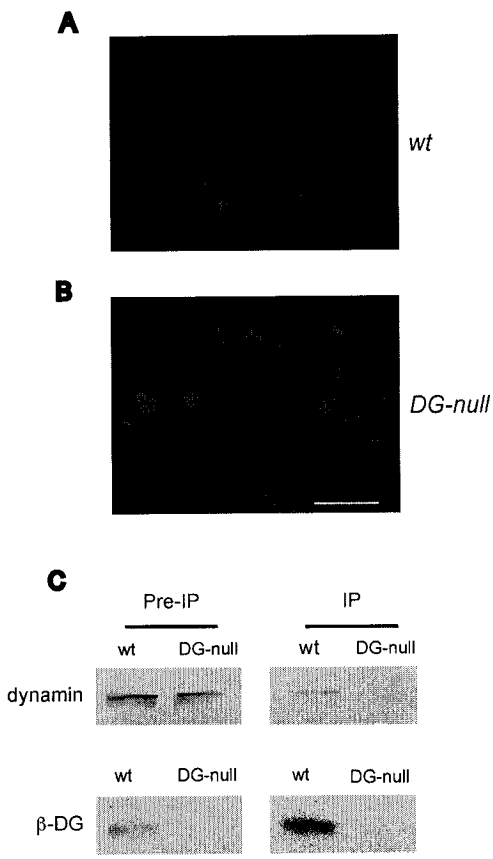

A
wt
B
DG-null
C
Pre-IP
IP
wt
DG-null
wt
DG-null
dynamin
wt
DG-null
wt
DG-null
β-DG

Figure 2-8: β-DG is associated with dynamin in fibroblastic cells isolated from differentiated ES cells. A and B show a prominent increase in transferrin uptake (as in Fig.2-7) in DG-null fibroblasts (DG-null, B) compared with wild-type (wt) fibroblastic cells (wt, A) when cells are incubated with transferrin at 37° for 15 min before fixation. C: Immunoprecipitation (IP) of βDG-dynamin complex from fibroblastic cells. Fibroblastic cell lysates from both wt and DG-null were electrophoresed, and blotted with antibody to dynamin (upper panel, Pre-IP) and β-DG (lower panel, Pre-IP). Antiserum to β-DG was used for immunoprecipitation from both fibroblastic cell lysates. The immunoprecipitated proteins were subjected to SDS-PAGE and probed with antibodies to dynamin (upper panel, IP) and β-DG (lower panel, IP). Note dynamin was co-immunoprecipitated from wt cell lysates (lane wt, upper panel, IP) but not DG-null cell lysates (lane DG-null, upper panel, IP). All images in C were grouped from a single gel and composed into a figure under uniform conditions of exposure. Bar, 50 microns.

Figure 2-9

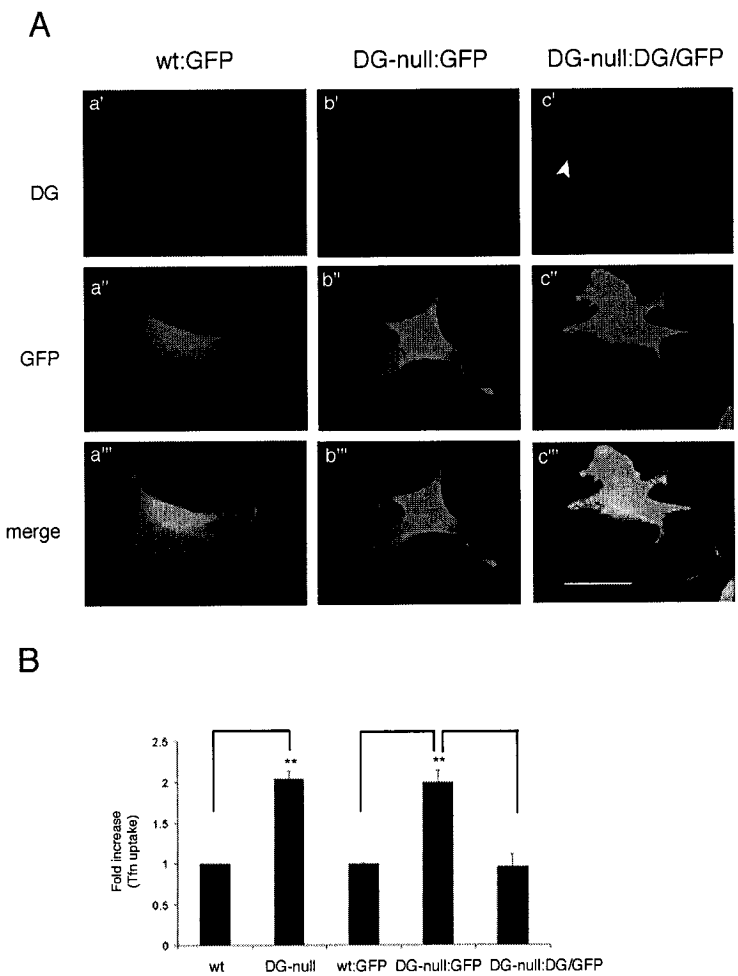

A
wt:GFP
DG-null:GFP
DG-null:DG/GFP
DG
GFP
merge
a'
b'
c'
a''
b''
c''
a'''
b'''
c'''
B
Fold increase (Tfn uptake)
2.5
2
1.5
1
0.5
0
**
**
wt
DG-null
wt:GFP
DG-null:GFP
DG-null:DG/GFP

Figure 2-9: Expression of DG in DG-null cells restores normal levels of transferrin uptake. A: Fibroblasts derived from both wild-type (wt) (a'-a''') and DG-null (b'-b''' and c'-c''') ES cells were infected with an adenovirus containing GFP (a'-a''', b'-b''') or DG/GFP (c'-c'''), and stained with antiserum to DG followed by RITC-labeled secondary antibody(a', b', c'). DG was observed at the cell surface and ruffles in DG-null cells infected with DG/GFP (arrowhead, c'), similar to labeling in wt cells (Fig. 2-6) or wt cells infected with GFP (a'). B: Rhodamine-conjugated transferrin (Tfn) was incubated with fibroblastic cells for 15 min 24hr after infection with an adenovirus containing GFP or DG/GFP. DG-null cells infected with virus containing DG/GFP (DG-null:DG/GFP) had decreased Tfn uptake compared with non-infected (DG-null) cells or cells infected with GFP alone (DG-null:GFP). The level of uptake was essentially the same as in wt cells infected with GFP (wt:GFP) or wt cells without viral infection (wt). Fluorescence was quantified (Methods) in cells 25-35 um in diameter to control for variation in cell size. Transferrin fluorescence is often concentrated in a portion of the cell so the full size of the cell was determined from GFP fluorescence in infected cells (A) or by phase imaging of uninfected cells. More than 20 cells were quantified for each treatment and fluorescence intensity was normalized to that in wild-type cells. Bar in A is 50 um.

Chapter 3

Dystroglycan expression in photoreceptors is essential for *Drosophila* eye development

By

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Abstract

Mutations in genes affecting dystrophin glycoprotein complex (DGC) result in Duchenne/Becker and other forms of muscular dystrophy. Dystroglycan (DG) is the core member of the DGC linking the cell cytoskeleton to the extracellular matrix. Targeted deletion of DG gene leads to muscular dystrophy and impaired brain development, which resembles several human congenital muscular dystrophies that are defective in glycosylation of α-DG. DMD/BMD and congenital muscular dystrophy not only affect muscle and brain but it also causes retinal defects. Here we use the *Drosophila* eye as a model to demonstrate that DG is required cell-autonomously for photoreceptor differentiation. Homozygous eyes containing severe hypomorphic DG alleles show severe disruption of retinal structure, aberrant lens formation and abolishment of electroretinogram in the adult fly eye. These adult defects appear derived from autonomous photoreceptor cell (PRC) defects in the early pupa that include size arrest, loss of polarity and progressive degeneration. In the 3rd instar larvae DG is present in the apical tips and the basal membranes of photoreceptor cells, two polarized locations opposing the extracellular matrix. At the pupal stage it continues to mainly distribute at the apical rhabdomere and basal membrane of PRCs. Expression of wild-type DG in DG-deficeint PRCs that would ortherwise be disrupted reverses the defects in the entire eye including the lens, suggesting DG function cell-autonomously for PRCs and non-autonomously for the lens. Over-expression of DG leads to larger ommatidia but the PRC number remains unchanged, suggesting that DG is both necessary and sufficient for PRC expansion. In rescue experiments, we found that extracellular DG alone can not rescue DG-deficient eye defects, whereas the

intracellular DG can substantially ameliorate PRC degeneration and structural defects while some PRCs remain disoriented, a sign of disrupted PRC planar polarity. Therefore, our data suggest that the degeneration and planar polarity disruption in DG-deficient PRCs are two independent processes that appear to attribute to respective function of the intracellular and the extracellular DG. Our data provide a great model to study DG-related congenital muscular dystrophies and DG biology.

INTRODUCTION

Muscular dystrophies are a group of diseases often caused by loss-of-function mutations affecting the dystrophin glycoprotein complex (DGC). The DGC-related muscular dystrophies involve not only muscle degeneration, but some of them also display mental retardation, brain defects and retina disruptions.

Dystroglycan (DG) is a core member of the DGC that spans the muscle sarcolemma and links the cytoskeleton to the extracellular matrix. DG is encoded by a single gene and post-translationally cleaved into tightly connected α and β-subunits (Holt et al., 2000). β-DG directly binds to multiple proteins including dystrophin, Grb2 and caveolin3 (Winder, 2001), whereas carbohydrate chains in mucin-like region of α-DG confer the ability to bind to the extracellular ligands laminin, agrin and perlecan in the matrix (Bowe et al., 1994; Campanelli et al., 1994; Gee et al., 1993; Gee et al., 1994; Ido et al., 2004; Jacobson et al., 2001; Peng et al., 1998; Sugiyama et al., 1994; Talts et al., 2000). Mutations in the gene encoding dystrophin cause Duchenne and Becker muscular dystrophies (Hoffman and Kunkel, 1989; Koenig et al., 1988; Monaco et al., 1986) that are often associated with defects in synaptic transmission in the retina, (Pillers et al., 1990; Pillers et al., 1993; Cibis et al., 1993; Pillers et al., 1995) and mental retardation (Campbell, 1995). Targeted deletion of the DG gene in mice results in embryonic lethality at day 6.5 or 7 (Williamson et al., 1997), and chimaeric mice deficient in DG and mice with specific deletion of DG in skeletal muscle develop a progressive muscular dystrophy and loss of dystrophin and sarcoglycans from sarcolemma (Cohn et al., 2002; Cote et al., 1999). Interestingly, expression of DG constructs in DG-null skeletal muscle that lack the mucin-like region is not sufficient to

prevent the muscle dystrophies, although the intracellular DG targets dystrophin, sarcoglycans and sarcospan to the muscle sarcolemma to form the complex (Barresi et al., 2004; Kanagawa et al., 2004). DG is also required to stabilize acetylcholine receptors in the postsynaptic muscle membrane and loss of DG causes the disruption of synaptic but not extrasynaptic basement membrane (Cohn et al., 2002; Grady et al., 2000; Cote et al., 1999; Jacobson et al., 2001; Li et al., 2002).

Recent data show mutations in several glycosyltransferases that cause a group of congenital muscular dystrophies named as Fukuyama congenital muscular dystrophy (FCMD), Muscle eye brain disease (MEB) and Walker-Warburg syndrome (WWS), all have defective glycosylations of α-DG, and, loss of its ability to bind to ligands (Hayashi et al., 2001; Michele et al., 2002; Moore et al., 2002; Muntoni et al., 2002). Similar defective glycosylation of α-DG are also found in dystrophic mice (*myd* mice) and human congenital muscular type 1D that carry a mutant *LARGE* gene normally encoding a protein necessary for glycosylation of α-DG DG (Barresi et al., 2004; Longman et al., 2003; Grewal et al., 2001; Kanagawa et al., 2004). Patients with congenital muscular dystrophies and the *myd* mice all have severely disrupted brain morphogenesis resembling the phenotype in the brain-specific DG-null mice, including fusion of cerebral hemisphere, disrupted layering of cortex and aberrant neuronal migration (Grewal et al., 2001; Holzfeind et al., 2002; Michele et al., 2002; Moore et al., 2002). As well, patients with FCMD, MEB and WWS display retinal defects including corneal opacities, retinal detachment, mis-folding and fusion and degeneration that can result in blindness at a very young age (Fahnehjelm et al., 2001; Hino et al., 2001; Takeda et al., 2003; Vervoort et al., 2004; Zervos et al., 2002).

However, the *myd* mice only display disrupted b-waves in electroretinogram (ERG) but no sign of abnormal neuronal migration, detachment or misfolding in the retina (Grewal et al., 2001; Holzfeind et al., 2002). Thus, despite the similar defects in brain, eyes have a wide spectrum of defects among these different types of muscular dystrophies, raising the questions about the biological roles of DG in the eye and the specificity of glycosylation of α-DG in pathogenesis of congenital muscular dystrophy.

Taking advantage of the powerful *Drosophila* genetics, we have used the *Drosophila* eye as a model to study the roles of DG. Because of the convenient FLP/FRT system to generate mutant clones, the UAS-Gal4 system to drive expression of gene of interest and the molecular and genetic markers available in the eye development, we can more precisely analyze the function of DG. In addition to advantage in genetics, *Drosophila* DG is conserved in the functional domains. Comparing with human DG, *Drosophila* DG is 31% identical in the intracellular domain and 51% identical in the last 15 amino acids that interact with dystrophin. The extracellular mucin-like region in the *Drosophila* DG is poorly conserved in core amino acids, but it contains many serine and theronine residues, among them fifteen consensus sites exist for putative O-glycosylation similar to the mucin-like region in vertebrate DG (Fig.1-2). Recent data have demonstrated that DG is required for a polarity formation in both follicle epithelial cells and oocytes (Deng et al., 2003), but the role of DG in the eye development remains unknown.

The *Drosophila* compound eye contains ~800 regularly repeated units called ommatidia. Each ommatidium is comprised of eight photoreceptor cells (R1-R8), four non-neuronal cone cells, three classes of surrounding pigment cells and a bristle

complex (Longley and Ready, 1995; Ready et al., 1976; Tomlinson and Ready, 1987). Photoreceptor cells differentiate from the monolayer of epithelial cells in the 3rd instar imaginal disc (Baker and Rubin, 1989; Freeman, 1996; Reinke and Zipursky, 1988), followed by cone cell and pigment cell differentiation (Cagan and Ready, 1989; Tomlinson and Ready, 1987). While cell specification is complete in the 3rd instar larvae, the patterning and differentiation of the ommatidia continue until mid-pupal life (Cagan and Ready, 1989; Longley and Ready, 1995; Tomlinson, 1988). This later differentiation involves PRC rotation, apical rhabdomere formation, PRC elongation, differentiation and assembly of cone and pigment cells in the ommatidia. Many genes have been shown to be essential for the pupal development, such as the polarity determinant crumbs, stardust and bazooka (Hong et al., 2003; Izaddoost et al., 2002; Pellikka et al., 2002), the actin cytoskeleton organizer moesin and Rac1 (Chang and Ready, 2000; Karagiosis and Ready, 2004), rhodopsin (Kumar and Ready, 1995) and neuronal membrane specific glycoprotein chaoptin as well (Van Vactor et al., 1988). Here, we show that DG is essential for cell-autonomous PRC differentiation in pupal development.

Materials and Methods

Fly strains and induction of DG-deficient clones

The generation of DG^{248} allele, a DG-hypomorphic allele, was described previously (Deng et al., 2003). Homozygous DG^{248} clones in the eyes were generated by mitotic recombination using ey-FLP/FRT system (Newsome et al., 2000), which efficiently generates large clones (>85% total eye tissue). The genotypes of flies that have DG-deficient clones in the eyes were *y w ey-FLP; FRT42D, LW^{+}/FRT42D, DG^{248}*. w^{1118}, *ey-FLP; FRT42D, GMR-myr-GFP* flies (Bloomington stock center) were crossed with *FRT42D, DG^{248}/Bc* flies to generate small GFP-negative DG-deficient clones, whereas wild-type cells in the eye disc express GFP in the plasma membranes. The genotypes of flies which have GFP-negative DG-null clones in the eyes are w^{1118}, *ey-FLP; FRT42D, GMR-myr-GFP/FRT42D, DG^{248}*.

Electroretinograms

Flies were immobilized by gently taping them onto slides with one eye facing up. Glass microelectrodes were filled with a solution containing 130 mM NaCl and 50 mM KCl and inserted inside the eye. The ground electrodes were placed on the back of the head in contact with the solution. The ERGs were tested first on the depth of insertion in the eye and final ERGs were recorded after 5-10 min dark adaptation followed by a 2 sec bright-light purse.

Rescue analysis and generation of UAS-DGextra-mRFP transgenic flies

To carry out rescue experiments, three DG transgenic fly lines containing *UAS-DG, UAS-DG$_{cyto}$* and *UAS-DG$_{extra}$-mRFP* driven by *ELAV-GAL4* (Luo et al., 1994) were used in combination with ey-FLP/FRT system to rescue DG-deficient phenotypes in the eye. The genotypes of the flies in rescue experiments are *ey-FLP; FRT42D, LW$^+$/FRT42D, DG^{248}; ELAV-GAL4, UAS-DG/+* for rescue with the full-length DG; *ey-FLP; FRT42D, LW$^+$/FRT42D, DG^{248}; ELAV-GAL4, UAS-DGextra-mRFP/+* for rescue with the extracellular and transmembrane DG and *ey-FLP; FRT42D, LW$^+$/FRT42D,DG^{248}, UAS-DG$_{cyto}$; ELAV-GAL4/+* for rescue with the intracellular and transmembrane DG. UAS-DG and UAS-DG$_{cyto}$ fly lines were generated and characterized previously (Deng et al., 2003) and over-expression of proteins in PRCs was confirmed by staining with 1:1000 diluted anti-*Drosophila* DG antiserum (Fig. 3-7 and Fig. 3-9a' respectively). cDNA of DG$_{extra}$ encoding extracellular and transmembrane domains of *Drosophila* DG was PCR-amplified using sense 5'-GAG TAA GCT TAT GAG ATT CCA GTG GTT CTT ATC GGC-3' and antisense primer 5'-GAG TAG ATC TAG CCA GTG CAG GCA ACA GGC AAT G-3' from genomic DNAs of UAS-DG flies, and inserted in frame into HindIII and BglII sites 5' upstream of mRFP in pcDNA3 vector (provided by Keith Murai). DG$_{extra}$-mRFP was digested with HindIII, blunt-ended, followed by digestion with XbaI, and then inserted into blunt-ended BglII and Xba I sites of pUAST vector. The sequence was confirmed by The McGill University and Genome Québec Innovation Centre. The UAS-DG$_{extra}$-mRFP transgene were micro-injected into fly embryos by Genetic Services, Inc. (Cambridge, MA) and transgenic lines were generated using standard procedures. The over-expression of DG$_{extro}$-mRFP driven by Elav-gal4 was confirmed by live imaging

of mRFP without fixation and permeabilization in the nervous system and pupal eye discs.

Immunostaining and histology

Eye discs of 3rd instar larvae and pupa were dissected in PBS, fixed in 4% paraformaldehyde, permeabilized in 0.5% Triton X-100 and stained with following primary antisera: rabbit polyclonal anti *Drosophila* DG (1:250 or 1:1000), mouse and rabbit anti-GFP (1:500, Molecular Probes), anti-chaoptin (24B10, 1:100, DSHB), anti-Boss (1:2000, DSHB), anti-armadillo (1:400, DSHB), anti-elav (1:200, DSHB) and mouse anti-α-DG (IIH6, 1:1000, Upstate Biotechnology). Secondary antibodies conjugated with rhodamine or fluorescein (Jackson ImmunoResearch) were used at a dilution of 1:200. Rhabdomeres were labeled with rhodamine-conjugated phalloidin (Sigma, 1:500). DNA was stained with 4'-6-Diamidino-2-phenylindole (DAPI) (1:5000, Sigma). Fluorescent images were captured using a confocal imaging system (*LEICA DM LFSA*) or a high-resolution fluorescence imaging system (*Canberra Packard)* and analyzed by 2D deconvolution using MetaMorph imaging software (Universal Imaging, Brandywine, PA).

For plastic sections of adult or pupal fly eyes, adult eyes or pupal discs were dissected, fixed in 4% paraformaldehyde overnight, sequentially dehydrated in 50%-100% ethanol and embedded in plastic for sectioning as described (Van Vactor et al., 1991). The samples in epon were trimmed and cut for 80 sections per eye, 1μm per section in thickness, mounted on glass slides and stained by toluidine blue dye.

DNA transfection and Immunocytochemistry

To determine whether fly DG could be functionally glycosylated, DG-null 3C12 embryonic stem cells engineered previously (Cote et al., 1999; Jacobson et al., 2001) were differentiated in cultures as described (Zhan et al., 2005) and transfected with DG_{extra}-mRFP and mRFP under control of CMV promoter in pcDNA3 vector by Fugene transfection reagent (Roche) according to manufacturer's recommendations. A mouse antibody IIH6, which recognizes the functional carbohydrates of α-DG in vertebrates, was used to visualize the glycosylation of the transfected *Drosophila* DG containing the extracellular and transmembrane sequences fused to mRFP (DG_{extra}-mRFP). Procedures for immunocytochemistry were similar to the described previously (Zhan et al., 2005). Briefly transfected cells were fixed in 4% paraformaldehyde, washed and incubated with IIH6 without permeablization and then incubated with fluorescein-labeled secondary antibody. Cells were observed by a Zeiss Axioskop epifluorescent microscope and images were captured with a Retiga 1300 monochrome 10 bit digital camera (*Qimaging Corp.*) and analyzed with Northern Eclipse software (*Empix*).

Scanning electron microscopy and DG over-expression analysis

Adult flies were immersed in 2% glutaraldehyde for 2 days, and sequentially dehydrated 10 minutes in 50%, 75%, 80%, 90%, 95%, three times in 100% ethanol, and further substituted with amyl acetate by placing flies 15 minutes into amyl acetate/ethanol (25/75), (50/50), 75/25 and 100% amyl acetate sequentially. The flies then went through critical point drying and the eyes were coated with gold palladium,

and observed with scanning electron microscope (McGill Facility for Electron Microscopy Research).

To observe the DG over-expression phenotypes, Elav-gal4 flies were crossed with UAS-DG flies. Wild-type flies and flies containing Elav-gal/+ and Uas-DG/+ were used as the controls. The genotype of DG over-expression flies is Elav-gal4/UAS-DG. The plastic sections were made as described above and at least 10 eyes have been prepared for each DG over-expression and control sample. To minimize the error in measurement resulting from samples at different location in the eye, sections were chosen from similar depths within the retina as judged by the size of the section. The perimeter and area of each ommatidium were measured from ommatidia localized in the center of each section. Measurements were done with Northern Eclipse software (Empix) and tabulated with Microsoft Excel and analyzed statistically using StatView (Abacus Concepts, Inc.).

Results

Loss of DG causes severe disruption of adult eye

Severe hypomorphic allele of DG in *Drosophila melanogaster* was previously generated in the laboratory of Dr. Hannele Ruohola-Baker (Deng et al., 2003). Using Ey FLP/FRT-induced mitotic recombination (Newsome et al., 2000), we induced DG-deficient (DG^{248}) cell clones in developing eyes. The DG-null clones do not contain pigment granules and thus could be identified as the white-colored patches that often have more than 85% of the eye surface (Fig, 3-1a'). Eyes with DG-deficient cells appear glossy because the eye surface is flattened and the lens facets and bristles of each ommotidium are missing (Fig. 3-1a'-c'). Irregular shapes of the entire external eye morphology are also observed (Fig. 3-1a'-c'). Analysis of the internal retinal structures in the adult revealed that DG-deficient clones, marked by absence of pigment granules around each ommatidia because of the loss of white gene in DG-deficient clones, have no rhabdomere in the longitudinal sections (Fig. 3-1d') that would otherwise extend from the distal to the proximal in the wild type (Fig. 3-1d). The thickness of the retina is also reduced 40-50% in the mutants compared with wild type retina (Fig. 3-1d and d'). The lenticular shape of the lens is also disrupted and is flattened on the external face (Fig. 3-1d' and e'). Cross sections of adult eyes revealed that areas containing DG-null ommatidia, marked by absence of pigment granules, lack PRCs and non-neuronal accessory cells (arrow, Fig. 3-1f') and some cell debris are present in the area (red arrowhead in Fig. 3-1f'). Some DG-deficient ommatidia (white arrowhead, Fig. 3-1f') have small, disorganized PRCs and appear degenerating. Within a separate ommatidium (red arrow, Fig. 3-1f'), one wild-type PRC with pigment granules appears

normally present (red arrow pointed) but other PRCs that do not contain pigment granules are missing, indicating a cell-autonomous role. To analyze the function of DG-null cells, electroretinograms (ERGs) were recorded from the DG-null areas upon light stimulation after dark adaptation. The DG-null clones demonstrated a nearly abolished receptor potential and a complete disappearance of on/off transients (Fig. 3-8a and 3-8b). The receptor potential is the depolarization of PRCs in response to light and on- and off-transients are synaptic components of ERG. The ERG results confirm that there are almost no phototransduction in the DG-null retina. Small residue response is likely due to current spread from neighboring normal tissues. ERGs from non-DG-deficient eye patches remain normal (data not shown). We conclude that DG is essential for the adult development and deficiency of DG leads to loss of normal ommatidial structural, loss of PRCs and diminished ERG in the adult eye.

DG is required for PRC growth and organization

Analysis of homologous recombination-induced DG-deficiency in vertebrate adults has revealed that DG is required for muscle survival and has complex roles in the central nervous system. To determine the roles of DG in *Drosophila* eye development, we analyzed the DG-deficient cells at various developmental stages. As mentioned, DG-deficient clones are efficiently induced by the EyFlp/FRT system and are usually accounted for >85% (Fig. 3-10h) of the eye disc area (Cafferty et al., 2004; Newsome et al., 2000). In the 3rd instar larvae larval, DG-deficient eye discs do not show abnormalities (Fig. 3-2) in 24B10 and Elav stainings, which specifically mark PRC plasma membrane protein chaoptin and nuclei protein Elav, indicating that the neuronal

specification and patterning are normal. Moreover, bride of sevenless (Boss), a ligand of sevenless on R8 membrane that is required for R7 specification is also normal (a-f, Fig. 3-2) (Reinke and Zipursky, 1988; Van Vactor et al., 1991). These data suggest that DG deficiency does not disrupt cell specification of PRCs and PRCs are normally present in the 3rd instar larvae.

A requirement for DG in PRC development was first observed in early pupa. Staining of DG-deficient mosaic discs in ~20% pupa with neuronal specific mAb 24B10, that specifically lebel the PRC membrane, revealed aberrant PRC morphology. Some ommatidia contain no or few PRCs. Arrows in Fig. 3-3a point to four cell clusters that are significantly smaller than adjacent, normal PRCs. Normally, the average length of lateral membrane of normal PRC at this stage is about 4.59±0.52 μm and the length of the basal membrane of the PRC is about 4.79±0.49 μm, however, the average lengths of small PRCs are reduced to about 1.22±0.45 μm for both lateral and basal membranes (Fig. 3-3b). This suggests that cells in the small ommatidia are retarded in their differentiation while normal cells are undergoing expansion in cell volume during the early pupa.

In a separate experiment, DG-deficient mosaic eye discs are generated by Ey FLP/FRT system that favors production of small clones where DG-deficient cells are GFP-negative but DG-expressing cells are positive for membrane-targeted GFP (methods). In these flies, DG-deficient cells in the 3rd instar larvae form normal zonula adherens (ZA) as revealed by antibodies to the apical ZA marker armadillo (g-i, Fig. 3-2). This indicates that, in addition to the normal specification, ommatidial cells in the 3rd instar larvae are properly polarized. Proceed to ~20% pupa, however, DG-deficient

PRCs, revealed by a negative GFP labeling, show a profound irregular spatial organization and lose their polarity within an ommatidium (white arrowhead, Fig. 3-3c-e). In this DG-deficient ommatidium, there are still seven PRCs labeled with 24B10 but they occupy only half space of the ommatidium and are shrunken and disorganized. Next to this ommatidium, we can see a devoid of six out of seven PRCs (stars in Fig. 3-3c-e) and the only remaining PRC is a non-DG-deficient cell (arrow, Fig. 3-3c-e). As loss of DG does not cause concordant loss of chaoptin protein as shown in larvae and in Fig. 3-3c-e, the absent PRCs have likely degenerated. Strikingly, as pointed out by arrows (Fig. 3-3c-e) this single non DG-deficient PRC within a mosaic ommatidium with its accessory cells develops fairly well while the rest of PRCs have degenerated or disrupted. The morphology of the cell appears normal and the apical membrane begins to form microvilli that will later become a mature rhabdomere (arrows, Fig. 3-3c-d) (n=25 in 25 ommatidia). This result indicates that cell-cell contacts may not be essential for PRC differentiation or maintenance of polarity in the early pupal development once it has been initiated and loss of DG in one PRC does not affect neighboring PRCs at this early stage. In view of this data and the severe disruption of the retina in the adult flies (Fig. 3-1), it appears that PRCs are degenerating first and this affects the surrounding accessory cells in the ommatidium and even the lens. Indeed, we can see wild type accessory cells that have migrated out of position in ommatidia lacking PRCs (top to the star in Fig. 3-3d-e) similar to that seen following induced-death of PRCs (Ryoo et al., 2004). To precisely determine if PRC defects disrupt the accessory cells or contrarily DG-deficient accessory cells are sufficient to lead to PRC defects, we did DG expression and rescue experiments (described below).

Progressive degeneration of PRCs

The cellular phenotypes observed in the small DG-deficient clones at ~20% and ~40% pupal development were quantified in Table 2. Briefly, at ~20% pupa 45% of PRCs in 120 DG-deficient clones (one clone usually covers more than one PRC) are not detectable as revealed by no stainings on antisera 24B10 and fluorescence-labeled phalloidin that binds to actin (Fig. 3-4b, c), whereas 55% of PRCs in DG-deficient clones (n=120) are still present, which includes 31% null cells showing irregular organization and small cell size, and 21% null cells without any observed phenotypes (Fig. 3-4c,d,e) (Table 2). By ~40% pupal life, 74% PRCs in DG-deficient clones (n=103) are undetectable, whereas 26% DG-deficient PRCs are still present, within which 15% display phenotypes and 11% appear normal (Fig. 3-4a, b, c) (Table 2). Because increased numbers in 24B10 negative staining cells and corresponding high percentage in phalloidin-negative staining cells, these data suggest a progressive degeneration of PRCs following cell disorganization and growth arrest.

To achieve evidence of cell death, at 40% papa DAPI was used to visualize nuclear DNA in the DG-deficient mosaic eye discs. Normal ommatidia have nuclei with clusters of sharply stained DNA that is localized in the center of 24B10-stained PRCs (arrowhead in Fig. 3-4e-f). However, in the area where PRCs have cellular defects revealed by aberrant 24B10 staining (delineated region in Fig. 3-4d-f), DNA labeling is often diffuse (arrows, Fig. 3-4d-f) that probably represents cleaved DNA. Therefore, these data suggest that nuclear breakdown and cell death occur in DG-deficient disc. Cross sections of eye discs with large DG-deficient clones in ~70% pupa show few recognizable ommatidia (asterisk, Fig.3-4h) while one ommatidium is present

and appear normal (arrow, Fig. 3-4h) that looks like a non-DG-deficient ommatidium because of surrounding granules. Also, in contrast to earlier pupal stages in which most accessory cells surrounding the DG-deficient PRCs appear normal (open arrowhead, Fig. 3-3d-e), the ommatidial accessory cells at this stage are grossly disorganized and PRCs virtually absent. The disc appears filled with cell debris and tissue (asterisk, Fig. 3-4h). Interestingly, some ommatidia have accumulations of macrophage-like cells (arrowheads, Fig.3-4h). Hemocytes or macrophages in *Drosophila* are known to accumulate and engulf apoptotic cells (Stramer et al., 2005; Tepass et al., 1994), which is consistent with event of cell death happening in DG-deficient cells. Together, our data suggest that, while normal PRCs undergo dramatic expansion and morphogenesis during pupal development, loss of DG leads to arrest of PRC growth and disorganization of PRCs, which may contribute to cell degeneration.

Distribution of DG in the eye discs

To better understand the roles of DG, the distributions of DG in the 3rd instar and early pupa were mapped with antisera to *Drosophila* DG (Deng et al., 2003). In the late 3rd instar larvae imaginal disc, DG is found as "dots" at the very apical eye disc (Fig. 3-5b) that co-localizes with 24B10 staining (Fig. 3-5a-c, s). Because 24B10 staining is neuron-specific, this suggests that DG is expressed at PRC tips. Benzer and colleagues (1987) showed that these apical tips are decroted with microvilli that project into the extracellular matrix, which lies between the disc surface and the peripodial membrane (Banerjee et al., 1987), a membrane formed by epithelial cells that lies outside of ommatidial cells. On the basal side of PRCs in the 3rd instar larvae where

axons project to the brain, DG is expressed as a "ring" (Fig. 3-5h) that surrounds and overlaps with 24B10 immunoreactivity in the periphery (Fig. 3-5g-i, s). In the DG-deficient discs in which cells are DG-deficient, anti-DG immunoreactivity is absent from the apical and basal domains of eye discs (Fig. 3-5e, k). Therefore, in the 3rd instar larvae, DG is expressed at the apical microvilli and basal subdomains of PRCs, two locations that are known next to the extracellular matrix (Banerjee et al., 1987; Longley and Ready, 1995).

Beginning with pupation, following PRC 90° rotation the apical surfaces of PRC become toward the center of ommatidia and the apical-basal axis is perpendicular to and the distal-proximal axis is parallel to the light entering the lens. In the distal part of 40% pupal eye discs, as in the 3rd instar DG is expressed in the apical membrane of PRCs (arrowheads in Fig. 3-5n-o and left panel of t), where the rhabdomere faces the inter-rhabdomere space and is recognizable by enriched 24B10 immunoreactivity (Fig. 3-5m and o). DG is also expressed in the basal membrane of PRC (arrows in Fig. 3-5n-o and left panel of t) that makes contact with the accessory cells. In addition, DG also appears in non-PRC cells that are adjacent to PRCs (red arrows Fig. 3-5o and left panel in t) which are probably interommatidial cells. In the proximal part of 40% pupa eye discs, where axonal bundles penetrate the eye discs (arrowheads, Fig. 3-5p-r), DG is again found as a "ring" in the retinal floor (arrowhead in q, Fig. 3-5) that surrounds and partially overlaps with the 24B10-staining (arrowhead in Fig. 3-5q, r and right panel in t). In addition DG is also found in the glial sheath along the axon bundles (arrows in Fig. 3-5q, r and right panel in t), in which its expression is increased in response to DG-deficiency (unpublished observation). This "ring" could either be the basal domain of

PRCs or pigment cells that are anchored to the "grommet", an extracellular matrix membrane formed at the retinal floor around ~37% pupa (Cagan and Ready, 1989; Longley and Ready, 1995), or it could be the enrichment site of glial cells similar to vertebrate DG localization at the microvilli of Schwann cells at the nodes of ranvier (Occhi et al., 2005; Saito et al., 2003). Nevertheless, our data demonstrate that DG is localized at both apical and basal membranes of PRCs as early as the 3rd instar larvae and continues to express so during pupal development, where DG could make contact with the extracellular matrix or other cells, and DG deficiency leads to loss of the contact which causes cell growth arrest, loss of polarity and ultimate cell death.

Over-expression of DG increases PRC size

We further investigate the roles of DG by over-expressing it in fly eyes. In some instances, over-expression of DG has been reported to act as a dominant inhibitor (Heathcote et al., 2000; Deng et al., 2003). However, when full-length DG is over-expressed in all the posterior cells of eye discs by crossing the GMR-gal4 flies with UAS-DG flies, we do not observe a phenotype comparable to the "glossy" DG-deficient adult eye (Fig. 3-1). Instead, eyes of GMR-Gal4/+, UAS-DG/+ flies are slightly larger than eyes of wild type and GMR-Gal4/+, +/+ flies (data not shown). Next, we determine to examine the internal ommatidia in the DG over-expression eyes. Full-length DG was specifically over-expressed in PRCs by a neuron-specific driver Elav-gal4 (Homyk et al., 1985; Campos et a;l., 1985; Koushika et al., 1996; Luo et al., 1994) and the individual ommatidia are examined in plastic sections. The over-expressed DG is confirmed in Fig. 3-7a-b. Similar to the larger external eye, trans-

heterozygous flies containing Elav-gal4/+, UAS-DG/+ have larger ommatidia with expanded membranes and cell bodies (Fig. 3-6d). The individual ommatidia in DG over-expressed eyes have significantly larger areas (226.3±4.8 μm^2, n=146) and longer perimeters (55.2±0.6 μm, n=146) compared with those in wild type eyes (area: 196.3±5.7 μm^2, n=138; perimeter: 50.1±0.5 μm, n=138) and with two controls that contain Elav-gal4 alone or UAS-DG alone (Fig.3-6e, f), but the PRC numbers remain unchanged. Thus, DG appears directly involved in regulating PRC growth and does not simply sustain cells that would otherwise degenerate.

Full-length DG rescues the defects in DG deficient eyes

To ascertain if the observed phenotypes reflect a role of DG in PRCs or accessory cells, full-length DG transgene was re-introduced into DG-deficient PRCs under control of a neuronal driver Elav-gal4. The expression of DG transgene in PRCs was evident. For example, when antisera to *Drosophila* DG protein (Deng et al., 2003) was diluted 1 to 1000 it is unable to detect DG in the wild type disc (Fig. 3-7a) but easily detected the over-expressed DG at the apical tips of PRCs (Fig. 3-7b). When DG is expressed in the PRCs, 247/248 eyes examined appeared indistinguishable from wild type eyes (Fig. 3-7c). Histological analysis showed that PRCs are normally organized and the retina structure appears as in the wild type flies (Fig. 3-7e-f). Furthermore ERGs are fully restored to the levels comparable to the wild type eyes (Fig. 3-8c). The external eye morphology is also normal with normal lens and organization (Fig. 3-7d), indicating that non-neuronal lens defects observed in the DG-deficient adult are

secondary to loss of DG in PRCs. These data indicates that DG is required in PRCs for eye morphogenesis.

Gene replacement and functional domain requirements for DG

Conceivable with required cleavage on the polypeptide for normal DG function (Jayasinha et al., 2003; Esapa et al., 2003), recent studies in the vertebrate muscles have suggested independent functions for α- and β-DG. α-DG by virtue of its interaction with ligands via its carbohydrates is required for muscle integrity and survival (Grewal et al., 2001; Holzfeind et al., 2002; Kanagawa et al., 2004) and modulate AChR aggregates independently of β-DG at the NMJs (Trembly and Carbonetto, 2006), whereas β-DG is required and sufficient to target dystrophin and the sarcoglycan complex to the muscle sarcolemma (Cote et al., 1999; Cohn et al., 2002; Kanagawa et al., 2004). In this study, we have shown that DG is required for PRC expansion, organization and survival. To test the roles of DG domains in PRCs in view of these functions, we performed the rescue experiments with the intracellular and extracellular DG using Gal4/UAS system. The intracellular DG (DG_{cyto}) containing the intracellular and transmembrane sequence was first used to rescue the DG-deficient eye phenotypes. Genetic crosses were performed to express DG_{cyto} transgene in DG-deficient PRCs in mosaic eyes, which contains one copy of Elav-gal4 and UAS-DG_{cyto} in Ey; FLP/FRT induced DG-deficient mosaic eyes. Crosses also generated another group of flies that contains one copy of UAS-DG_{cyto} alone in the DG-deficient mosaic eyes to serve as a control. The expression of DG_{cyto} was confirmed on the membrane of PRCs, using the anti-*Drosophila* DG antisera diluted at 1:1000, in the mosaic eyes containing one copy

of Elav-gal4-and UAS-DG_{cyto} (Fig. 3-9a') but not in UAS-DG_{cyto} control eyes (Fig. 3-9a). We examined the glossy eye phenotypes of DG_{cyto}-containing (Fig. 3-9c) and control (Fig. 3-9b) flies, based on the scope of glossy patch on the eye surface area, and over 300 eyes have been examined. We found that DG-deficiency causes much smaller and less frequent glossy patches in eyes containing DG_{cyto} transgene than control eyes (Fig. 3-9d). Statistically, the big glossy patches in DG-deficient mosaic eyes containing DG_{cyto} transgene appear much less frequently comparing with the control (Fig. 3-9d). For example, we could not see any 100% normal eyes in the control (Fig. 3-9b, a representative), but near one third of mosaic eyes looks almost entirely normal in the flies containing DG_{cyto} transgene (Fig. 3-9c, a representative). Thus, it appears that the intracellular DG substantially ameliorates the DG-deficient phenotype in the external eye. Next, we examined the histology of DG_{cyto}-containing eyes that do not show glossy patches and the histology of control eyes with small patches (~5%). Not surprisingly, DG-deficient eyes containing DG_{cyto} transgene have largely preserved retinal structures and most PRCs and accessory cells are present (Fig. 3-9g) comparing with DG-deficient eyes (Fig. 3-1f' and Fig. 3-10f) and with DG-deficient control eyes containing UAS-DGcyto alone (Fig. 3-9h). However, retinae of these expected rescued eyes still show disorganization of PRCs but the size and number of PRC appear normal (star, Fig. 3-9g), and occasionally ommatidia have only six visible PRCs (arrows, Fig. 3-9g). This disorganization of PRCs appears as disrupted PRC planar polarity. In the wild type eyes, PRCs are recruited and oriented beginning in the 3rd instar larvae so that at one part of disc PRC clusters are uniformly oriented PRCs (Fig. 3-9e'), whereas in DG-deficient eyes that containing DG_{cyto} transgene some ommatidia show dis-oriented

PRCs with R3 and R4 have chaotic directions (red, Fig. 3-9g') comparing with the neighboring ommatidia (blue, Fig. 3-9g'). In the control eyes containing UAS-DG_{cyto} alone (Fig. 3-9h), DG-deficient eyes show strong phenotypes similar to the DG-deficient eyes containing no UAS-DG_{cyto} (Fig. 3-1f' and Fig. 3-10f), which have a degenerated retina and disrupted ommatidia (arrow, Fig. 3-9h) and an autonomous degeneration of single PRCs (arrowhead, Fig. 3-9h). Furthermore, over-expression of DG_{cyto} in heterozygous PRCs does not cause visible defects in the histology (Fig. 3-9f), planar polarity (Fig. 3-9f') and the external morphology (data not shown). Therefore, these data suggest that the intracellular DG partially rescues PRC degeneration, but not the organization of PRCs. A disrupted planar polarity of PRCs remains in these eyes, suggesting an independent function for the extracellular domain of DG.

In vertebrates carbohydrates of α-DG in the mucin-region are the ligand binding domains which is required for muscle survival, brain development and synaptic transmission. The amino acids of *Drosophila* extracellular DG are poorly conserved but contain 15 consensus O-linked glycosylation sites (Fig.1-2). To find out if the functional carbohydrates are conserved in *Drosophila* DG, the extracellular *Drosophila* DG (DG_{extra}-mRFP), containing the extracellular and transmembrane regions of DG that are fused with mRFP in the C-terminus, was expressed in differentiated DG-null embryonic stem cells and mAb IIH6 was used to identify the functional carbohydrates. The DG-null mouse embryonic stem cells were engineered (Cote et al., 1999) and differentiated as described previously (Zhan et al., 2005). In the transfected DG-null cells, DG_{extra}-mRFP protein is clustered at the cell membrane and can be recognized by antibody IIH6 without permeabilizing the membrane (Fig. 3-10b-b''). IIH6 is known to

recognize the functional O-linked carbohydrate chains of α-DG, thus, *Drosophila* DG can be glycosylated to contain the same functional carbohydrate epitope as the vertebrates, which suggests that ligand binding appears to be a conserved function of DG and glycosylation and transport of extracellular DG do not need the participation of intracellular DG same as in the vertebrates (Trembley and Carbonetto, 2006). Additional experiments also show that IIH6 recognizes the endogenous DG in the western blots of *Drosophila* homogenates (Shah and Carbonetto, unpublished result).

To test the necessity of the extracellular domain of DG in the development, genetic crosses were performed to express the extracellular DG (DG_{extra}-mRFP) in the DG-deficient PRCs using Gal4-UAS system. The crosses resulted in one group of flies containing one copy of Elav-gal4 and UAS-DG_{extra}-mRFP in DG-deficient mosaic eyes and another group containing neither Elav-gal4 nor Uas-DG_{extra}-mRFP in the DG-deficient mosaic eyes to serve as a control. The red fluorescence generated by DG_{extra}-mRFP was found in all the nervous system in living animals (data not shown) and in plasma membranes and rhabdomeres of DG-deficient PRCs in pupal eye discs containing one copy of Elav-gal4 and UAS-DG_{extra}-mRFP (Fig. 3-10a), without fixation and permeabilization. This confirmed that DG_{extra}-mRFP transgene is indeed expressed in the PRCs. We next examined the scope of glossy patches on DG-deficient eyes containing DG_{extra}-mRFP transgene and those containing no transgene in a same way as in the DG_{cyto} rescue experiment. About three hundred eyes were examined on both groups. DG-deficient eyes containing the transgene (Fig. 3-10d) showed no difference in the size and frequency of glossy patches comparing with eyes containing no DG_{extra} transgene (Fig. 3-10c) and Figures 3-10c and 3-10d are the representatives.

Statistically, the size and frequency of glossy patches in eyes containing DG_{extra}-mRFP show a same trait comparing with the fly eyes containing no DG_{extra} transgene (Fig. 3-10h). For example, about half of eyes examined on both types of flies show greater than 70% areas with glossy patches (Fig. 3-10h). Histology of retina was examined from flies with small patches on both types, which demonstrates the same retinal degeneration and disruption (Fig. 3-10f, g). As control, over-expression of DG_{extra}-mRFP on PRCs does not cause disruption in the retina of wild type flies (Fig. 3-10e) and in the external morphology (data not shown). Therefore, different from intracellular DG, the extracellular DG is unable to rescue the loss of PRC phenotype. These results suggest that role of DG in cell survival is independent from its action in cell polarity.

Discussion

Muscular dystrophy is a group of diseases that are often caused by loss-of-function mutations affecting dystroglycan-dystrophin complex. The major feature of the disease is muscle degeneration, but this can be associated with mental retardation and various retinal defects ranging from defective retinal b-wave (Holzfeind et al., 2002; Pillers et al., 1993), abolished ERG, retinal degeneration and blindness (Fahnehjelm et al., 2001; Hino et al., 2001; Takeda et al., 2003; Vervoort et al., 2004; Zervos et al., 2002). However, mechanisms of the disease remain to be understood, especially for those in the CNS. Using *Drosophila* eye as the model, I demonstrated an essential role of DG in autonomous PRC development and non-autonomous requirement for the entire retina, and gene replacement experiments revealed distinct functions for DG domains in eye development.

DG-deficient eyes don't have retinae. Adult eyes without DG show disrupted ommatidia, loss of photoreceptors and accessory cells with replacement by connective tissue as well as abolishment of ERG. These severe phenotypes begin with disorganization of PRC or loss of polarity and simultaneous retardation in PRC growth in the early pupa when ommatidial cells undergo early pupal differentiation. With pupal development in DG-deficient eyes progressive PRC degeneration occurs, DNA disorganization and dispersion and macrophage-like cell infiltration are also observed. Similar events occur in DG-null dystrophic muscle (Cote et al., 1999; Cohn et al., 2002).

DG controls PRC growth and spatial organization. DG-deficient PRCs in 20% pupal development show a growth arrest with smaller cell size in addition to the

abnormal spatial organization. This disrupted organization demonstrated that PRCs lose their polarity, including the planar polarity that was initiated earlier in the larval stage (Klein and Mlodzik, 2005). Therefore, DG appears to play a role for PRC planar polarity, in addition for the apical-basal polarity in epithelial cells of *Drosophila* embryos (Deng et al., 2003). Does loss of cell organization cause the growth arrest and final degeneration of PRCs? Loss of polarity is not necessarily associated with arrest of cell growth and degeneration since mutations in many polarity genes such as scribble, discs-large, and lethal giant larvae disrupt cell polarity but cause simultaneous cell proliferation and tissue overgrowth (Bilder, 2004). Moreover, mutant Frizzled causes disruptions in planar cell polarity within a tissue but those cells do not show growth arrest and degeneration (Vinson and Adler, 1987; Zheng et al., 1995; Adler and Lee, 2001); also mutant Crumbs in pupal PRCs disrupts the adherens junctions but PRCs show no sign of degeneration (Izaddoost et al., 2002; Pellikka et al., 2002). In our data, over-expression of one copy of DG driven by GMR-Gal4 or Elav-Gal4 in the wild-type flies significantly increases the size of eye and individual PRCs, suggesting that DG is not only necessary but also sufficient to promote postmitotic PRC growth. More importantly, our data demonstrate that intracellular DG can largely rescue the PRC degeneration and cell size but PRCs remain disorganized. Therefore, DG is required for two different cellular processes: growth of PRC and organization of PRC. As DG has been shown to associate with signaling adaptor Grb2 (Yang et al., 1995; Zhan et al., 2005), MAPK pathway (Jones et al., 2005), PIK3/Akt pathway (Langenbach and Rando, 2002) and other intracellular signaling (Li et al., 2005), involvement of DG in

cell size growth that is largely dependent upon protein synthesis (Stocker and Hafen, 2000; Kozma and Thomas, 2002) deserves further investigation.

Our data demonstrate an autonomous effect for DG in PRCs, but non-autonomous effect for other cells including accessory cells and lens. First, when DG-deficient PRCs show defects in organization and cell size in ~20% pupal development the surrounding cells appear normal (open arrows, Fig. 3-3d, e). Interestingly, degeneration of PRCs at 20% pupa is not accompanied by degeneration of accessory cells, instead, there is an invasive behavior for wild-type accessory cells that protrude into the "empty" ommatidial space (Fig. 3-3d, e). It appears that PRC defects arise independent of and begins prior to ommatidial accessory cell defects. Second, DG is expressed in PRCs with an apical localization at tips of PRCs in the 3rd instar where PRCs form the apical microvilli that project into the extracellular matrix and at rhabdomere microvilli in the pupa which face the inter-rhabdomere space. As well, DG is localized at the basal membrane of PRCs as a "ring' at the late 3rd instar (Fig. 3-5h, i), which is formed by PRCs because it overlaps with PRC-specific 24B10 staining at the periphery (Fig. 3-5i, s). One thing should be noted that this "ring" in the 3rd instar larvae appears like the axon-surrounding grommet that is formed by pigment cells and the cone cell plates at the pupal retina floor (Longley and Ready, 1995), but it actually can not be the grommet because grommet is not formed until 37% pupal development (Cagan and Ready, 1989; Longley and Ready, 1995). Although the basal cone cell processes are adjacent to the basal domain of PRC and surrounds the axons (Longley and Ready, 1995), this basal DG immunoreactivity belong most likely to PRCs because it overlaps with 24B10 staining in the periphery (Fig. 3-5i). Therefore, this "ring" at the

basal disc of 3rd instar larvae could be the site where PRCs make contact with the extracellular matrix or with the accessory cells (Fig. 3-5s). When PRCs rotate 90°, expand and become surrounded by accessory cells at the pupal stage (Cagan and Ready, 1989; Longley and Ready, 1995; Tomlinson and Ready, 1987), our pupal staining has indeed shown that DG continues to be expressed at the basal membrane of the PRCs (Fig. 3-5n, o, t). Therefore, similar to vertebrate DG that connects cells to the extracellular matrix or to other cells, *Drosophila* DG containing the same functional carbohydrate chains (Fig. 3-10b-b") could make contact of a photoreceptor with the extracellular matrix at both apical and basal membranes. Thus, loss of DG could cause loss of PRC contact with the extracellular matrix or with other cells, which could be the reason why PRCs are disrupted. Zonula adherens among the ommatidial cells are the best known cell-cell contact involved in maintaining PRC polarity. Our data show that individual DG-positive PRC shows no sign of defects when neighboring photoreceptors are degenerated or disorganized (Fig. 3-3d, e). This is very interesting and suggests that DG is sufficient to maintain PRC organization and DG-negative PRCs do not maintain the neighboring PRCs within one ommatidium. Third, re-introduction of DG specifically in the PRCs can rescue the phenotypes of the whole eye (Fig.3-7) including those in PRCs, in accessory cells and lens and the ERG is also restored to the normal (Fig. 3-8c). This confirms that the structural and functional phenotypes are mainly resulted from loss of DG in PRCs, and the entire retinal defects including those of accessory cells observed in ~70% pupa (Fig. 3-4h) and adult (Fig. 3-1f') are secondary to failure of PRC development. Also, it demonstrated that non-PRC expression of DG in pupal development is not functionally important. And, lens defects observed in

adults are secondary to PRC defects and are most likely caused by lens-secreting cone cell defects prior to ~45% pupal development (Cagan and Ready, 1989).

Our results demonstrate that intracellular DG is sufficient to largely rescue the eye phenotypes, especially PRC degeneration, but not the organization of PRC or planar polarity of PRCs (Fig. 3-9g). Therefore, it is the function of extracellular domain of DG to organize PRCs and disorganization or disrupted PRC planar polarity is not sufficient to induce PRC degeneration. Indeed, seven PRCs exist in these rescued ommatidia while their polarity is disrupted (stars, Fig. 3-9g), suggesting cell survival and planar polarity are two uncoupled processes and intracellular DG is largely required for cell survival and extracellular DG is required for planar polarity. This rescue data also suggests that an intracellular signaling(s) is coupled to DG and sufficient to keep PRC alive, which is consistent with the over-expression data that DG is involved in regulating PRC expansion (Fig. 3-3a and Fig. 3-6). On the other hand, by sequence comparison, extracellular *Drosophila* DG contains a mucin-like region with 15 putative O-linked glycosylation sites and our data show that extracellular DG can be glycosylated to contain same carbohydrate epitope that is recognized by IIH6 (Fig. 3-10b-b"), suggesting that *Drosophila* DG has the functional ligand-binding ability. However, when extracellular DG is used to rescue the DG-deficient phenotypes, the retina shows same phenotypes as eyes containing no transgene. Therefore, keeping cell in contact and organization with extracellular DG does not sustain the challenge of loss of intracellular DG, and PRC degeneration caused by absence of intracellular DG is sufficient to cause the eye phenotypes similar to loss of entire DG protein. These data are consistent with the previous evidence that DG polypeptide must be cleaved to

function properly (Jayasinha et al., 2003; Esapa et al., 2003) and α- and β-DG subunits play coordinate but also distinct roles in vertebrate muscles (Kahl and Campanelli, 2003; Kanagawa et al., 2004; Trembley and Carbonetto, 2006). Moreover, extending the previous evidence, our data suggests that α-DG is required for but appear not sufficient to rescue DG-deficient phenotype in PRCs.

In summary, our data demonstrated for the first time an autonomous requirement of DG in postmitotic PRC differentiation and showed that cell growth arrest, disrupted PRC polarity and progressive PRC degeneration could be the reasons leading to the severe disruption in adult DG-deficient eye. Extending previous evidence that α and β-DG could play distinct roles in muscle, using rescue experiments we demonstrated PRC planar polarity disruption and cell degeneration are independent processes which are attributed to respective function of extracellular and intracellular DG.

ACKNOWLEDGEMENTS

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Table 2. Progressive degeneration of PRCs in DG-deficient clones.

	24B10 negative cells	phalloidin negative cells	24B10 positive cells	
			misorganized/small	normal morphology
20% pupa (n=120 null clones*)	45%	N.D.	34%	21%
40% pupa (n=102 null clones)	74%	74%	15%	11%

*a clone usually contains multiple PRCs. N.D. not done.

Figure 3-1

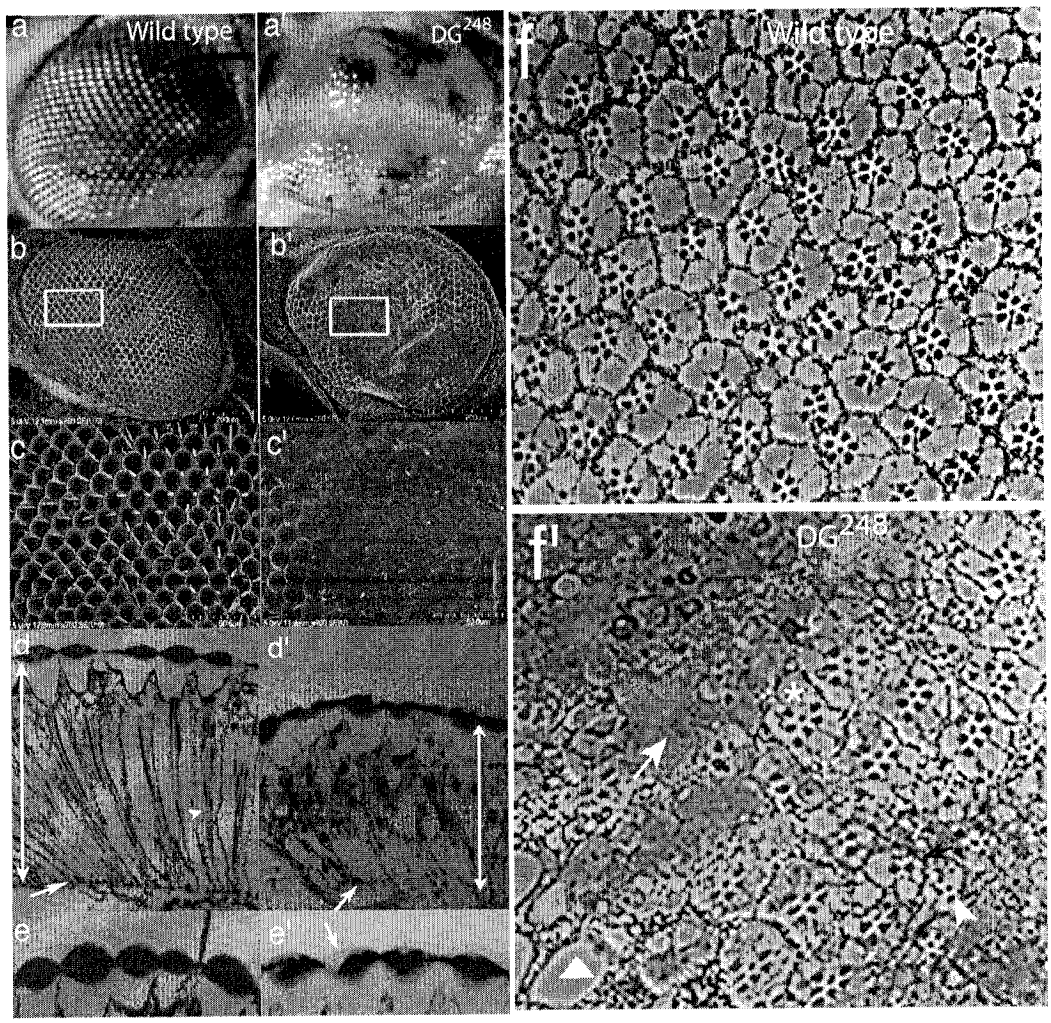


Figure 3-1: Deletion of DG in the eye disrupts the structure of the adult eye

Light and scanning EM micrographs of the wild type fly eye (**a**-**c**) show the classical ommatidial facets and bristles of the external eye. **a'**-**c'** show equivalent micrographs of flies expressing DG^{248} generated by the FLP/FRT system which induces typically 80-90% DG-deficient (DG^{248}) tissue. The DG-deficient tissue in **a'** is white while tissue with heterozygous expression of the wild type DG gene is red. The DG-deficient (white) regions appear flattened and glossy (**a'**). Electron micrographs reveal regions where the external eye has collapsed (**b'**) and the facets have been obliterated (**c'**). Histology of the wild-type eye stained with toluidine blue show regularly arranged rhabdomeres (**d**, arrowhead) and surrounding pigment cells that span the thickness of the eye and rest on a retinal floor (**d**, arrow). In DG-deficient eyes the internal structure is disorganized and the eye thinner (**d'**). Interestingly in **e'** the lenses which normally (**e**) form a biconvex disc are flattened on their external, but not internal, faces though cuticle that forms the external boundary is visible within an flattened and partially "empty" lens (**e'**, arrow). Cross sections of the internal eye reveal that the normal (**f**) array of PRC with central rhabdomeres is disrupted (**f'**, arrow) and there appears to be macrophage-like cells (**f'**, top of the arrow) in these regions. Open arrow points to an ommatidium that has only two pigment-containing PRCs visible while other non-pigment-containing PRCs appear disrupted. Therefore, DG appears function autonomously. Heterozygous ommatidia contain normal rhabdomeres and photoreceptor cells (**f'**, asterisk). Triangle marks the pseudocone on the edge of the eye.

Figure 3-2

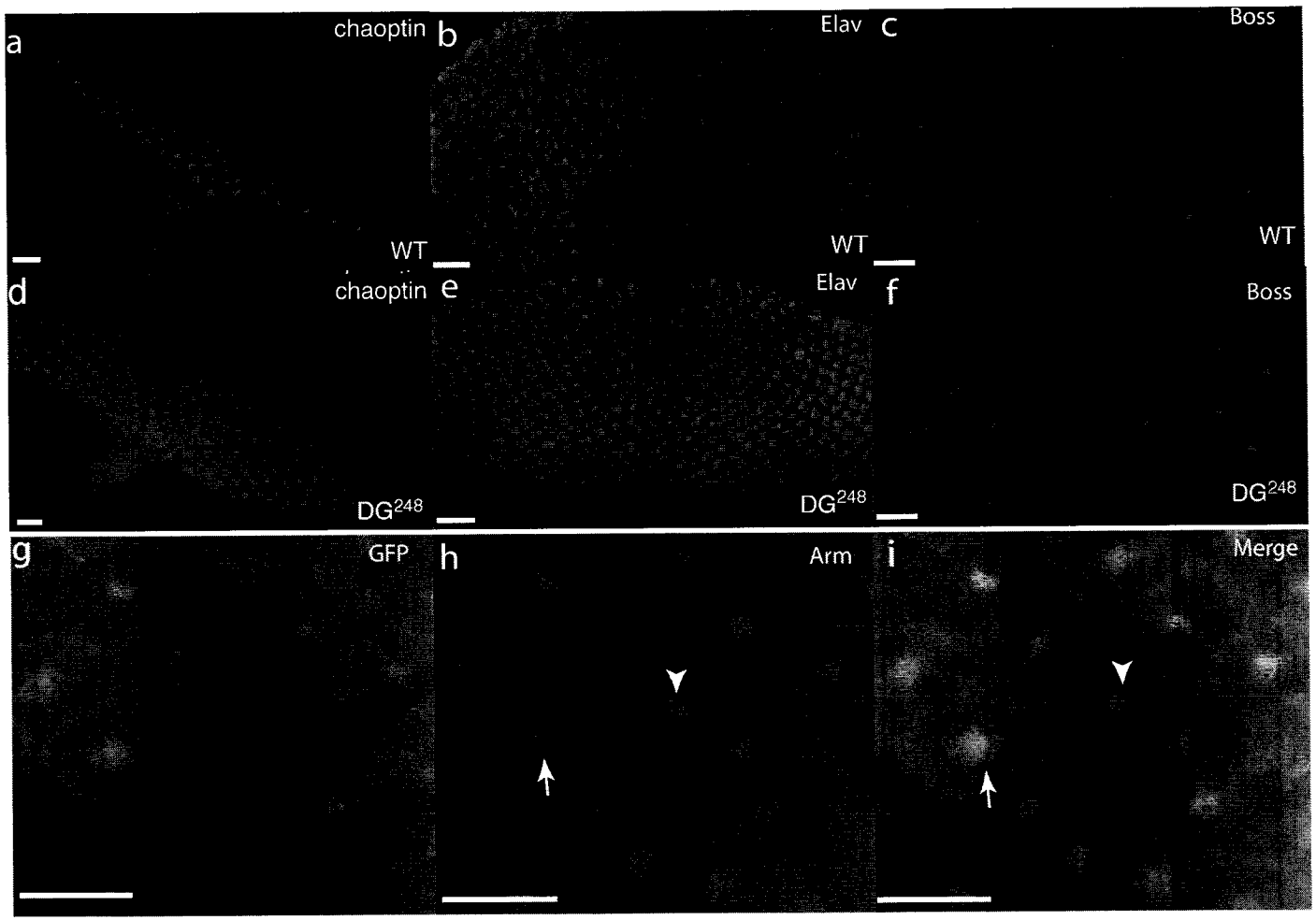

a
chaoptin
b
Elav
c
Boss
WT
WT
WT
d
chaoptin
e
Elav
f
Boss
DG248
DG248
DG248
g
GFP
h
Arm
i
Merge

Figure 3-2. DG deficient photoreceptor cells develop normally through the 3rd instar larvae.

Immunohistochemistry with antibodies to chaoptin (**a**, **d**), Elav (**b**, **e**) and Boss (Bride of Sevenless) (**c**, **f**) were used to identify R cells in wild type (**a-c**) and DG-deficient regions of the eye imaginal disc (**d-f**) of late 3rd instar larvae. Chaoptin (Fig.1) and Elav, a neuron specific nuclear protein, is localized normally (**a**, **b**) in DG deficient eye discs (**d**, **e**). Similarly Boss, which is expressed on the apical surface of R8 and necessary for formation of R7, is localized normally (**c**) in DG deficient eye discs (**f**). In small, DG-deficient clones generated by the ELP/FRT system wild-type cells can be identified by membrane-targeted GFP (**g-i**). In this instance both wild type ommatidia (h, i; arrow) and DG-deficient ommatidia appear normal and have well-organized zonula adherens (h, i; arrowhead) between neighboring R cells marked by armadillo (Arm). Bar, 10 microns.

Figure 3-3

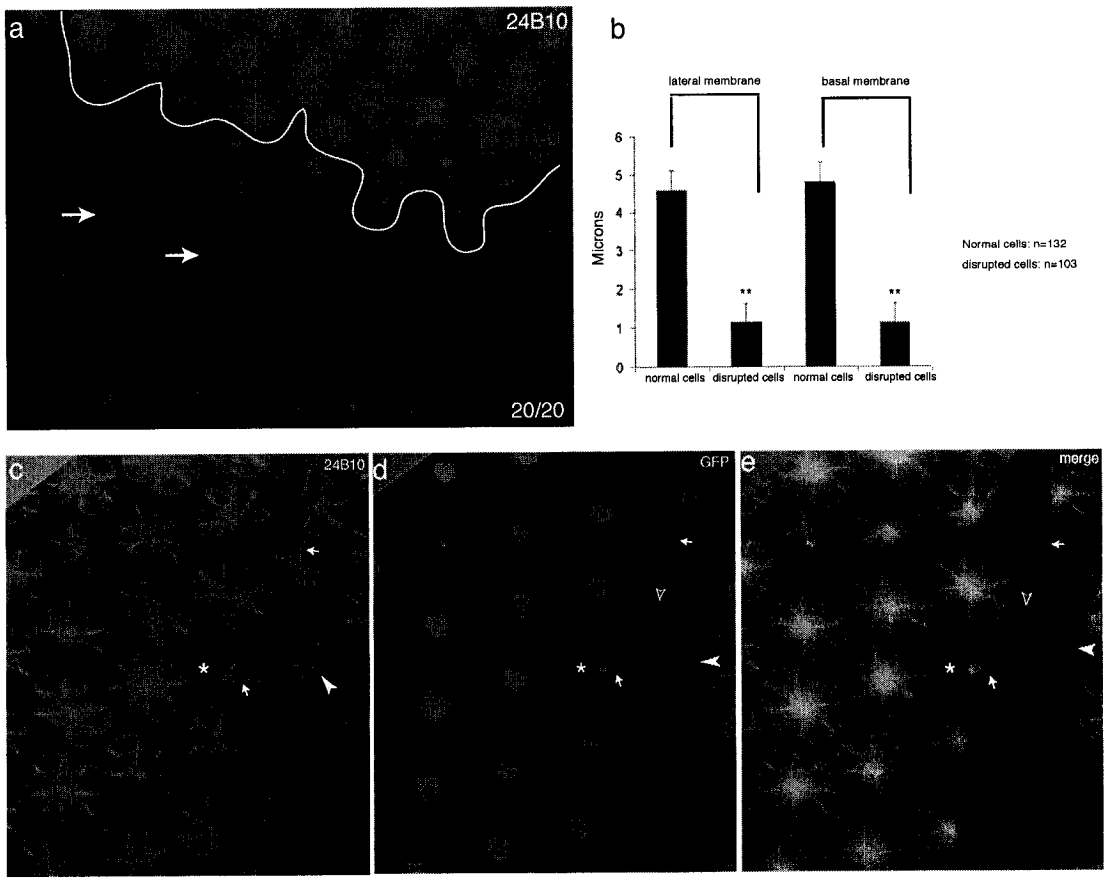

a
24B10
20/20
b
lateral membrane
basal membrane
Microns
6
5
4
3
2
1
0
**
**
normal cells
disrupted cells
normal cells
disrupted cells
Normal cells: n=132
disrupted cells: n=103
c
24B10
d
GFP
e
merge

Figure 3-3. DG is required for photoreceptor size increase and organization during early pupal development.

Antibody 24B10 was used to identify PRC in pupal eye discs (20% of pupation) mosaic for wild type and DG deficient cells generated by the FLP/FRT system. A line in (**a**) separates a disrupted region of the eye from a normal region. In 20 such preparations there were many small PRC (arrows) adjacent to patches with normal ommatidia. **b**, Quantification of the lengths of R cell lateral (apical to basal axis) membranes and (circumferential) basal membranes showed that those in disrupted regions were significantly shorter in lengths (**, $P<0.01$). **c-e**, In pupal eye discs with small clones of DG-deficient cells (Fig.3-2) individual PRCs, identified with antibody 24B10 to chaoptin, are absent from some ommatidia (asterisk). These cells are invariably deficient for DG as reflected in the lack of GFP labeling (**d**). The arrowhead points to an ommatidium (**c**) with DG-deficient PRCs (**d**) that have pulled away from the supporting cells of the ommatidium and are misoriented and shrunken. Conversely, adjacent to this ommatidium, one sees a single wild type PRC (arrow) within an otherwise empty ommatidium (**c**, **e**). This DG positive cell appears normal, is attached to adjacent supporting cells and has elaborated a rhabdomere (**d**, **e**). In the same ommatidium (top to the asterisk, **d**,**e**) supporting cells appear to be moving into a vacated ommatidial cavity (**d**, **e**). Open arrowheads point to normal appearing accessory cells that surround the misoriented DG-deficient PRCs.

Figure 3-4

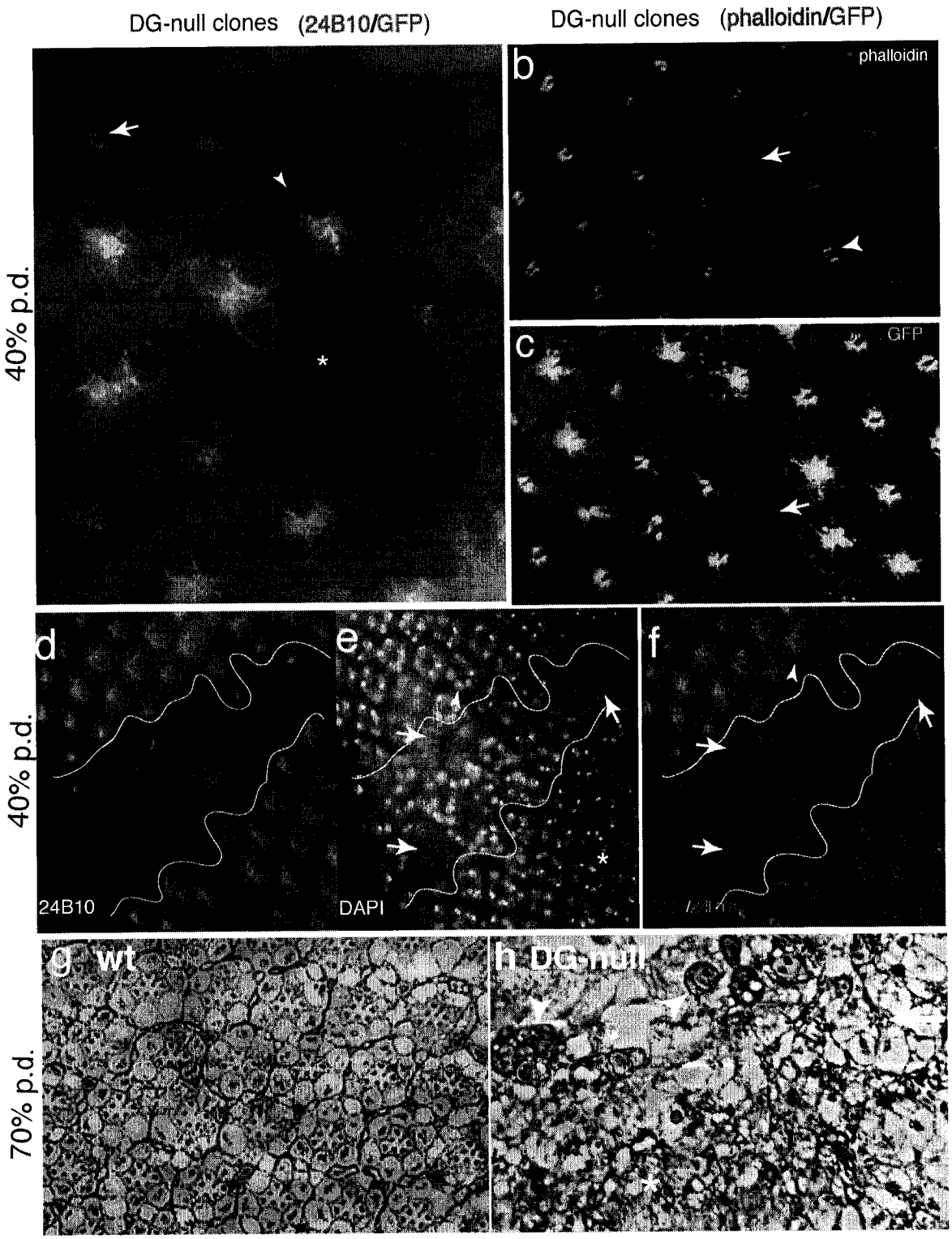

DG-null clones (24B10/GFP)
DG-null clones (phalloidin/GFP)
40% p.d.
b
phalloidin
c
GFP
40% p.d.
d
e
f
24B10
DAPI
70% p.d.
g wt
h DG-null

Figure 3-4. Progressive degeneration of photoreceptor cells.

a, Stainings of 24B10(red) and GFP (green) in clones at 40% pupa show same phenotypes as in the 20% pupa. Some PRC in DG-deficient clones are lost (asterisk), some are smaller and disorganized (arrow), while some are still normal (arrowhead). **b-c**, Phalloidin staining in 40% pupa is mostly lost in DG-deficient ommatidia (arrow, **b,c**) but strongly present in the normal ommatidia, which is concentrated at rhabdomeres (arrowhead, **b**). This phalloidin staining is merged with GFP stainings (**c**). Statistics of these stainings in 20% and 40% pupa were summaried in (Table 2). **d-f**, Affected DNA in 40% pupal DG-deficient mosaic disc. 24B10 was used to mark PRCs in the disc (**d**). DAPI was used to recognize nuclear DNA (**e**). Normal ommatidia contain sharply-stained DNA clusters while each dot is localized in the center of PRC (arrowhead, **e** and **f**). Delineated area shows cellular defects by 24B10 staining and also has diffused DNA (arrows, **e**, **f**), suggesting a cell death is happening. **g-h,** Cross sections of wild type (**g**) and DG-deficient mosaic (**h**) discs in 70% pupa. Most areas of retina (asterisk, **h**) are disrupted and show tissue deposit and cell debris. Macrophage-like cells are accumulated in some ommatidia (arrowheads, **h**). The arrow points to one ommatidium, surrounded by normal pigment granules, thus, probably a non-DG-deficient ommatidium, has relatively normal rhabdomeres and PRCs.

Figure 3-5

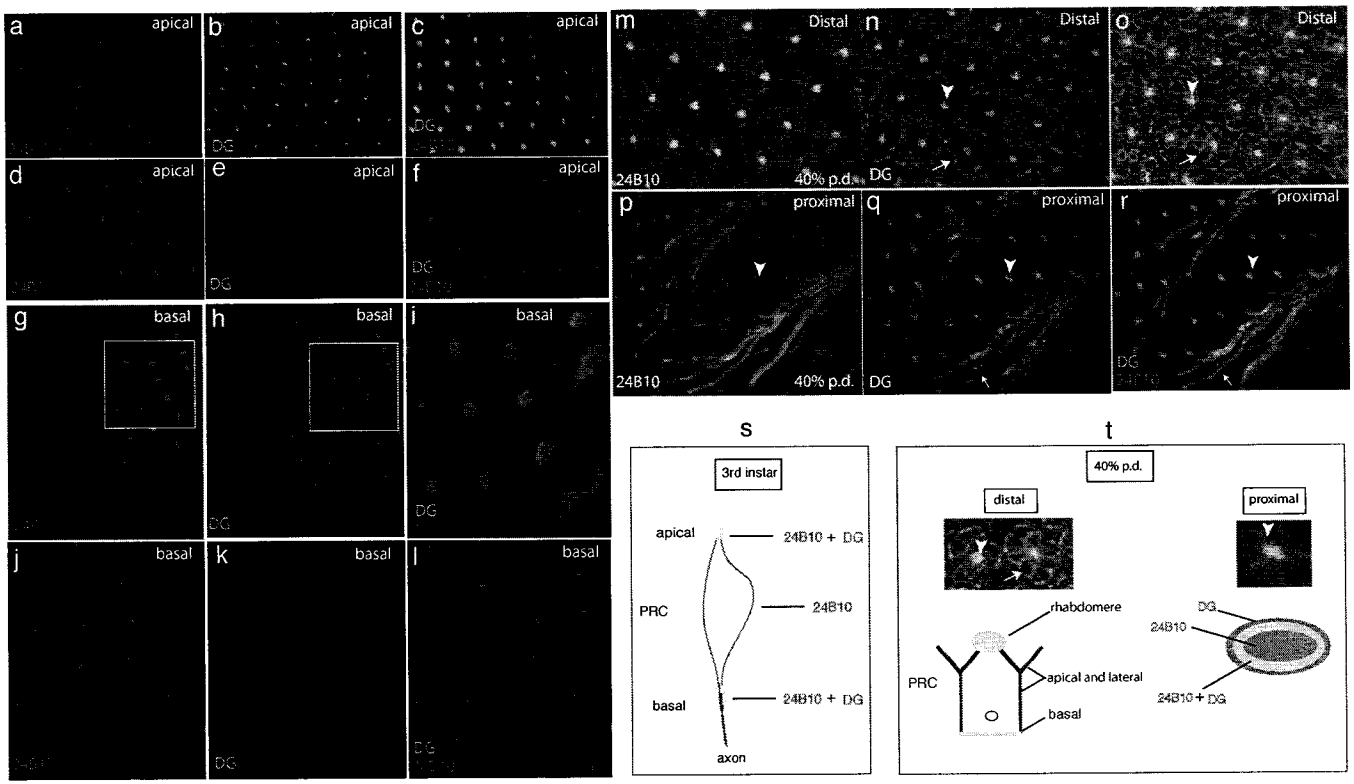
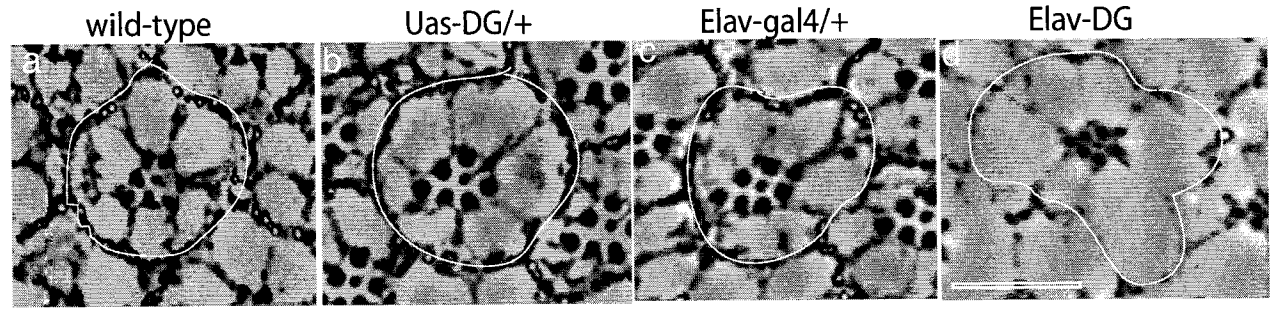
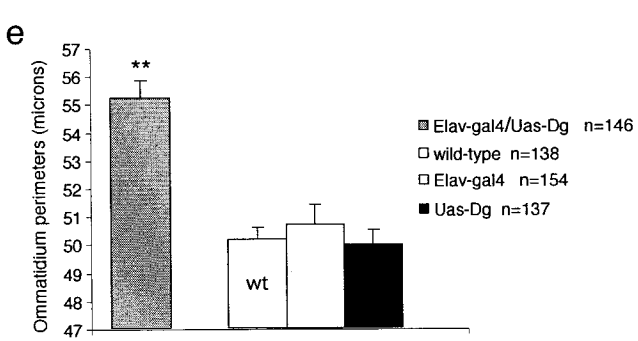

a apical
b apical
c apical
d apical
e apical
f apical
g basal
h basal
i basal
j basal
k basal
l basal
DG
m Distal
n Distal
o Distal
24B10
40% p.d.
DG
p proximal
q proximal
r proximal
s
3rd instar
apical
24B10 + DG
PRC
24B10
basal
24B10 + DG
axon
t
40% p.d.
distal
proximal
rhabdomere
apical and lateral
basal
PRC
DG
24B10
24B10 + DG

Figure 3-5. Distribution of DG in the developing fly eye

The eye imaginal disc of the 3rd instar larvae is immunolabeled with mab 24B10, that recognizes chaoptin, a membrane protein that is concentrated in the apical tips of PRCs in these apical, optical sections (**a-f**). Antiserum to Drosophila DG (Deng et al., 2003) labels the apical tips of wild-type (**b**) but not DG-deficient R cells (**e**). In basal regions of the imaginal disc (**g-l**), chaoptin-positive axons can be seen exiting the retina surrounded by an area that also expresses DG. An amplified image shows the peripheral co-staining of 24B10 and DG (**i**). In DG-deficient imaginal discs the bundling of axons in the basal region appears normal as revealed by chaoptin labeling (**j**) despite the absence of DG (**k**). In the 40% pupa DG is found along with chaoptin in the rhabodomere at the centre of each ommatidium (**m-o**, arrowhead) and at the basal aspect of each PRC (**n-o**, arrow), as well within axons (**p-r**, arrow) and surrounding glia that exit the retina in the proximal side of the disc (**p-r**, arrowhead). **s,** Carton shows the distribution of DG in the 3rd instar PRCs. DG is distributed in the apical and basal parts of PRCs where it is co-stained with chaoptin, while chaoptin is uniformly distributed on PRC surface and axon. **t,** Top of the left panel is two amplified PRCs in **o**. Bottom is a cartoon showing the distribution of DG in PRCs; Top of the right panel is an amplified sites where axons exit the pupal eye disc (arrowhead, **r**). Bottom is a cartoon showing the distribution of DG at this site.

Figure 3-6


wild-type
Uas-DG/+
Elav-gal4/+
Elav-DG
a
b
c
d


e
Ommatidium perimeters (microns)
57
56
55
54
53
52
51
50
49
48
47
**
wt
Elav-gal4/Uas-Dg n=146
wild-type n=138
Elav-gal4 n=154
Uas-Dg n=137

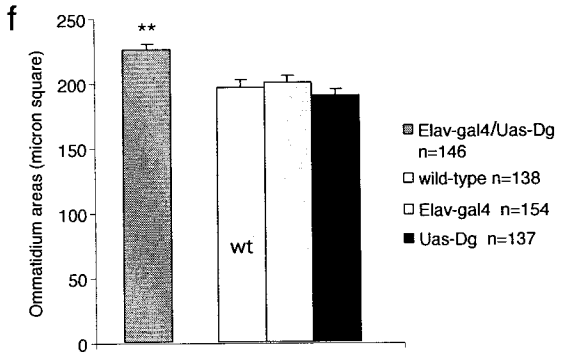

f
Ommatidium areas (micron square)
250
200
150
100
50
0
**
wt
Elav-gal4/Uas-Dg n=146
wild-type n=138
Elav-gal4 n=154
Uas-Dg n=137

Figure 3-6. Over-expression of DG in photoreceptor cells increases ommatidial size.

Cross sections of ommatidium (encircled) in wild type flies (**a**) and flies containing Uas-DG/+ alone (**b**), Elav-Gal4/+ alone (**c**) and Elav-gal4/Uas-DG (*Elav-DG*, **d**) showed that over-expression of DG in PCRs induces larger ommatidia with expansion of PRCs (**d**) as compared with the controls (**a-c**), but the number of PRCs remains unchanged (**d**). **e**, Ommatidia perimeters are significantly increased when DG is over-expressed in PRCs driven by Elav-Gal4. **f**, Ommatidial areas are significantly increased when DG is over-expressed. Over-expression of the DG transgene was confirmed immunohistochemically in Fig. 3-7b. **, $P<0.01$. Bar, 10 microns.

Figure 3-7

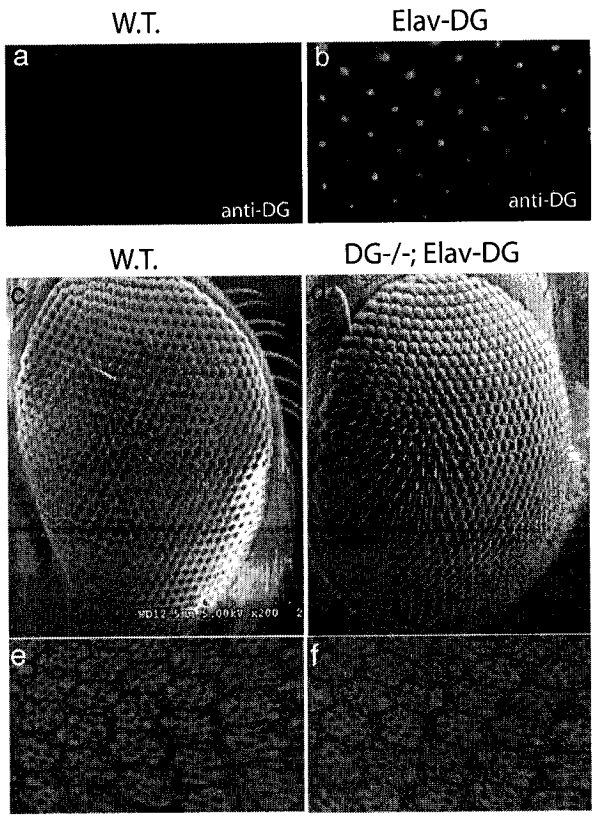


Figure 3-7. DG expression in photoreceptors is sufficient to restore the normal eye structure

a-b, Diluted antisera (1:1000) to *Drosophila* DG were used to visualize the expression of DG in wild type (**a**) and DG-over-expressed (*Elav-DG*) flies (**b**) driven by Elav-gal4. Although the antisera at 1:250 dilutions recognize the wild type DG (Fig. 3-5b,h), the antisera at 1:1000 dilutions barely detect the wild type DG (**a**), however, it easily detected DG at the apical tips of 3rd instar PRCs containing Elav-gal4 and Uas-DG (*Elav-DG*) (**b**). As the DG transgene driven by Elav was induced in PRCs of the DG mosaic eyes generated by FLP/FRT system (Fig.3-1) (*DG-/-; Elav-DG*), the external eye, visualized by scanning electron microscopy, looks completely normal (**d**) as does the internal structure in histological section (**f**).

Figure 3-8

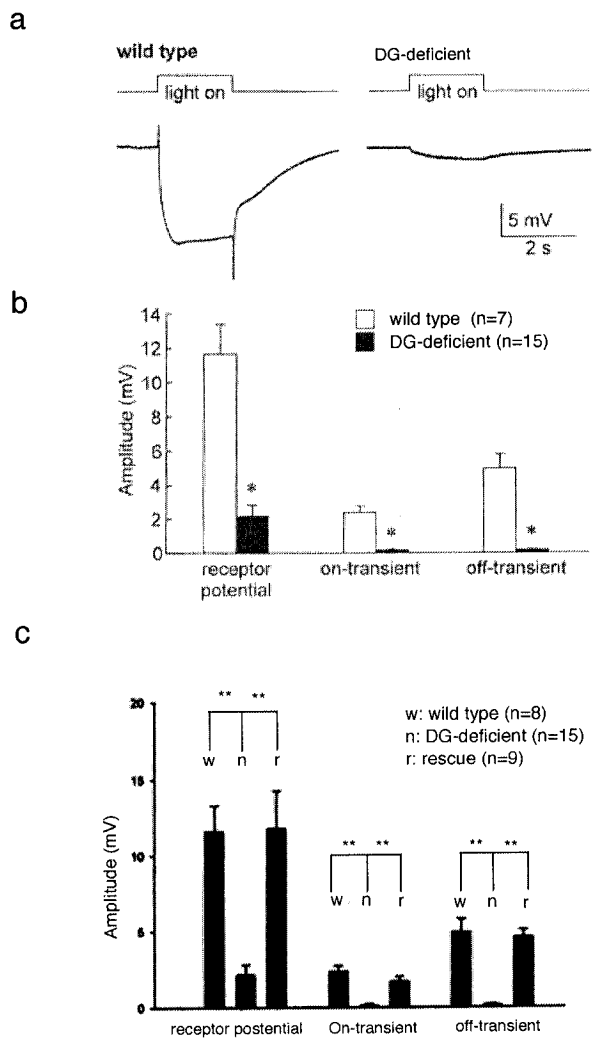

a
wild type
light on
DG-deficient
light on
5 mV
2 s
b
wild type (n=7)
DG-deficient (n=15)
Amplitude (mV)
14
12
10
8
6
4
2
0
receptor potential
on-transient
off-transient
c
w: wild type (n=8)
n: DG-deficient (n=15)
r: rescue (n=9)
Amplitude (mV)
20
15
10
5
0
w n r
receptor postential
On-transient
off-transient

Figure 3-8. DG expression in photoreceptor cells is sufficient for development of a functional eye.

Electroretinograms (ERGs) were recorded from wild-type flies, DG-deficient mosaics and mosaics expressing a DG transgene in their PRCs (Fig. 3-7b). (**a**) ERGs were recorded after a 5 min dark adaptation followed by a 2 sec bright-light pulses (**a**, Top trace). The bottom trace shows representative ERGs of wild type (left) and DG-null regions (white) of mosaic eyes (right). In DG-deficient regions the 9-14mV PRC depolarization (left) was greatly diminished and the early and late synaptic transients (left) completely abolished. (**b**) Quantification of ERG parameters in wild type and DG null patches showed significant differences ($P<0.01$) between wild-type and DG null eyes. (**c**) ERGs from wild type eyes (N=8) DG-deficient mosaic eyes (N=15) or those expressing the DG transgene in PRCs (N=9) again shown significant differences in the receptor potential and synaptic transients between wild type and DG-deficient eyes. Mosaic deficient eyes expressing the DG transgene also had significantly greater ERG responses that were indistinguishable from wild-type eyes. **, $p<0.01$.

Figure 3-9

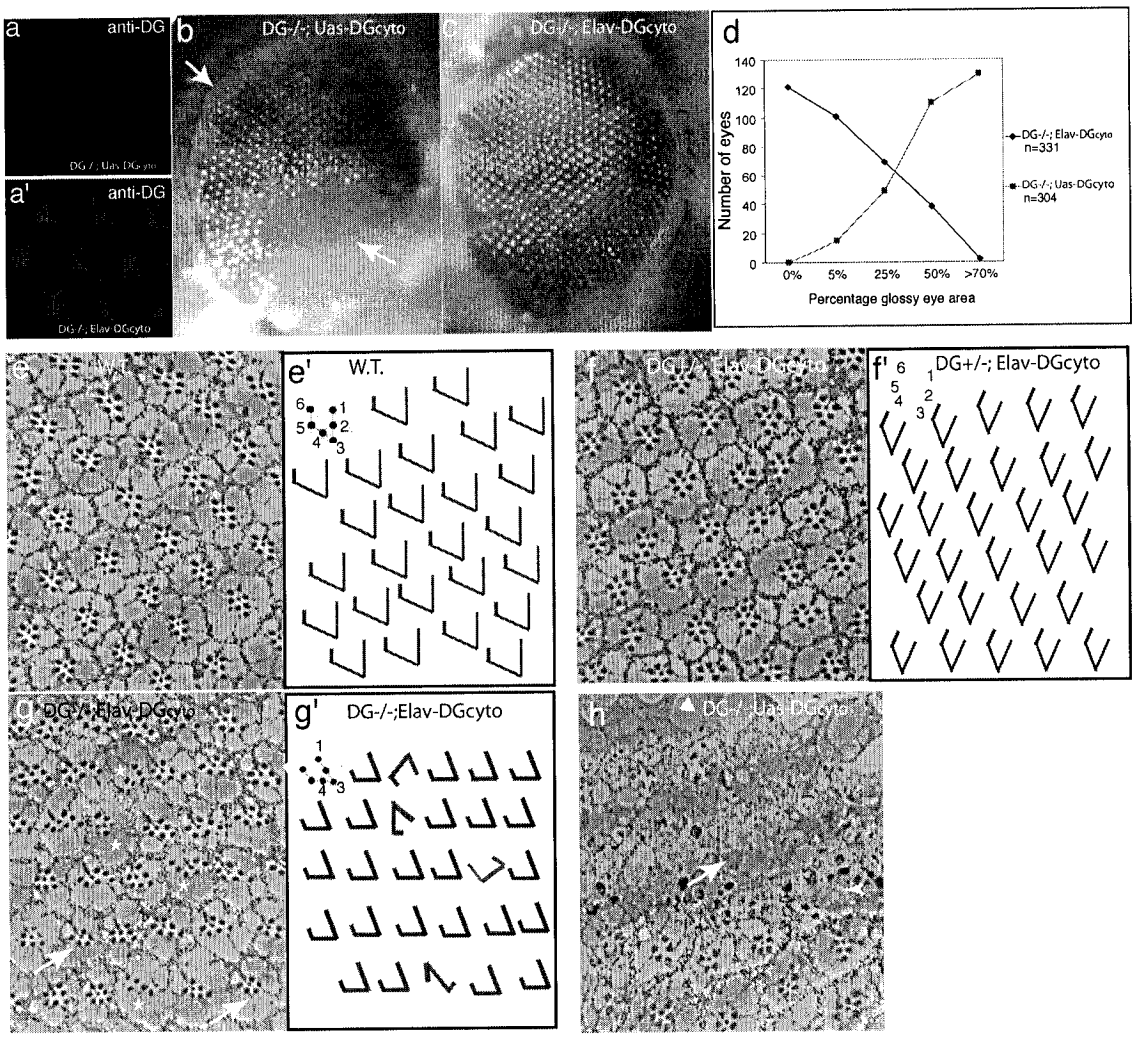

a
anti-DG
a'
anti-DG
b
DG-/-; Uas-DGcyto
c
DG-/-; Elav-DGcyto
d
Number of eyes
Percentage glossy eye area
0%
5%
25%
50%
>70%
DG-/-; Elav-DGcyto n=331
DG-/-; Uas-DGcyto n=304
e'
W.T.
f'
DG+/-; Elav-DGcyto
g'
DG-/-;Elav-DGcyto

Figure 3-9. Expression of cytoplasmic domain of DG in DG-deficient PRCs partially rescues PRC defects and show disrupted PRC planar polarity.

a, PRCs in DG-deficient mosaic eye discs (Fig.3-5) are unlabeled by antisera to DG. **a'**, In DG-deficient mosaic discs containing Elav-gal4/+; Uas-DGcyto/+ (DG-/-; Elav-DGcyto) that drives expression of a construct consisting of the transmembrane and cytoplasmic domain of DG (DG_{cyto}), the DG labeling is evident on the cell surface and in the rhabdomere when the antiserum was used at 1:1000 dilutions. **b**) The external structure of a DG-deficient mosaic eye (genotype: EyFlp; FRT42D, LW+ / FRT42D, DG^{248}, Uas-DG_{cyto}; +/Sb) has an obvious glossy patch of disrupted ommatidia. **c**) In contrast, in mosaic eyes expressing the membrane anchored cytoplasmic domain of DG (genotype: EyFlp; FRT42D, LW+ / FRT42D, DG^{248}, Uas-DG_{cyto}; Elav-gal4/+) the size of the glossy patch in the mosaic eyes is greatly reduced. **d**) Fly eyes were categorized by the percent of the surface that was glossy for these two genotypes. More than 120 of the DG deficient mosaic eyes (**b**) have glossy patches that cover greater than 70% of the eye and none have completely normal eyes. In contrast in DG-deficient mosaic eyes expressing DG_{cyto} (**c**) more than 120 eyes are completely normal and none have large glossy patches. **e-e'**, Cross section of wild type adult fly eyes (**e**) showing normal structure and normal PRC planar polarity with R3 pointing to a uniform direction in this part of eye (**e'**). **f**, Cross section of heterozygous ($DG^{+/-}$) eyes containing DG_{cyto} transgene (genotype: FRT42D, DG^{248}, Uas-DG_{cyto}/+; Elav-gal4/+) show normal PRCs (**f**) and planar polarity (**f'**) similar as the wild type (**e**, **e'**). **g**, DG-deficient eyes containing DG_{cyto} transgene (genotype: EyFlp; FRT42D, LW^{+} / FRT42D, DG^{248}, Uas-DG_{cyto}; Elav-gal4/+) show that the retina is largely preserved and most

ommatidia have seven PRCs (**g**) comparing with the DG-deficient eyes without DG_{cyto} transgene (**h**) (genotype: EyFlp; FRT42D, LW^+ / FRT42D, DG^{248}, Uas-DG_{cyto}; +/Sb). In two ommatidia (**g**), there are only six PRCs (arrows), and some ommatidia have disoriented PRCs, a sign of disrupted planar polarity (asterisks, **g**). **g'**, A drawing shows that PRCs with disrupted planar polarity (red, **g'**) have different direction comparing with the neighboring normal ommatidia (blue, **g'**). The number in **e'**, **f'**and **g'** represents individual R1-R6 and their orientation in one ommatidium. **h**, Control eye that contains Uas-DG_{cyto} but no driver show severe disruption of retina and PRC defects (arrow) similar to observed in DG-deficient retina (Fig. 3-1f'). Arrowhead in **h** points to disrupted PRCs in a mosaic ommatidium indicating an autonomous effect of DG in PRCs. Triangle in **h** points to pseudocones on the edge of retina. Ten eyes were sectioned from each genotype and shown here are the representatives.

Figure 3-10

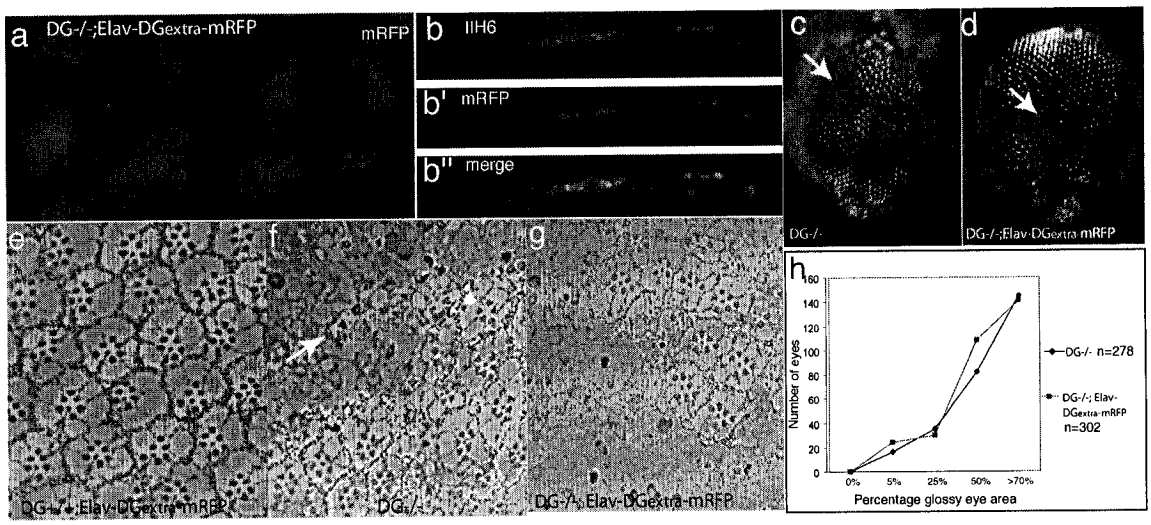

a DG-/-;Elav-DGextra-mRFP
mRFP
b IIH6
b' mRFP
b'' merge
c
d
DG-/-
DG-/-;Elav-DGextra-mRFP
e
f
g
DG-/+;Elav-DGextra-mRFP
DG-/-
DG-/-;Elav-DGextra-mRFP
h
Number of eyes
0
20
40
60
80
100
120
140
160
0%
5%
25%
50%
>70%
Percentage glossy eye area
DG-/- n=278
DG-/-; Elav-DGextra-mRFP n=302

Figure 3-10. Expression of extracellular domain of DG in DG-deficient PRCs does not rescues PRC defects.

a, DG-deficient mosaic discs containing Elav-gal4/+; Uas-DG_{extra}-mRFP (DG-/- ; Elav-DG_{extra}-mRFP) that drives expression of a construct consisting of the transmembrane and extracellular domain of DG fused with mono red fluorescence protein (mRFP) in its C-terminus. The expression of the fusion protein (DG_{extra}-mRFP) was found in the plasma membrane and rhabdomeres of PRCs (**a**) when discs were dissected and directly visualized by fluorescence microscope. **b-b''**, DG_{extra}-mRFP was transfected in DG-null differentiated ES cells and stained with IIH6, an antisera recognizing the functional carbohydrates of vertebrate DG. mRFP (**b**) was found to co-cluster with IIH6 staining (**b'**) in the non-permeabilized cells on the plasma membrane (**b''**). **c-d**, Glossy patches were similarly estimated as percentage of eye area in two kinds of flies. One contains no DG_{extra}-mRFP transgene (**c**) and the other contains the transgene (**d**). In ~300 eyes examined there was no difference in size of glossy patches in flies expressing the extracellular portion of DG compared with DG-deficient mosaic eyes (**h**). Cross sections of eyes from flies containing over-expressed DG_{extra}-mRFP in wild-type (**e**) show normal retina and PRCs; Expression of DG_{extra}-mRFP in DG-deficient mosaic flies (**f**) show similar retina disruption as those in DG-deficient eyes (**f**). Genotypes: **e**, Elav-gal4, Uas-DG_{extra}-mRFP/+; **c** and **f**, EyFlp; FRT42D, LW^+/ FRT42D, DG^{248}; +/Sb; **g** and **d**, EyFlp; FRT42D, LW^+/ FRT42D, DG^{248}; Elav-gal4, Uas-DG_{extra}-mRFP/+. Ten eyes were sectioned on each genotype and shown here are the representatives.

Chapter 4

General Discussion

DG is the core member of the DGC linking the cytoskeleton to the extracellular matrix. DG contains the transmembrane β-DG and the tightly attached extracellular α-DG, both of which are derived from a single polypeptide but post-translationally cleaved and modified by glycosylation. Direct mutations in DG genes have not been found in human most likely due to essential roles of DG in embryonic development (Williamson et al., 1997). Targeted deletion of DG gene in mice, however, leads to muscle degeneration and cell death (Cote et al., 1999; Cohn et al., 2002) resembling those human muscular dystrophies caused by mutations in dystrophin, sarcoglycans and laminin. Mutations in genes encoding several glycosyltransferases that lead to hypo-glycosylation of α-DG, and, thus, loss of binding to extracellular laminin, cause several congenital muscular dystrophies (Barresi and Campbell, 2006; Muntoni et al., 2004). The main cause of the muscle degeneration is due to the disruption of the linkage between the cell and extracellular matrix. However, the detailed mechanisms are unclear.

Using the *Drosophila* visual system as the focus of my work I have found that loss of DG in retina leads to a failure in retinal development that results from cell autonomous defects in PRC growth, organization and a progressive PRC degeneration beginning when photoreceptor cells initiate the early pupal development. Moreover, gene replacement experiments show that the intracellular DG is sufficient to partially rescue the PRC survival and retinal structural defect (Chapter 3) but not the organization of PRCs in an ommatidium.

In contrast to the functional and anatomical defects in DG-deficient retina of *Drosophila* melanogaster, loss of dystrophin isoform Dp260 in human and mouse retina

causes synaptic transmission defects, the disrupted b-wave in the ERG, but the retinal structure is well maintained (Pillers et al., 1990; Pillers et al., 1993, Cibis et al., 1993; Pillers et al., 1995). The DGC, including Dp260 and DG, in retina is localized to the presynaptic terminal of photoreceptor cells in the outer plexiform layer (Koulen et al., 1998; Schmitz and Drenckhahn, 1997b; Ueda et al., 1997; Ueda et al., 1995) and the b-wave defects in DMD patients and mouse models are most likely the consequence of loss of DGC members in the presynaptic terminal (Blake and Kroger, 2000). Although specific deletion of DG in the eye causes aberrant structure and function in *Drosophila* (Chapter 3), recent data showed that mutations of *LARGE* gene in mice that lead to the disruption of α-DG glycosylation cause reduced and delayed b-waves but no gross abnormalities in the eyeball or in the retinal structure (Grewal et al., 2001; Holzfeind et al., 2002). Therefore, DG and its complex members appear to be required presynaptically for normal photoreceptor synaptic function; however, the mechanism for this requirement is unknown. In chapter 2, using proteomics methods a novel DG-associated protein, the GTPase dynamin 1, was found to directly associate with β-DG in the brain and the retina where they are colocalized in the outer plexiform layer (Chapter 2). Dynamin is the well-known protein required for vesicle endocytosis and we showed that loss of DG affects dynamin-mediated transferrin uptake in heterologous cells (Chapter 2). These data indicate that DG may regulate synaptic vesicle endocytosis at the photoreceptor terminals and the disrupted b-wave in the DMD/BMD patients may be caused by failure in this regulation as the consequence of DG disruption in photoreceptor terminal of muscular dystrophy patients.

DG Distribution

DG has a very broad and complicated distribution dependant on the tissue and cell type. DG is concentrated at postsynaptic sites of peripheral (Gingras and Ferns, 2001; Leschziner et al., 2000; Ohlendieck et al., 1991b) (Cohen et al., 1995) and inhibitory central synapses (Brunig et al., 2002; Levi et al., 2002), and is uniformly localized at the extra-synaptic sarcolemma (Ohlendieck et al., 1991b), neuronal cell bodies and glial endfeet around the blood vessels (Tian et al., 1996). In the vertebrate retina, DG is expressed at the outer plexiform layer, the inner limiting membrane, around the blood vesicles (Fig 2-5B, chapter 2), and at the retinal pigment epithelium during development (Lunardi et al., 2006; Ueda et al., 2000). As presented previously, DG is colocalized with dynamin 1 in the outer plexiform layer and biochemically DG and dynamin 1 can be co-immunoprecipitated from retina extracts. Dynamin is widely distributed in all kinds of synapses and dynamin 1 is localized presynaptically (De Camilli et al., 1995; Praefcke and McMahon, 2004; Warnock et al., 1997). In retina dynamin is expressed in the outer and inner plexiform layers (Fig. 2-5B), both of which are synaptic layers containing glutamatergic synapses (Sterling and Matthews, 2005). But, only in the outer plexiform layer is DG colocalized with dynamin 1, which is consistent with the biochemical results in brain that association of DG and dynamin 1 is not in 1:1 stoichiometry (Fig. 2-2), implying that DG is only expressed and colocalized with dynamin 1 at subpopulations of synapses in the CNS including the ribbon synapse of the retina.

DG has been shown to express in the actin-enriched microvilli in many cell types. In several vertebrate cell lines DG is distributed at the microvilli, the actin-

enriched membrane tips (Spence et al., 2004) as well as in the microvilli of Schwann cells at nodes of Ranvier (Occhi et al., 2005; Saito et al., 2003). In the cultured ES cells, DG is codistributed with dynamin 1 and cortactin in the membrane ruffles (Fig.2-6), an actin-riched membrane protrusion (Faix and Rottner, 2005). In *Drosophila* PRCs, DG is expressed at the apical tips, the microvilli, of PRCs at the 3rd instar larvae (Fig. 3-5a-c). The microvilli at the apical larval discs are the membrane-enriched structure of cells that project into the extracellular matrix (Banerjee et al., 1987), and, thus, DG could connect PRCs to this matrix. In the pupal development DG continues to be expressed at the apical membrane, in rhabdomeres, facing the extracellular molecules in the inter-rhabdomere space (Figure 6, chapter 3).

DG is also localized at the basal side of epithelial cells opposing to the BM in both vertebrate and *Drosophila* embryos (Deng et al., 2003; Durbeej et al., 1995). In the 3rd instar larvae eye disc, DG is also distributed at the basal subdomain of *Drosophila* PRCs in a "ring" (Fig. 3-5) and continues the pattern in the basal surface of PRCs in pupal development opposing the surrounding ommatidial accessory cells (Fig. 3-5). Because this "ring" surrounds and partially overlaps with neuron and axon specific 24B10 staining (Fig. 3-5f) in the peripheral but not in the central axonal (Fg. 3-5f), we conclude that DG is expressed at the basal domain of PRC. This basal localization is the site that PRC makes contact with the extracellular matrix or possibly with the accessory cells (Longley and Ready, 1995). Ultrastructurally, whether DG connects the basal membrane of PRC to the basement membrane or to the accessory cells remains to be answered. Nevertheless, DG displays a polarized distribution at the both apical and basal sides of PRCs, where PRCs are in contact with the extracellular

matrix or to other cells. In addition, we suggested in Chapter 3 that *Drosophila* DG contains the same functional carbohydrates as the vertebrate DG that are required to bind to the G domain-containing molecules in the extracellular matrix or in other cells, therefore, these carbohydrates could be the units linking PRCs to extracellular matrix or to other cells.

DG regulate endocytosis

As mentioned previously, β-DG forms an intracellular complex with Dp260, β-dystrobrevin, syntrophin in the periactive zone of photoreceptor terminal (Blank et a., 1999; Claudepierre et al., 1999; Dalloz etal., 2001; Jastrow et al., 2006), where it connected to α-DG that could in turn bind to synaptic agrin in the cleft (Koulen et al., 1999). As shown in chapter 2, β-DG is also associated and colocalized with dynamin in the presynaptic terminals (Fig. 2-5). Thus, dynamin could be anchored through DG complex to the extracellular matrix in this ribbon synapse although direct evidence is lacking.

Dynamin is required for endocytosis and a temperature-sensitive dynamin mutant called *shibire* in *Drosophila* leads to a severe impairment in pre-synaptic transmission in the restrictive temperature due to failure in synaptic vesicle "pinching off" and depletion of vesicles (Koenig and Ikeda, 1989; Poodry and Edgar, 1979; Poodry et al., 1973; van der Bliek and Meyerowitz, 1991). The function of dynamin in endocytosis results from its ability to self-assemble into rings and spirals on the "neck" of vesicle and its ability to hydrolyze guanosine triphosphate (GTP) (De Camilli et al., 1995). The latter is dependant on the self-assembly (Warnock et al., 1996), the amino

terminal GTPase domain and the GTP effector domain (GED) of the dynamin molecule (Muhlberg et al., 1997; Sever et al., 1999). In our transferrin-uptake assay, we showed that DG-deficient cells have a two-fold increase in the transferrin-uptake compared to that in wild-type cells (Fig. 2-7, 8, 9), which could be reversed by re-introduction of DG (Fig. 2-9). Thus, DG appears to negatively control transferrin-uptake in normal conditions. In DG-deficient cells, we could not observe change in dynamin distribution under immunofluorescence microscopy (Zhan and Carbonetto, unpublished observation) due to the abundant presence of dynamin in the cytoplasm. Nevertheless, DG could more likely affect dynamin functionally in addition to serving as an anchor site. For example, the direct interaction of DG with dynamin (Fig. 2-4) could limit the ability of dynamin to self-assemble or hydrolyze GTP. Therefore, future experiments would investigate the domain requirement for interaction of DG and dynamin and test its impact in the assembly and GTP hydrolysis.

Transferrin-uptake utilizes many of the same molecules, including dynamin and clathrin, and shares many basic endocytic mechanisms as the synaptic vesicle endocytosis although it is not tightly regulated by synaptic transmission (Jarousse and Kelly, 2001). DG, by coupling dynamin to the DGC scaffold and the extracellular matrix at the peri-active zone where vesicle endocytosis often occurs (Jarousse and Kelly, 2001), could regulate synaptic vesicle endocytosis at the ribbon synapse of outer plexiform layer. This hypothesis can be tested in animal models. As suggested from chapter 3, DG is essential for development of the whole eye, so it is very difficult to utilize DG-deficient animals to analyze the synaptic function. However, *myd* mice have a defective glycosylation of α-DG and the synaptic transmission defect is seen in

disrupted b-waves whereas the reported retinal structure is conserved (Grewal et al., 2001; Holzfeind et al., 2002), therefore, it would be interesting to determine if the defective glycosylation of α-DG affects the interaction of DG and dynamin, and, thus, a changed endocytosis. On the other hand, DMD/BMD patients and *mdx*^{3cv} mice that have normal retinal structures show disrupted b-waves and reduced DG distributions in the outer plexiform layer (D'Souza et al., 1995; Kameya et al., 1997; Pillers, 1999; Pillers et al., 1999), thus, future experiments could determine whether increased endocytosis occurs in the photoreceptor terminals of *mdx*^{3cv} mice.

Possible mechanisms of DG function in the eye

Using *Drosophila* eye as a model loss-of-function, gain-of-function studies as well as rescue experiment suggested a possible mechanism of DG function. Loss of DG leads to defective PRC contact with its ligands in the extracellular matrix or in other cells and results in simultaneous disruptions of cell polarity and intracellular DG-mediated signaling that appears essential for the cell growth and survival. Below, I first discuss the possible intracellular mechanisms that could be involved in the cell growth and death, and then I discuss if dynamin-mediated endocytosis could contribute to defects in DG-deficient eye.

Cell growth is a distinct process from proliferation especially in postmitotic cells, and size control in cell mass and dimensions is linked to protein synthesis (Kozma and Thomas, 2002; Neufeld et al., 1998; Stocker and Hafen, 2000). DG is known to associate directly with an adaptor protein Grb2 in both muscle and neuron (Yang et al., 1995; Chapter 2), thus, loss of DG may affect Grb2-mediated, Sos (son of

sevenless)-induced activation of the small GTPase Ras in the growth factor receptor-Ras-MAPK-Myc pathway, a known pathway leading to protein synthesis and cell growth (Kozma and Thomas, 2002; Neufeld et al., 1998; Stocker and Hafen, 2000). Loss-of-function mutations in Ras cause poor cell growth with small cell size and over-expression leads to increased cell size with normal cell cycle rate (Prober and Edgar, 2000). Moreover, Ras also participates in the PI3K (phosphatidylinositol 3-kinase)-Akt/PKB (protein kinase B)-mTOR (mammalian target of rapamycin) pathway, a key regulator of cell size in many species and a suppressor of apoptosis (Cantley, 2002; Franke et al., 2003; Schmelzle and Hall, 2000; Stocker and Hafen, 2000). Therefore, in future experiments it would be interesting to determine how DG could affect the Ras and the downstream molecules in the two signaling pathways. On the other hand, independently from my *Drosophila* studies, Sgambato et al. (2006) recently showed that suppression of DG in cultured dividing epithelial cells leads to a growth arrest that is caused by accumulation of cells in S-phase of the cell cycle. Moreover, they showed that down-regulation of DG is accompanied by down-regulation of PTEN, upregulation of AKT, and increased apoptosis (Sgambato et al., 2006). Therefore, it would be very interesting to further investigate whether intracellular DG is coupled to cell cycle or cell growth in vivo using *Drosophila* as a model.

Could dynamin play a role in DG-deficiency induced defects in the eye? In addition to the requirement for endocytosis in synapses, flies carrying the temperature-sensitive allele of dynamin, *shibire*, when heated to inactivate *shirbire* during early embryo stage, die (Poodry et al., 1973). Heating for 3-6 hours during the period from 48 hrs before to 3 hrs after pupariation results in a scar in the eye while the remaining

areas are normal, which was said to reflect a disruption of the morphogenic furrow (Deitcher, 2002; Poodry et al., 1973). Longer heating (12 hrs) in this period gives a roughened small eye resembling *eyeless* (Poodry et al., 1973), a master gene controlling eye development (Halder et al., 1995). It was found that Notch acts upstream of *eyeless* (Kumar and Moses, 2001a) and notch and delta signaling requires endocytosis (Parks et al., 2000), which could explain why *shibire* resembles *eyeless*. These data demonstrate that dynamin is critical for eye development in the 3rd instar larvae stage. Unfortunately, it is not documented what are the phenotypes if the *shibire* flies are heated after pupation starts, during which DG plays a critical function. In other instances, when endocytosis is blocked by over-expression of a dominant-negative dynamin that is defective in GTP hydrolysis, heterozygous eyes show a rough eye phenotype with disorganized morphology, but the external ommatidia or lens exist (Chang et al., 2002). Moreover, when endocytosis is blocked in homozygous mutant for Hsc70, a protein required for clathrin uncoating, the photoreceptors and ommatidia are essentially normal (Chang et al., 2002). Thus, defective endocytosis appears to lead to less severe phenotypes than DG homozygous. But, since intracellular portion of DG has multiple interacting proteins, defects in dynamin-mediated endocytosis could still contribute to the phenotypes we observed in DG-deficient retina. For example, dynamin is required for Grb2-mediated EGF receptor internalization (Huang et al., 2004; Vieira et al., 1996; Wang and Moran, 1996; Yamazaki et al., 2002), loss of DG could lead to higher rate of EGF receptor internalization, thus, affecting the PRC growth and survival.

From *Drosophila* to human muscular dystrophy

Congenital muscular dystrophy type 1D is caused by mutations in LARGE gene, and its animal model is *myd* mice. LARGE directly participates in synthesis of carbohydrates of α-DG and transfer of LARGE into cells isolated from FCMD, MEB and WWS patients can functionally restore the defective glycosylation of α-DG (Kanagawa et al., 2004; Barresi et al., 2004). *myd* mice have similar phenotypes in brain as the DG-null mice, which also resemble the brain defects in FCMD, MEB and WWS patients (Moore et al., 2002; Michele et al., 2002). These brain data suggest that α-DG or its carbohydrate chains is functionally essential for the DG molecule. However, in the retina the situation appears more complex. In FCMD, MEB and WWS patients, retina show severe defects including disorganized retinal structure, retinal degeneration and blindness (Fahnehjelm et al., 2001; Hino et al., 2001; Vervoort et al., 2004; Zervos et al., 2002). In fukutin-null mosaic mice, there are complete loss of ERG, retinal misfold and lens defects (Takeda et al., 2003). However, in the well-characterized *myd* mice, retinae show mainly b-wave defects without defective neuron migration or misfolding (Grewal et al., 2001; Holzfiend et al., 2002), and only mild structural defects have been recently reported in the outer plexiform layer and the inner limiting membrane (Lee et al., 2005). In our DG-deficient *Drosophila* eye, the retinal phenotypes are quite severe and more resemble those in fukutin-null mice eyes or those in MEB patients with retina degeneration and early blindness (Fahnehjelm et al., 2001; Vervoort et al., 2004), but not the *myd* mice. Since LARGE protein is known to directly bind to α-DG and participate in synthesis of the functional carbohydrates, phenotypes in *myd* mice more likely demonstrate the function of α-DG. Therefore, if the severe

phenotypes in *Drosophila* DG-deficient eyes are compared to the milder phenotypes in *myd* mice eye, one would conclude that, in retina, α-DG only represents part of the DG functions, which appears different from the brain. Indeed, in our rescue experiments, our data show that extracellular and intracellular DG play distinct functions, while the extracellular DG appears responsible for PRC organization or planar polarity, the intracellular DG largely rescues the DG-deficient eye phenotype but extracellular DG could not. However, we should consider the retina data very carefully because the DG-deficient phenotypes in *Drosophila* eye are in some degree similar to those in fukutin-null mosaic mice and in the FCMD, MEB and WWS patients. More experiments aiming to understand how the individual mutant glycosyltransferase in these congenital muscular dystrophies affects glycosylation of α-DG or other protein(s) should be address. And, due to the huge difference in retina structures between *Drosophila* and mammals, a mouse model with eye-specific DG deletion could be more decisive to answer some of these questions.

In skeletal muscle, loss of DG cause muscle degeneration, without defects in the extrasynaptic basement membrane (Cote et al., 1999), and apoptotic cell death appear obvious in DG-null cells (Montanaro et al., 1999; Li et al., 2002). This resembles the phenotypes in DG-deficent retina of *Drosophila*. Therefore, it is possible that DG deficiency causes muscle disorientation and growth defect. Kanagawa et al., (2004) showed that DG mutant constructs containing no mucin-like region are not sufficient to rescue the DG-null skeletal muscle phenotypes, my data imply that, unlike in retina, loss of ligand binding to the extracellular matrix could lead to autonomous disorientation of muscle cells, which under the constant contraction challenge is

sufficient to cause muscle fiber damage even if the intracellular functions of DG is conserved. In addition to that, my data also suggest an important function for β-DG and its associated complex in contribution to the DG-null dystrophy. I propose that although β-DG in muscle is not sufficient to rescue the phenotype, loss of dystrophin, sarcoglycans/sarcospan or other signals from the sarcolemma in response to DG deficiency contribute to DG-null muscular dystrophy.

Conclusion

My experiments demonstrate two novel DG functions in the nervous system. First, they show DG interacts directly with the GTPase dynamin 1 in brain and retina and regulates dynamin-mediated endocytosis. Thus, it suggests one of the mechanisms in DG and dystrophin-related muscular dystrophies could be the disruption of synaptic transmission in the nervous system, especially in the retina where defective transmission has been known for decades. Second, using *Drosophila* as the model, show that DG is autonomously required for PRC development, which in turn causes severe disruption of whole eye, and provide more detail analysis of DG functions, thus, it can serve as a great model to further investigate the molecular mechanisms of muscular dystrophies and DG biology in general.

Chapter 5

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Appendix

Research Compliance Certificate

A part of this thesis research is involved in using rodent as the research subject. According to the rule set by Faculty of Graduate and Postdoctoral Studies, McGill University, the following page is attached to this thesis and shows that the animal usage is approved by McGill University and the research involved in using animals was carried out according to the approved "Animal Use Protocol".