Determination of Genetic Interactions Required for Dystrophin-Dystroglycan Function and Regulation in a *Drosophila* Model of Muscular Dystrophy

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1. Zusammenfassung

Im Menschen hängen genetisch diverse Formen von Muskeldystrophie (MD) mit einer Schädigung des Dystrophin-Glykoprotein Komplexes (DGC) zusammen. Der DGC besteht aus Dystrophin, Dystroglykanen, Sarkoglykanen, Sarkospan, Syntrophinen und Dystrobrevin. Die Charakterisierung des *Drosophila melanogaster* DGC zeigt, dass die Fliege alle wesentlichen Bestandteile des DGC aufweist, jedoch in wesentlich geringerer Komplexität. Mutationen in den *Dystrophin* (*Dys*), *Dystroglykan* (*Dg*) und *Sarkoglykan* Genen der Fliege verursachen Symptome ähnlich denen beim Menschen: verminderte Mobilität, verkürzte Lebenserwartung sowie altersabhängige Muskeldegeneration. Des Weiteren beeinflussen Mutationen in *Dys* und *Dg* die Freisetzung von Neurotransmittern in der neuromuskulären Endplatte und rufen axonale Migrationsdefekte hervor. Weiterhin sind *Dys* und *Dg* wichtig bei der Festlegung der Zellpolarität in Keimbahn- und Follikelzellen. Da eine Störung der Interaktion zwischen Bestandteilen des DGC im Menschen Muskeldystrophie hervorruft, haben wir uns entschieden die Wechselwirkung zwischen den beiden DGC Hauptkomponenten, DYS und DG, genauer zu untersuchen. Der C-Terminus von DG enthält einige Bindungsstellen für SH3-, SH2- und WW-Domänen. Es wurde gezeigt, dass das C-terminale PPxY-Motiv in DG die WW-Domäne von DYS binden kann. Unsere *in vitro* Studien zeigen, dass beide PPxY-Motive in *Drosophila* DG, WWbsI und WWbsII, an die WW-Domäne in DYS binden können und dass eine Phosophorylierung des Tyrosins in jedem der PPxY-Motive zur Aufhebung der DG-DYS Bindung führt. Dies deutet auf einen möglichen Regulationsmechanismus hin. Außerdem haben wir gezeigt, dass beide WW-Bindungsstellen erforderlich sind um die DG Funktion bei der Festlegung der Polarität der Eizelle aufrecht zu erhalten. Der Vergleich der Sequenzen von WW-Bindungsstellen in 12 *Drosophila* Spezies und dem Menschen zeigt außerdem, dass diese Stellen hochgradig konserviert sind. Daher schlagen wir vor, dass das Vorhandensein von zwei WW-Bindungsstellen in DG die essentielle Bindung zwischen DG und DYS sicherstellt und eventuell eine weitere Regulationsebene für die Zytoskelettumlagerungen darstellen könnte, an denen der DGC beteiligt ist. Um neue Komponenten zu finden, die mit dem DGC interagieren oder seine Funktion regulieren, haben wir ein *Drosophila* Modell für MD benutzt und genetische „Screens“ durchgeführt bei denen wir dominante Modulatoren von *Dg* und *Dys* Mutationsphänotypen identifiziert haben. Im groß angelegten „Primär-Screen“ haben wir nach Modifikationen von einfach zu bewertenden Phänotypen gesucht, wie zum Beispiel der Veränderung der posterioren Flügel-Querader. Mit dieser Vorhergehensweise haben wir Modulatoren gefunden, die zu unterschiedlichen funktionellen Gruppen gehören: Gene die Funktionen im Muskelgewebe, bei neuronaler/zellulärer Migration oder als Motorproteine besitzen, sowie Komponenten des Zytoskeletts und Mitglieder der TGF-β-, EGFR- und Notch-Signalwege. Um die Mechanismen der Muskeldegeneration aufzuklären die durch eine verminderte *Dys* und *Dg* Funktion verursacht werden, wurde ein „Sekundär-Screen“ in Muskelgewebe durchgeführt. Dabei wurden Modulatoren identifiziert, die den Phänotyp der Muskeldegeneration jeweils unterdrückten oder verstärkten. Weiterführende Untersuchungen der identifizierten Komponenten zeigten, dass diese entweder im Muskel- oder Nervengewebe benötigt werden, wo

dann spezifische Interaktionen mit Bestandteilen des DGC auftreten können. Diese neuen Komponenten, die zu DGC abhängiger Muskelerhaltung beitragen, werden gegenwärtig analysiert.

1. Summary

In humans genetically diverse forms of muscular dystrophy (MD) are associated with a disrupted Dystrophin-Glycoprotein Complex (DGC). The DGC consists of dystrophin, dystroglycans, sarcoglycans, sarcospan, syntrophins and dystrobrevin. Characterization of the *Drosophila melanogaster* DGC shows that the fly retains all essential components of DGC, but with substantially less diversity. Similarly to humans, mutations in the fly *Dystrophin* (*Dys*), *Dystroglycan* (*Dg*) and *Sarcoglycan* genes cause reduced mobility, shortened lifespan and age-dependent muscle degeneration. In addition, mutations in *Dys* and *Dg* affect neurotransmitter release in the neuromuscular junctions and cause the axon migration defects. Furthermore, Dys and Dg are important for the establishment of cell polarity. Since disruption of the connection between the components of DGC causes muscular dystrophy in humans, I decided to study the interaction between two major components of the DGC, Dys and Dg, in more detail. The C-terminal end of Dg contains a number of putative SH3, SH2 and WW domain binding sites. The most C-terminal PPxY motif has been established as a binding site for Dys WW-domain. Now my *in vitro* studies indicate that both *Drosophila* Dg PPxY motifs, WWbsI and WWbsII can bind to the WW domain of Dys and phosphorylation of the tyrosine of each PPxY motif abolishes Dg-Dys binding, suggesting a possible regulatory mechanism. Moreover, I have shown that both WW binding sites are required for maintaining the Dg function *in vivo* for the establishment of oocyte polarity. Additionally, the sequence comparison of WW binding sites in 12 species of *Drosophila*, as well as in humans, reveal a high level of conservation at these sites. Therefore, it has been proposed that the presence of the two WW binding sites in Dg secures the essential interaction between Dg and Dys and might further provide additional regulation of the cytoskeletal rearrangements involving DGC. To reveal new components that interact with DGC or regulate its function I used a *Drosophila* model for MD and performed genetic screens to identify dominant modifiers of *Dg* and *Dys* related mutant phenotypes. In the primary large scale screen I have looked for modifications of easily score-able phenotype such as alterations in the posterior crossvein. Using this screening strategy I have found modifiers that belong to different functional groups: genes involved in the muscle function, neuronal/cell migration and motor function factors as well as cytoskeletal components and members of the TGF-beta, EGFR and Notch signaling pathways. In order to shed light on the mechanisms of muscle degeneration caused by *Dys* and *Dg* down-regulation the secondary screen in muscle tissue was performed. As a result, the modifiers that suppressed/enhanced muscle degeneration phenotype were identified. Further studies of identified components showed their requirement in either muscle or nervous tissue, where specific interaction with the DGC components may occur. The novel components that contribute to DGC-dependent muscle maintenance are being analyzed.

2. Introduction

2.1. Muscular dystrophies involving the Dystrophin-Glycoprotein Complex

Muscular dystrophy is a general term that describes a group of inherited and gradually progressing myogenic disorders. Genetically, the pattern of inheritance can be X-linked recessive as in Duchenne or Becker muscular dystrophies (DMD/BMD), autosomal dominant as in limb-girdle muscular dystrophy type 1 (LGMD type 1), or autosomal recessive as in limb-girdle muscular dystrophy type 2 (LGMD type 2) (Campbell, 1995; Groh et al., 2009; Moore et al., 2008; Straub and Campbell, 1997). DMD is a severe progressive muscle-wasting disease affecting approximately 1 out of 3500 males (Blake et al., 2002). Patients with DMD have a childhood onset phenotype and die before their early twenties as a result of either respiratory or cardiac failure, whereas patients with BMD have moderate weakness in adulthood and may have normal lifespan (Campbell, 1995; Wagner et al., 2007). The limb-girdle muscular dystrophies initially involve the shoulder and pelvic girdle muscles. Moreover, muscular dystrophies may or may not be associated with cardiomyopathy (Bushby, 1999). One of the most important advances in understanding the molecular genetics of neuromuscular diseases has been the cloning of the gene encoding dystrophin, the protein absent in muscle of DMD patients (reviewed in (Davies et al., 1983)). In the last few years the role of the dystrophin in skeletal muscle has been studied, and several dystrophin-associated proteins have been identified, which are the members of Dystrophin-Glycoprotein Complex (DGC). The DGC is a large complex of membrane-associated proteins that is critical for integrity of skeletal muscle fibers. This complex consists of dystrophin, the dystroglycans (α and β), the sarcoglycans (α, β, γ and δ), sarcospan, the syntrophins (α1, β1, β2, γ1- and γ2) and α-dystrobrevin (Figure 1, (Durbeej and Campbell, 2002)). Components of the DGC are now being characterized and evidence is beginning to indicate that proteins of this complex could be responsible for other forms of muscular dystrophy (Figure 1). Members of the dystrophin protein family perform a critical but incompletely characterized role in the maintenance of membrane-associated complexes at points of intercellular contact in many vertebrate cell types. Dystrophin is a large (427 kDa) protein, which is highly conserved in vertebrates. Its largest isoform comprises an N-terminal domain, which binds to F-actin, 24 spectrin-like repeats and a characteristic C-terminal set of domains. Dystrophin is functionally similar to cytolinkers, as it links the multiple components of the cellular cytoskeleton to the transmembrane dystroglycan complex. The direct link between dystrophin and microtubules

(MTs) was reported recently (Prins et al., 2009). A number of dystrophin-related vertebrate proteins, whose function is even less clear, have been described (reviewed in (Ilsley et al., 2002)).

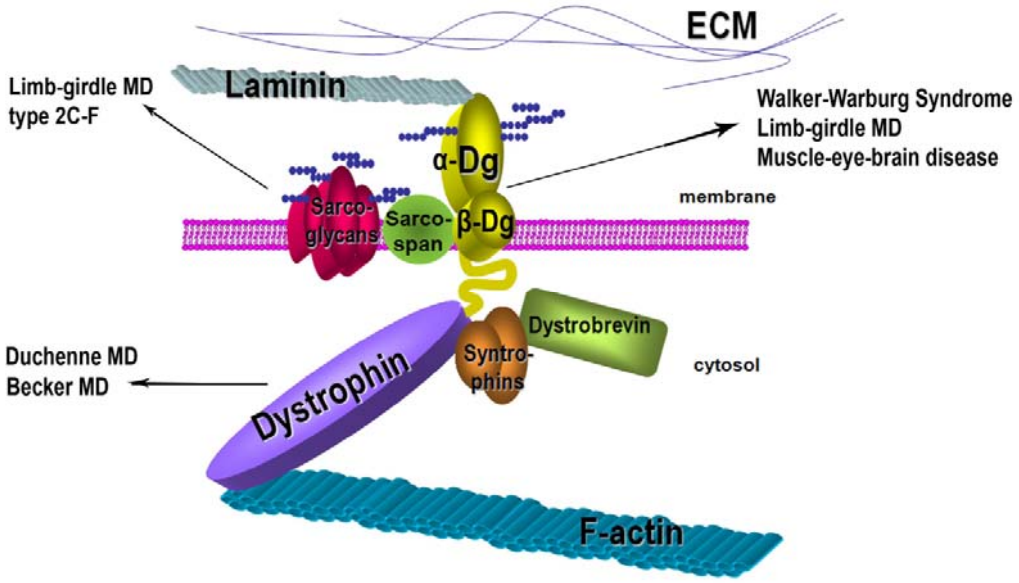

Figure 1. Structure of Dystrophin-Glycoprotein Complex. Arrows indicate disorders caused by the disruption in corresponding component.

Utrophin is very similar in overall structure to dystrophin itself, and its disruption in mice causes subtle abnormalities of the neuromuscular junction. Dystrophin-related protein 2 (DRP 2) resembles certain small (approximately 110 kDa) isoforms of dystrophin and utrophin in that it comprises the last two spectrin-like repeats and the C-terminal region. Dystrophin, utrophin and DRP 2 are expressed in distinct, but partially overlapping regions of the vertebrate body. A number of motifs are recognizable in the C-terminal region of this family of proteins. The WW domain has been implicated in mediating the interaction between dystrophin and the transmembrane protein β-dystroglycan. Motifs in the remaining C-terminal sequences comprise of two EF hands, a ZZ domain with the ability to bind Zn^{2+}, and two leucine heptads, which form a region involved in binding members of the syntrophin family of PDZ-domain containing proteins, which in turn bind to neuronal nitric oxide synthase (reviewed in (Ilsley et al., 2002)).

Another major component of the DGC is dystroglycan. Interestingly, the expression of dystroglycan is ubiquitous and not simply restricted to muscle cells. Besides muscle, dystroglycan is expressed at high levels in both, developing and adult tissues. Typically dystroglycan is present in the cell types that adjoin basal membranes such as epithelial and neural tissues (Durbeej and

Campbell, 2002). Dystroglycan provides a continuous link between laminin-2 in the extracellular matrix and dystrophin that is attached to the intracellular cytoskeleton. Dystroglycan is synthesized as a precursor protein and later it is proteolytically cleaved into two interacting subunits, α- and β-dystroglycan. α-dystroglycan is a heavily glycosylated membrane protein that interacts directly with laminin-2; in contrast, β-dystroglycan is an integral membrane glycoprotein that can bind both dystrophin and utrophin. The dystrophin-anchoring site on β-dystroglycan is localized to the extreme C-terminus at amino acids 880-895 (Huang et al., 2000). The knock-down of dystroglycan in mouse embryos leads to early embryonic lethality prior to gastrulation, long before any muscle has formed (reviewed in (Durbeej and Campbell, 2002)). Therefore it is not possible to analyze the consequences of dystroglycan deficiency in muscle. To overcome this Carbonetto and co-workers generated chimeric mice, lacking dystroglycan in skeletal muscles (Cote et al., 1999). Interestingly, these mice develop progressive muscle pathology and have disrupted neuromuscular junctions. Thus, dystroglycan is necessary for myofiber stability and differentiation.

Recently a group of human muscular dystrophies have been demonstrated to have defective dystroglycan glycosylation and are hence termed dystroglycanopathies. Thus far, six dystroglycanopathy genes have been identified: *POMT1, POMT2, POMGnT1, fukutin, FKRP* and *LARGE.* Although a paralogue of LARGE, (LARGE2) has been identified and is likely to be involved in dystroglycan glycosylation, no mutations have yet been identified in this gene (Muntoni, 2004). The dystroglycanopathies can be divided into several clinical disorders that range in severity from Walker-Warburg syndrome (WWS), a severe form of congenital muscular dystrophy (CMD), which is also associated with ocular abnormalities and CNS defects, to forms of LGMD that have an onset later in life and have no CNS involvement. Muscle-eye-brain disease (MEB) is a type of CMD associated with loss-of-function mutations in the gene encoding a glycosyltransferase, *POMGnT1* and resulting in eye defects, epilepsy and lissencephaly (smooth brain appearance as a result of abnormal neuronal migration) (reviewed in (Moore et al., 2008)). The mechanistic bases for these disorders are yet to be determined.

The sarcoglycan complex is a group of single-pass transmembrane proteins (α-, β-, γ- and δ-sarcoglycans) that are tightly associated with the sarcospan to form the subcomplex within the DGC (Crosbie et al., 1999). Although the exact function of the sarcoglycan-sarcospan complex is not known, it is well established that mutations in any of α-, β-, γ- and δ-sarcoglycan genes result in the distinct forms of muscular dystrophy now collectively called sarcoglycanopaties (LGMD2C-F) (Groh et al., 2009). The sarcoglycans are primary expressed in muscles and correct expression of sarcoglycans in skeletal muscle is a necessity for their normal function. Furthermore, the proper

expression of sarcoglycans in smooth muscles is important for normal skeletal and cardiac muscle function (Straub et al., 1999).

2.2. The role of DGC in the signal transduction

Muscle contraction results in changing of cell shape and its shortening. Throughout this process, the contractile machinery inside the myofibers must remain intimately connected with the membrane and extracellular matrix. Without this association, movement will be improperly transmitted and myocytes would cause damage to their membranes. The Dystrophin-Glycoprotein Complex provides a strong mechanical link from the intracellular cytoskeleton to the extracellular matrix. Dystroglycan is an important cell adhesion receptor linking the actin cytoskeleton via dystrophin to laminin in the extracellular matrix. Nowadays the notion of dystroglycan as a simple laminin-binding receptor is increasingly being challenged. New roles and new binding partners are continually emerging. Recent structural advances have provided exciting new insights into the precise molecular interactions between Dystroglycan and other key components of the DGC (reviewed in (Winder, 2001)). The latest studies on C_2C_{12} muscle cell culture showed nuclear and nuclear envelope localization of dystrophin and dystrophin-associated proteins (dystroglycan, the sarcoglycans, syntrophins and dystrobrevin) suggesting their new role in the nuclear envelope associated function, such as nuclear structure integrity and underplaying of regulatory nuclear processes (Gonzalez-Ramirez et al., 2008). Coupled with increasing understanding of DGC function at the molecular level, we are finally beginning to probe the complexities of the complex, not only in disease, but in development, adhesion and signaling. Evidence for a signaling function of the DGC has emerged from the identification of neuronal nitric oxide synthase (nNOS), associated with the DGC via dystrobrevin and loss of nNOS from the sarcolemma in DMD (reviewed in (Bredt, 1999)). The association of the DGC of skeletal muscle and brain with voltage-gated Na+ channels and acethylcholine receptors at the NMJ is indirectly mediated by dystroglycan (reviewed in (Winder, 2001)). Dystroglycan interacts with the cysteine-rich domain of dystrophin through the carboxyl-terminus, where it contains a number of putative binding motifs: two WW binding sites, two SH3 (Src homology 3) binding sites and three SH2 (Src homology 2) binding sites. *In vitro* studies have suggested that the interaction between two major components of the DGC, Dystroglycan and Dystrophin, is mediated by the most C-terminal WW domain binding motif, PPxY, on Dystroglycan and Dystrophin WW and EF-hand domains (Blake et al., 2002; Huang et al., 2000; Ilsley et al., 2001; Jung et al., 1995; Rentschler et al., 1999). However, the role of second WW binding site on the C-terminus of Dystroglycan has never been addressed. *In vitro* experiments

have also shown that when the tyrosine of the PPxY motif is phosphorylated, the binding between Dg and Dys is abolished (Ilsley et al., 2001; Sotgia et al., 2001). This suggests a potential mechanism to regulate the Dg and Dys interaction in a tyrosine phosphorylation-dependent manner. In the search for a potential regulator, recent studies have revealed several proteins that interact with Dystroglycan. Both the Grb2 (growth factor receptor-bound protein 2) adaptor protein, as well as MEK1, and ERK of the Ras-Raf mitogen-activated protein kinase (MAPK) cascade have been shown to interact, *in vitro* and *in vivo*, with the C-terminus of Dg (Spence et al., 2004; Yang et al., 1995) However, Dystroglycan appears to be only an anchor for MEK1 and ERK rather than a substrate (Spence et al., 2004), and Dg might not have a direct involvement in this signaling pathway. Independently, recent work has revealed that laminin and Dystroglycan-dependent phosphorylation of Syntrophin affects the Grb2-SOS1-Rac1-PAK1-c-Jun N-terminal kinase (JNK) pathway and ultimately results in the phosphorylation of c-Jun on Ser-65 (Zhou et al., 2007). Moreover, the latest studies demonstrate the existence of a specific link between the laminin-DGC-Gβγ-PI3K-Akt signaling in skeletal muscle (Xiong et al., 2009). Gβγ binding activates PI3K/Akt signaling in laminin-dependent manner, and phosphorylation of Akt and GSK results from activation of PI3K. This laminin-DGC-Gβγ-PI3K-Akt signaling is likely to be important on the pathogenesis of muscular dystrophies (Xiong et al., 2009). Thus, although studies suggest a clear role for Dg in signaling, the regulation of Dg by signaling and the specific regions of the Dg C-terminus involved in this process are unknown. An understanding of the relevant signal transduction and interactions between these pathways in the skeletal muscle cell will facilitate efforts to elucidate the pathogenesis of muscular dystrophies.

2.3. The *Drosophila* model for DGC-dependent muscular dystrophy

A number of animal models have been established for Duchenne muscular dystrophy, but severe muscular dystrophy in the absence of *dystrophin* alone has only been observed in dogs (reviewed in (Collins and Morgan, 2003)). Mice and *C.elegans* exhibit muscle degeneration in the absence of *dystrophin* when also lacking *myoD* (Gieseler et al., 2000; Megeney et al., 1996), a gene required for muscle regeneration. The differences in severity of muscle degeneration in the diverse animal models can most likely be explained by the different strategies organisms have adopted to regenerate muscle after damage. In addition, the *mdx* mouse is a very poor model of the DMD phenotype**.** It is well recognized that the *mdx* mouse does not model the progressive and severe weakness, joint contractures, respiratory failure, and cardiomyopathy that are hallmarks of the

human disease (reviewed in (Wagner et al., 2007)). All above facts make it difficult to study the mechanisms of MD utilizing such models, hence development of a new remarkably good model for genetic manipulations remains an open task. Fly models have been generated for a wide spectrum of human diseases such as developmental disorders, neurological disorders, cancer, metabolic disorders and cardiovascular disease (Bier, 2005; Bier and Bodmer, 2004; Bonini and Fortini, 2003). It is known that the fruit fly has a lot of advantages as a model organism for human diseases: a large number of developmental processes is conserved between flies and vertebrates and 197 out of 287 known human disease genes have *Drosophila* homologues. In addition, the fly has a short lifespan, a lot of progeny and available tools for genetic studies. One of the biggest advantages of *Drosophila* as a model system is a capability to perform the genetic screens to identify new genes involved in the biological processes (reviewed in (St Johnston, 2002)). Seventeen known human components (three dystrophin-related proteins, two dystrobrevins, five sarcoglycans, five syntrophins, one dystroglycan and one sarcospan) appear to be reduced to eight in *Drosophila* (one dystrophin, one dystrobrevin, three sarcoglycans, two syntrophins and one dystroglycan) (Greener and Roberts, 2000). The simplicity of this system recommends it as a model for its human counterpart. The *DLP* (*Dys*) gene, the *Drosophila melanogaster* homologue of the vertebrate dystrophin and utrophin genes, encodes three full-length gene products, DLP 1, 2 and 3 and truncated proteins Dp186, Dp205 and Dp117. *Drosophila DLP* gene is as complex as the mammalian *dystrophin* gene. The structure of this large gene encoding several full-length products and several truncated products has been conserved for at least 600 million years, indicating that both types of products have important function (Neuman et al., 2005). All *Drosophila* dystrophin isoforms bear the conserved dystrophin carboxy-terminal region, but, as in mammals, each has a distinct amino-terminal domain. Furthermore, the *Drosophila* dystroglycan protein contains all the hallmarks of vertebrate Dg: a mucin-like domain, a transmembrane domain, and a C-terminal region with WW-, SH2- and SH3-binding domains. The last 12 amino acids of the C-terminus include the WW-domain binding motif (PPxY), which is the dystrophin binding site. Vertebrate Dg contains the second PPxY motif in its cytoplasmic domain, which is also conserved in *Drosophila* (Deng et al., 2003). Within the DGC, the sarcoglycan component is composed of a series of single pass transmembtrane proteins. In the *Drosophila* genome there are only three sarcoglycan subunits: a subunit related to α-sarcoglycan, a β-sarcoglycan-like subunit and one single subunit that is equally related to γ-, δ- and ζ-sarcoglycans, which in *Drosophila* is called δ-sarcoglycan (Allikian et al., 2007).

Characterization of the fly DGC shows that flies possess essentially the same components as vertebrates. Furthermore, regions and domains known to mediate interactions between members of the complex are highly conserved between human and fly, suggesting that the overall structure of the complex is identical (Neuman et al., 2005). Recent studies showed that the human dystrophin protein can bind *Drosophila* dystroglycan and vice versa, suggesting that dystrophin-dystroglycan interaction is evolutionary conserved from human to fly and that the insights from *D.melanogaster* should be transferable to humans (Shcherbata et al., 2007). The fruit fly (and presumably most metazoans) has a potential to form a complex almost identical to the well-characterized human skeletal muscle DGC and the reduced heterogeneity of the DGC components in this experimentally amenable organism makes it an ideal model for resolving the fundamental ancestral role of the DGC as well as for studying mechanisms of muscular dystrophy. Recently, the *Drosophila melanogaster* models for muscular dystrophies caused by dystrophin, dystroglycan and sarcoglycan deficiency were established and phenotypes similar to human neuromuscular diseases were described in the fruit fly (Allikian et al., 2007; Shcherbata et al., 2007). It has been shown that mutations in *Drosophila* Dystrophin (*Dys*), Dystroglycan (*Dg*), as well as in Sarcoglycans (*Sgc*) reduce fly lifespan and mobility. The detailed analysis of indirect flight muscle (IFM) structure showed that the reason of climbing defects is age-dependent muscle degeneration (Allikian et al., 2007; Shcherbata et al., 2007). Unlike mammalian muscles, where degeneration is coupled with ongoing regeneration, no evidence for regeneration was seen in *Drosophila Sgc* mutants (Allikian et al., 2007). Moreover, the transgenic RNA interference was used to examine the role of the different *Drosophila* Dystrophin isoforms in muscle and it was found that the Dp117 isoform is expressed in muscle and, in addition to DLP2, plays role in stabilizing the muscle structure. Reduction of Dp117 expression levels results in muscle degeneration and lethality (van der Plas et al., 2007).

Most members of the vertebrate DGC are concentrated at the neuromuscular junction (NMJ), where their deficiency is often associated with NMJ structural defects. Hence, synaptic dysfunction may also intervene in the pathology of dystrophic muscles. Recently, the synaptic role of *Drosophila* DGC components were dissected. It has been shown that Dys and Dg are expressed in the postsynapse and reduction of Dys and Dg level in the postsynaptic muscle cell affects neurotransmitter release from the presynaptic apparatus (Bogdanik et al., 2008; van der Plas et al., 2006). A similar function in the NMJ was indicated for the *Drosophila dPOMT1* mutants found in a screen for synaptic mutations (Wairkar et al., 2008). In humans, Protein-O-mannosyl transferase 1 (POMT1) is the first enzyme required for the glycosylation of Dystroglycan, and mutations in the *POMT1* gene can lead to both Walker-Warburg syndrome and limb girdle muscular dystrophy type

2K, which are associated with severe mental retardation and major structural abnormalities in the brain. Similarly to *Dystroglycan* mutations, defects in the *Drosophila dPOMT1* cause a decrease in the efficacy of synaptic transmission and changes in the subunit composition of the postsynaptic glutamate receptors at the neuromuscular junction (Wairkar et al., 2008). These results bring up the possibility that muscular dystrophies in humans might also, at least partially, be attributed to the altered kinetics of acetylcholine transmission through the neuromuscular junction. Furthermore, Dys and Dg in *Drosophila* are required for proper photoreceptor axon migration. The axonal projection of R-type sensory neurons to the brain optic lobes in *Drosophila* larvae are disrupted due to the lack of Dys and Dg; most of the axons migrate to the correct terminal zone in lamina, but form abnormal patches at the lamina plexus. Additionally, it has been shown that Dys and Dg are required both in neuron and glial cells for proper axonal growth and targeting (Shcherbata et al., 2007).

Correspondingly to humans, *Drosophila* Dystrophin plays an important role in maintaining heart morphology and function, which has been shown using the fly heart as an *in vivo* assay system. Both the long DLPs and the short Dp117 Dystrophin isoforms are expressed in the adult *Drosophila* heart. The *Dys* mutant, haploinsufficiency or knockdown flies, develops age-dependent cardiac abnormalities, reminiscent of *mdx* mice. The *Dys* mutant flies have dilated and abnormally performing hearts consistent with the mammalian phenotype of dilated cardiomyopathy (Taghli-Lamallem et al., 2008). Analogously reduced heart tube function was observed in the *Drosophila Sarcoglycan* mutants (Allikian et al., 2007).

In addition, both Dystrophin and Dystroglycan are required for cellular polarity in *Drosophila*. A gain-of-function screen for mutants defective in polarity in *Drosophila* oogenesis resulted in finding fly homologues of the DGC components: *Drosophila* Dystroglycan and Laminin A (Deng and Ruohola-Baker, 2000; Deng et al., 2003). Reduction of the Dys and Dg function in the germline resulted in mislocalization of the oocyte polarity marker Orb (Deng et al., 2003; Shcherbata et al., 2007). Loss of Dystroglycan function in follicle and discs epithelia results in expansion of the apical marker to the basal side of cells and overexpression results in a reduced apical localization of these markers (Deng et al., 2003). Dys and Dg also are required non-cell-autonomously to organize the planar polarity of basal actin in follicle cells (Deng et al., 2003; Mirouse et al., 2009).

Taken together, the phenotypes caused by mutations in *Drosophila* DGC are remarkably similar to phenotypes observed in human muscular dystrophy patients, and therefore this suggests that functional dissection of the DGC in *Drosophila* should provide a new insight into the origin and potential treatment of these neuromuscular disorders. Studies in this new model with easy-to-

manipulate genetics might reveal a mode of the DGC regulation by identifying key regulatory components through a modifiers screen. In addition, careful functional analysis of the complex in different cell types in model organisms might result in an unifying theme that will clarify molecular mechanisms of its function.

2.4. Research objectives

I Studying of Dystrophin-Dystroglycan interaction in vitro

- To perform *in vitro* analysis of Dystrophin-Dystroglycan interaction the part of the *Drosophila* Dystrophin protein required for the interaction domains (WW and EF hand) had to be expressed in *E.coli* and purified. Therefore the conditions of the protein expression and extraction had to be established.
- To measure the Kd for interaction of Dystrophin protein with different Dystroglycan peptides containing each of the WW binding motifs (wild type, mutated and phosphorylated), the fluorescent polarization assay (used previously by (Shcherbata et al., 2007)) had to be applied and the new technique had to be learned and practiced.

II Studying the function of the Dystroglycan C-terminal WW binding sites in vivo

- To generate transgenic flies expressing different forms of Dystroglycan protein, the full length and modified Dystroglycan PCR products, which can be expressed in the germline, had to be synthesized and cloned to a pUASp vector with subsequent injection to the fly embryo.
- While transgenic flies were generated, the expression of the *Dystroglycan* needed to be confirmed and quantified by immunohistochemical staining of follicle epithelium cells and by Western bloting.
- To perform functional analysis for both WW binding sites of Dystroglycan *in vivo*, the transgenic constructs had to be expressed in germline (utilizing *Mat-tub-Gal4* enhancer trap line), where Dystroglycan is required for establishment of the oocyte polarity. Next, the Orb (oocyte polarity marker) staining had to be analyzed.
- The constructs that showed their functionality, had to be used in a "rescue assay" to confirm that observed phenotypes are indeed due to the requirement of Dystroglycan function.

III Performing large scale screen to identify dominant modifiers of DGC-dependent wing vein phenotype

- To start screening, the *Drosophila* strains containing both RNAi and enhancer trap constructs had to be created to reduce level of Dys and Dg and to get viable flies with Dys/Dg phenotypes.
- Different screening strategies had to be learned, established and applied to find modifiers of Dys/Dg–dependent mutant phenotype in wing veins: EMS-induced mutagenesis, Deficiency screen and P-element lethal screen.
- To map mutations selected as modifiers of the wing vein phenotype in the EMS-induces mutagenesis screen, the "deficiency mapping" needed to be used.
- For further functional analysis of found modifiers in the process of oocyte polarity establishment the Flp/FRT technique had to be applied to generate mutant germline clones.

IV Performing a secondary screen to identify dominant modifiers of DGC-dependent muscle degeneration phenotype

- While the large scale screen was performed with analysis of an easily score-able wing vein phenotype, the pre-selected modifiers had to be used in the secondary screen. To analyze modifications of the muscle degeneration phenotype a technique for preparing and staining muscle sections had to be utilized.
- In order to investigate if new-found modulators have function in muscles and demonstrate their own muscle degeneration phenotype, the RNA interference mutants that target each component had to be used. *Tub-Gal4* and *act-Gal4* enhancer trap lines had to be applied to activate transgenic constructs ubiquitously in all tissues.
- For further tissue-specific analysis, the muscle- and the motor neuron-specific drivers (*24B-Gal4*, *MHC-Gal4*, and *D42-Gal4*) had to be utilized and the muscle architecture had to be analyzed.

3. Results

Every chapter within the results starts with a description of:

- the main aim of the particular manuscript in the context of the complete thesis
- the authors and their contribution to the practical work
- the status of the manuscript

3.1. *In vitro* analysis of *Drosophila* Dystrophin–Dystoglycan interaction

Dystroglycan and Dystrophin interaction is mediated by the most C-terminal WW domain binding motif, PPxY, of Dystroglycan and the Dystrophin WW and EF-hand domains (Huang et al., 2000; Jung et al., 1995; Rentschler et al., 1999). However, the role of the second WW binding site, which is present on the C-terminus of Dystroglycan has never been addressed. Now I have used a fluorescent polarization assay (Shcherbata et al., 2007) to detect *in vitro* binding between the part of *Drosophila* Dystrophin protein, which has WW and EF hand domains and Dystroglycan peptides containing each of the WW binding motifs (called DmWWbsI and DmWWbsII). Analysis of obtained results showed that both peptides DmWWbsI and DmWWbsII can bind Dystrophin *in vitro* with a K_d within the range of reported dissociation constants for class I WW domains (Kato et al., 2002). As expected mutations predicted to abolish the WW binding domains resulted in the reduction of binding. Previous *in vitro* experiments have shown that when the tyrosine of the PPxY motif on the most C-terminus of Dg is phosphorylated, the binding between Dg and Dys is abolished (Ilsley et al., 2001; Sotgia et al., 2001). Currently I have demonstrated that phosphorylation of tyrosine in both DmWWbsI and DmWWbsII can dramatically affect Dystrophin-Dystroglycan binding in *Drosophila* suggesting a potential mechanism to regulate the Dg and Dys interaction in a tyrosine phosphorylation-dependent manner.

A Putative Src Homology 3 Domain Binding Motif but Not the C-terminal Dystrophin WW Domain Binding Motif Is Required for Dystroglycan Function in Cellular Polarity in *Drosophila*

Andriy S.Yatsenko, Elizabeth E. Gray, Halyna R. Shcherbata, Larissa B. Petterson, Vanita D. Sood, **Mariya M. Kucherenko**, David Baker, and Hannele Ruohola-Baker

Author contribution to the practical work:

Mariya M. Kucherenko	Experiments for *in vitro* analysis of interaction between *Drosophila* Dystrophin and Dystroglycan
Andriy S. Yatsenko and *Elizabeth E. Gray*	All experiments except *in vitro* interaction analysis.
Halyna R. Shcherbata	Analysis of cell polarity
Larissa B. Petterson	Designing of transgenic constructs and *in vitro* analysis of human and *Drosophila* Dys- Dg interaction
Vanita D. Sood	Experiments for *in vitro* analysis of human Dys-Dg interaction

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A Putative Src Homology 3 Domain Binding Motif but Not the C-terminal Dystrophin WW Domain Binding Motif Is Required for Dystroglycan Function in Cellular Polarity in *Drosophila*[*] [S]

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The conserved dystroglycan-dystrophin (Dg·Dys) complex connects the extracellular matrix to the cytoskeleton. In humans as well as *Drosophila*, perturbation of this complex results in muscular dystrophies and brain malformations and in some cases cellular polarity defects. However, the regulation of the Dg·Dys complex is poorly understood in any cell type. We now find that in loss-of-function and overexpression studies more than half (34 residues) of the Dg proline-rich conserved C-terminal regions can be truncated without significantly compromising its function in regulating cellular polarity in *Drosophila*. Notably, the truncation eliminates the WW domain binding motif at the very C terminus of the protein thought to mediate interactions with dystrophin, suggesting that a second, internal WW binding motif can also mediate this interaction. We confirm this hypothesis by using a sensitive fluorescence polarization assay to show that both WW domain binding sites of Dg bind to Dys in humans (K_d = 7.6 and 81 μM, respectively) and *Drosophila* (K_d = 16 and 46 μM, respectively). In contrast to the large deletion mentioned above, a single proline to an alanine point mutation within a predicted Src homology 3 domain (SH3) binding site abolishes Dg function in cellular polarity. This suggests that an SH3-containing protein, which has yet to be identified, functionally interacts with Dg.

The dystroglycan-dystrophin complex contains multiple proteins, including the actin-binding protein dystrophin, the transmembrane protein dystroglycan, and a variety of extracellular proteins, including laminin, agrin, and perlecan (1). The Dg[3] protein is a crucial player in this complex acting as an anchor between the actin cytoskeleton and the extracellular matrix. Dg binds Dys at its proline-rich C-terminal end and laminin at its highly glycosylated N-terminal end (2) (Fig. 1*A*).

When the interactions between components of the Dg·Dys complex are disrupted, the muscle degenerative disease muscular dystrophy (MD) results (3–5). In mouse models, loss of Dg in muscle cells causes mild muscular dystrophy phenotypes (6). Furthermore, several human forms of MD, such as Fukuyama MD, result from mutations in the enzymes that glycosylate Dg. In addition to its role in maintaining the structural integrity of muscle cell membranes, Dg is also required in the brain. When it is knocked out in the mouse brain, disrupted neural migration and disorganized cortical layers are observed (7, 8). This is consistent with the fact that brain malformations as well as learning and memory difficulties are often observed in MD patients (9–12). Dg is not only important in the pathogenesis of MD and the associated brain malformations, but it also has an important role in cell adhesions and anchoring the cell to the extracellular matrix. Loss of Dg protein has been associated with the progression of various epithelial cancers (2, 13). Specifically, Dg is down-regulated in breast and prostate cancers (14, 15).

In vitro studies have suggested that the interaction between Dg and Dys is mediated by the most C-terminal WW domain binding motif, PP*X*Y, on Dg and the Dys WW and EF-hand domains (16–18). *In vitro* experiments have also shown that when the tyrosine of the PP*X*Y motif is phosphorylated, the binding between Dg and Dys is abolished (19, 20). This suggests a potential mechanism to regulate the Dg and Dys interaction, in which signaling proteins containing SH2 or SH3 domains may bind to Dg in a tyrosine phosphorylation-dependent manner. In the search for a potential regulator, recent studies have revealed several proteins that interact with Dg. Both the Grb2 (growth factor receptor-bound protein 2) adaptor protein, as well as MEK1, and ERK of the Ras-Raf mitogen-activated protein kinase (MAPK) cascade have been shown to interact, *in vitro* and *in vivo*, with the C terminus of Dg (21, 22). However, Dg appears to be only an anchor for MEK1 and ERK rather than a substrate (22), and Dg might not have a direct involvement in this signaling pathway. Independently, recent work has revealed that laminin and dystroglycan-dependent phosphoryl-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Results, Experimental Procedures, and Figs. S1 and S2.

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[3] The abbreviations used are: Dg, dystoglycan; Dys, dystrophin; MD, muscular dystrophy; SH3, Src homology 3; SH2, Src homology 2; MEK1, dual threonine/tyrosine kinase; ERK, extracellular signal-regulated kinase; DBR, dystroglycan binding region; bs, binding site; FL, full-length; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; GFP, green fluorescent protein.

ation of syntrophin affects the Grb2-SOS-Rac1-c-Jun N-terminal kinase (JNK) pathway and ultimately results in the phosphorylation of c-Jun on Ser-65 (23). Thus, although studies suggest a clear role for Dg in signaling, the regulation of Dg by signaling and the specific regions of the Dg C terminus involved in this process are unknown.

To shed light on the regulation of Dg and its role in signaling, we have analyzed the binding motifs that are required for the function of the Dg·Dys complex in cellular polarity in *Drosophila*. The proline-rich C terminus of Dg has several potential protein-binding motifs, which suggests that it may be involved in regulating the complex and potentially may have a signaling role. Proline-rich sequences have been shown to be the targets of several protein interaction domains involved in signal transduction. For example, SH3 domains have been shown to bind core P*X*X*P* motifs (where P is proline and *X* indicates any amino acid). *Drosophila* Dg contains two putative SH3-binding sites, consisting of the core P*X*X*P* motif. Proline-rich sequences also serve as targets for binding by WW domains (24). In particular, the class I WW domain ligand, PP*X*Y (where Y is tyrosine), appears twice in the C-terminal region of Dg. The more C-terminal PP*X*Y motif has been established as a binding site for the WW domain of dystrophin in humans (17, 18) and in *Drosophila* by *in vitro* binding studies (25). The role of a putative second, more N-terminal WW domain binding site or the potential SH3 domain binding sites are not yet understood.

Drosophila is an excellent system to study the Dg·Dys complex (25–29). In particular, Dg is required for cellular polarity in the oocyte and epithelial cells in *Drosophila* as well as in mouse mammary epithelial cells (26, 30). Furthermore, *Drosophila* is a model for the MD disease phenotype, as reduction of dystrophin and dystroglycan in the muscles leads to progressive muscle degeneration and loss of muscle function (25). In this study, we test which regions of the Dg C terminus are essential for Dg function in cellular polarity *in vivo*. Specifically, we show by a single amino acid substitution that a putative SH3 domain binding site is critical for Dg function in both loss-of-function and overexpression studies. However, the most C-terminal WW domain binding site previously shown to be essential for dystrophin binding is dispensable for cellular polarity in *Drosophila*.

EXPERIMENTAL PROCEDURES

Fly Stocks—*Drosophila melanogaster* stocks were raised on standard cornmeal/yeast/agar medium at 25 °C. For overproduction of pUASp-Dg in the germ line, we used the following: *NGT40*; *P(w+:nanosGal4:VP-16)Ab-2* (31, 32) and *Mat-α4-Tub > Gal4-VP16/CyO* (33). For overproduction of pUASp-Dg in embryos we used *Daughterless-Gal4* (34). For overproduction of pUASp-Dg in the follicle cells, we used *hsFlp; act < FRT-CD2-FRT < Gal4; UAS-GFP* (35). For generation of dystroglycan clones, we used *FRT42D-Dg^{323}/CyO* (Dg^{323} is a dystroglycan loss-of-function mutant with a 3155-bp deletion between bp 32,345 and 35,669 of DS03910) (26) and *hsFLP; FRT42D Ubi-GFP/CyO*. For overproduction of pUASp-Dg in a *dystroglycan* mutant background, we used *FRT42DDg^{323}/CyO; P(w+ nos-Gal4:VP16)A4-2 III*, and *hsFLP; FRT42D Ubi-GFP/CyO; pUASp-Dg/TM3* or pUASp-Dg */FM7; FRT42DDg^{323}/CyO*, and *hsFLP; FRT42D Ubi-GFP/CyO; P(w+ nos-Gal4:VP16)A4-2 III* (*pUASp-Dg* refers to all *dystroglycan* constructs: FL, C1, C2, 4P, DC2, Pro → Ala, ALLP, AATA).

Generation of pUASp-Dg Transgenic Animals—Full-length and truncated dystroglycan PCR products that can be expressed in the germ line were synthesized from the template LD11619 using the forward primer GGGGTACCAACATGAGATTCCAGTGGTTCT in conjunction with one of the following reverse primers: FL, CTCTAGATTATGGCGACACATATGGCGGT; C1, GCTCTAGATTACTTCTCGTCCTTGAGTATGAC; C2, GCTCTAGATTAATATGGCGGTGGCTTCTCGTCCTTGAGTATAGAC; 4P, GCTCTAGATTATGGCGACACAGGTGGCGGT; DC2, GCTCTAGATTAGTCCACGTCGTTGTCAC (Invitrogen) and cloned into pUASp, a vector that allows efficient germ line expression (36).

To generate a construct with mutated SH3-binding sites (pUASp-2XSH3 knock-out, Pro → Ala), the QuickChange® XL site-directed mutagenesis kit (Stratagene) was used to introduce proline to alanine substitutions in the SH3bsI (PATP → AATA). LD11619 was used as a template with forward primer CGTGGCAAGTCGGCAGCCACGGCCTCTACCGCAAACC and reverse primer GGTTTGCGGTAGGAGGCCGTGGCTGCCGACTTGCCACG, generating the intermediate plasmid pBS-Dg AATA. pBS-Dg AATA then served as a template for PCR with the forward primer GGACGAGAAGCCGGCGCTGCTGCCACCATCCTACAATACC and the reverse primer GGTATTGTAGGATGGTGGCAGCAGCGCCGGCTTCTCGTCC, designed to substitute the first proline of the SH3bsII with an alanine (PLLP → ALLP), thus generating pBS-2XSH3 knockout. This served for the template for standard PCR performed with the forward primer GGGGTACCAACATGACATTCCAGTGGTTCT and reverse primer GCTCTAGATTATGGCGACACATATGGCGGT.

PCR products were digested with KpnI and XbaI and cloned into the pUASp vector (36). The constructs were injected into embryos to obtain at least two independent stable transformant lines.

Overproduction of Dystroglycan in the Germ Line, Follicle Cells, and Embryos—For Dg overproduction in germ line cells, balanced *pUASp-Dg/P(w+:nanosGal4:VP-16)Ab-2* or *Mat-α4-Tub > Gal4-UP16/CyO* animals were raised in yeasted vials at 25 °C for 3 days, dissected, and analyzed. For Dg overproduction in the follicle cells, *hsFlp; UAS-GFPact < FRTCD2FRT < Gal4/pUASpDg* animals were heat-shocked at 37 °C for 1 h, raised in yeasted vials at 25 °C for 3 days, dissected, and analyzed. All *pUASp*-Dg constructs used were crossed to these three Gal4 drivers to test for proper overproduction of protein and correct localization of protein to the membrane in the germ line and somatic cells. The following *pUASp-Dg* lines were used for germ line analysis: FL-1, 5; 4P-1, -2, -3, -4; DC2-1, -2; Pro → Ala-1-3; C2-1, 3; C1-1, -2; ALLP-1-3; AATA-1,2,4. For Dg overproduction in the embryo, *pUASp-Dg/Daughterless-Gal4* embryos were collected and left for 20 h at 18 °C to develop to stage 13, stained, and analyzed. The following *pUASp-Dg* lines were used to analyze embryos: FL-1, -2, -4; 4P-1, -3; DC2-1, 3; Pro → Ala-1, -2, -3; C2-1, -5, -10; C1-1, -2. For Dg rescue experiment the following lines were used: FL-1, -2, 3, -5, -6; 4P-1, -3, -4; DC2-2, 3-; Pro → Ala-1–3; C2-1, -3; C1-1, -2; ALLP-1–3; AATA-1,2,4. Importantly, the low level of Dg constructs driven by only one copy of the *nanosGal4*-driver used in the rescue

experiments does not generate significant overexpression phenotypes. Abnormal stage 7–8 clones (severe necrosis, no oocyte, or abnormal Orb staining) were not included in our calculations. For each construct, values for the proportion of ovaries or embryos with mislocalized polarity markers between independent insertion lines were averaged, and average deviations were calculated.

Antibody Staining Procedure—Ovaries were dissected in phosphate-buffered saline (PBS) and fixed while shaking on a nutator for 10 min in PBS containing 5% formaldehyde. Embryos were collected in (0.7% NaCl, 0.3% Triton X-100), dechorionated, and fixed for 20 min in (4% formaldehyde, 0.1 M sodium phosphate buffer, pH 7.2). Embryos were transferred to a 20-ml scintillation vial containing fixture and 100% *n*-heptane 1:1 and fixed for 20 min at room temperature on a shaker. Next, the fixture was removed, and an equal amount of 100% methanol was added. The vial was shaken vigorously to rupture the vitelline membrane. Embryos were rinsed with methanol and dehydrated through an ethanol series and rehydrated prior to antibody staining.

Ovaries and embryos were rinsed with PBT (PBS, 0.2% Triton X-100) four times (15 min each rinse) and blocked in PBTB (PBT, 0.2% bovine serum albumin, 5% normal goat serum) for 1 h at room temperature. The tissue was incubated with primary antibodies overnight at 4 °C and then incubated in secondary antibodies overnight at 4 °C. The next day they were rinsed with PBT for 15 min, stained with DAPI (1 μg/ml in PBT) for 10 min, and rinsed with PBT and mounted onto slides in 70% glycerol, 2% *n*-propyl gallate, 1× PBS. To analyze slides, a two-photon laser-scanning confocal microscope (Leica TCS SP/MP) was used.

The following primary antibodies were used at the following designated dilutions: rabbit anti-dystroglycan (1:3000 (26)), mouse anti-Orb and anti-Crb (1:20; Developmental Studies Hybridoma Bank), and rabbit anti-GFP directly conjugated AF488 (1:1000; Molecular Probes). The following secondary antibodies were used at the designated dilutions: Alexa 568 anti-rabbit and Alexa 568 anti-mouse (1:500; Molecular Probes) and 488 phalloidin (1:50; Molecular Probes).

Plasmid Construction for in Vitro Analysis and Protein Expression—The WW-EF hand region (DBR) of *Drosophila* dystrophin was amplified from the template LD11292 using PCR with forward primer GGAATTCCATATGACCATTGGACCACTGCCC and reverse primer CCGCTCGAGTTACTGGTGCTTGGCCGCCTC and cloned between the NdeI and XhoI restriction sites of the His tag expression vector pET-15b (Novagen). *Drosophila* DBR protein was expressed in *Escherichia coli* strain BL21(DE3) after induction by 1 mM isopropyl 1-thio-β-D-galactopyranoside in standard LB medium (Qbiogene/Bio 101, Inc.). Cell pellets were collected, resuspended in Binding Buffer solution (150 mM MOPS, 150 mM NaCl, 5 mM imidazole), and lysed by a French press. Protein was purified using nickel-nitrilotriacetic acid (Qiagen) affinity chromatography. Protein was concentrated using an Amicon ultracentrifugal device (Millipore), and imidazole was removed by dialysis. Purified DBR protein was stored in 50 mM MOPS, pH 6.5, 150 mM NaCl, 400 mM Na_2SO_4, 10 mM dithiothreitol.

The Drk (Dreadlock)-FL gene of *Drosophila* was amplified via PCR from the template LD12029 with forward primer CCGCTCGAGATGGAAGCGATTGCCAAACACG and reverse primer CGCGGATCCTTATGAATGATATGGCGTCACAT and then cloned into the His tag expression vector pET-15b (Novagen) using the XhoI and BamHI restriction sites. *Drosophila* Drk protein was expressed in *E. coli* strain BL21(DE3) after induction by 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Cell pellets were collected and lysed by a French press. Protein was purified using nickel-nitrilotriacetic acid (Qiagen) affinity chromatography. Protein was concentrated using an Amicon ultracentrifugal device (Millipore) and imidazole removed by dialysis. Purified Drk protein was stored in 20 mM Tris, pH 7.9, 150 mM NaCl, 150 mM Na_2SO_4.

The DBR of human dystrophin (18) was expressed as a glutathione *S*-transferase fusion protein and purified by glutathione affinity chromatography. Five hundred units of thrombin (Amersham Biosciences) were loaded onto the glutathione column with DBR bound, and the column was sealed and incubated overnight at 4 °C to cleave the glutathione *S*-transferase from the DBR. The DBR was washed off the column and concentrated, and the buffer was exchanged during concentration to the same storage buffer used for the *Drosophila* DBR.

Fluorescence Polarization Experiments—Synthesized dystroglycan peptides (Fig. 5*C*) were N-terminally tagged with tetramethylrhodamine by Invitrogen Evoquest Services (sequences DmWWbsI, GKSPATPSYRKPPPYVSP; HmWWbsI, KNMPTYRSPPPYVPP; DmWWbsII, PVI-LKDEKPPLLPPSYNT; HmWWbsII, PL-ILQEEKAPLPPPEYSN). Six additional tetramethylrhodamine-labeled peptides were ordered from Genemed Synthesis Inc. (DmWWbsI-pY, GKSPATPYRKPPPpY-VSP; DmWWbsII-pY, PVILKDEKPPLL-PPSpYNT; HmWWbsI-pY, KNMPTYRSPPPpYVPP; DmWWbsI-W, GKSPATPYRKWPPYVSP; DmWWbsII-G, PVI-LKDEKPPLLLPPSGNT; and DmSH3bsII-2A, PVILKDEKPALLPPSYNT). All peptides were over 95% pure based upon high pressure liquid chromatography and mass spectrometry analysis. Fluorescence polarization experiments were performed at 25 °C using a Wallac 1420 Victor3 fluorescence plate reader (PerkinElmer Life Sciences). Dystroglycan peptide (200 nM) was incubated with increasing concentrations of dystrophin protein in storage buffer to a final volume of 250 μl. Anisotropy values were measured at an excitation wavelength of 531 nm and an emission wavelength of 595 nm. Dissociation constants (K_d) were determined by plotting millianisotropy *versus* the concentration of Dys and fitting the data to the equilibrium binding Equation 1,

$$\frac{W\mathrm{Dg}}{\mathrm{Dg}} = \frac{\mathrm{Dg} + W + K_d - \sqrt{\mathrm{Dg}^2 + K_d^2 W^2 - 2 \times \mathrm{Dg} \times W + 2 \times W \times K_d + 2 \times \mathrm{Dg} \times K_d}}{2 \times \mathrm{Dg}} \quad \text{(Eq. 1)}$$

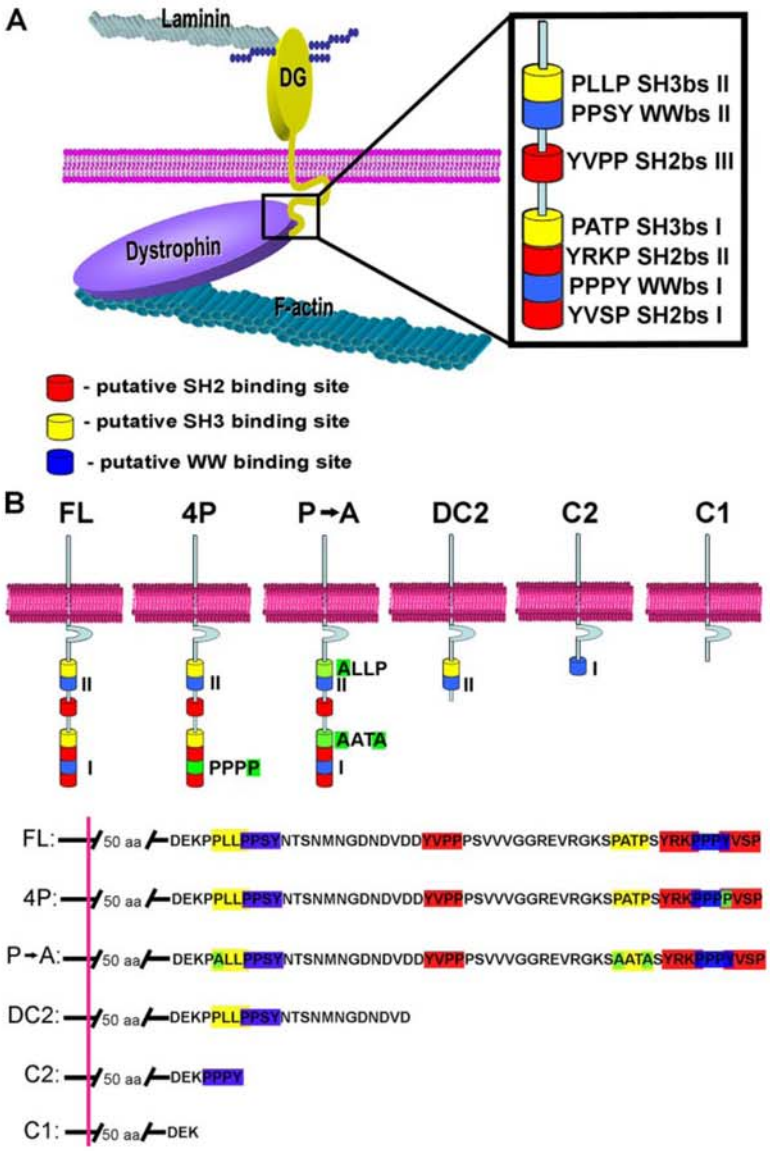


FIGURE 1. **The *Drosophila* dystroglycan-dystrophin complex.** *A*, Dg·Dys complex consists of the actin-binding protein dystrophin, the transmembrane protein Dg, and extracellular proteins, including laminin. Dg is a crucial player in this complex acting as a connector between the actin cytoskeleton and extracellular matrix. Dg binds Dys at its proline-rich C-terminal end, which contains several well conserved putative binding sites for signaling proteins. *B*, schematic drawing of pUASp-Dg constructs with various changes at the Dg C terminus (*FL*, full length; *4P*, Tyr to Pro substitution in the WWbsI; *P → A*, Pro to Ala substitutions in the SH3bsI and SH3bsII; *DC2*, *C2*, and *C1* = stepwise deletions of the proline-rich C terminus).

where Dg is the total concentration of dystroglycan peptide; *W* is the total concentration of dystrophin protein; *W*Dg is the concentration of dystroglycan-dystrophin complex, and K_d is the apparent dissociation constant for the complex. The assays using phosphorylated peptides were performed with (1:100; phosphatase inhibitor cocktail 2, Sigma) and without phosphatase inhibitors resulting into the same K_d values. This suggests that no obvious effects of contaminant phosphatases were observed in this assay.

RESULTS

Drosophila Dg is required for proper polarity of different cell types (25, 26). The C terminus of Dg contains several putative WW, SH2, and SH3 domain binding sites where signaling proteins may bind and participate in the regulation of the Dg·Dys complex (Fig. 1*A*) (26). Specifically, *Drosophila* Dg contains two class I WW domain binding sites, WWbsI and WwbsII; three putative SH2-binding sites, SH2bsI, SH2bsII, and SH2bsIII; and two putative SH3-binding sites, SH3bsI and SH3bsII (Fig. 1*A*). To analyze the function of each of these potential binding sites, we generated transgenic animals expressing a variety of Dg constructs, in which some of these binding sites are deleted, and we examined their capacity to affect polarity in overexpression and loss-of-function experiments (Figs. 3 and 4). We first generated the C1 construct, which lacks all C-terminal binding sites, and compared its function to full-length Dg (*C1*, *FL*; Fig. 1*B*). The most C-terminal PPPY motif (WWbsI) binds dystrophin (16, 18, 25). To test the importance of this interaction *in vivo* in *Drosophila*, we generated C2, which only contains this PPPY motif (Fig. 1*B*). When the tyrosine of the PPPY motif is phosphorylated, Dys binding to the Dg C terminus is reduced (Fig. 5) (19). To test whether tyrosine phosphorylation is an important component in the regulation of the Dg·Dys complex *in vivo*, we generated 4P, in which the PPPY motif (WWbsI) has been altered to PPPP. To analyze the importance of the second WW domain binding site, we generated DC2, which contains the second WW and SH3 domain binding sites (WWbsII and SH3bsII). Finally, to analyze whether the two SH3 domain binding sites are critical, we generated P → A, in which both sites (SH3bsI and SH3bsII) have been disrupted by proline to alanine substitutions (Fig. 1*B*). The Gal4-UAS system for protein expression was utilized to express the Dg constructs in the follicle cells, the germ line cells, and the embryos. To avoid problems because of positional effects, 2–6 independent lines were generated and

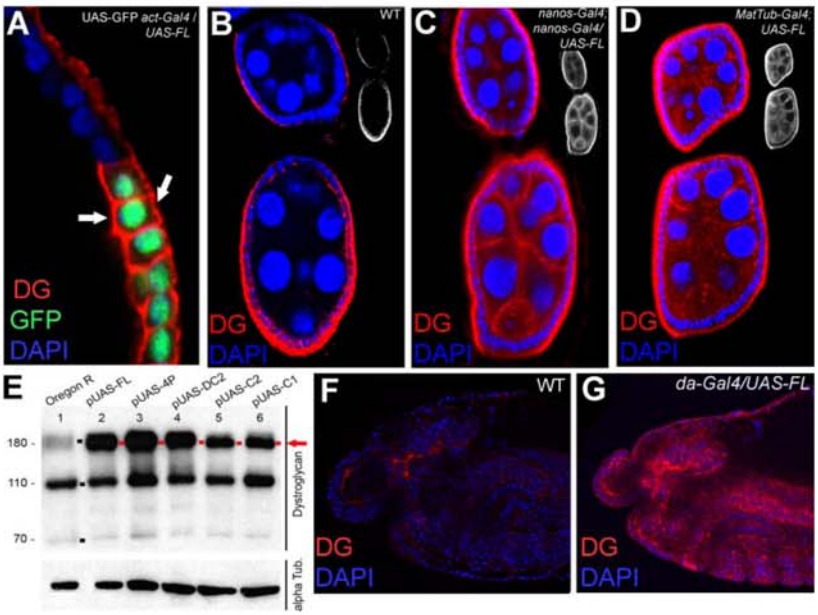


FIGURE 2. **Dg constructs used in this study and their expression in the follicle cells, germ line cells, and embryo.** Dg, *red*; GFP, *green*; DAPI, *blue*. *A*, Dg is localized to the basal membrane in wild type follicular epithelium, but when pUASp-Dg constructs are expressed in follicle cell clones, marked with GFP, Dg is found on both apical and basal membranes (*arrows, hsFlp; act < CD2 < Gal4/+; UAS-GFP/pUASp-FL*). *B*, wild type Dg expression in the germ line cells. *C*, *nanos-Gal4* driven pUASp-Dg expression in the germ line cells (*NGT40; P(w+: nanosGal4:VP-16)Ab-2/pUASp-FL*). *D*, *MatTub-Gal4*-driven pUASp-Dg expression in the germ line cells (*Mat-α4-Tub > Gal4-UP16; pUASp-FL*). *E*, Western blot of ovarian protein extracts probed with Dg antibody to detect overexpression in the germ line cells in different pUASp-Dg constructs driven by *Mat-α4-Tub > Gal4-UP16*. Three Dg bands of ~180, ~110, and ~70 kDa (marked by the *black dots*) are detected in wild type (OregonR, *lane 1*). When all Dg constructs are overexpressed, an ~180-kDa band is enriched (*lanes 2–6*, marked by *red dots, red arrow*). *F*, wild type Dg staining in the embryo at stage 13. *G*, *Daughterless-Gal4*-driven pUASp-Dg expression in the embryo at stage 13 (*Daughterless-Gal4/pUASp-FL*).

analyzed for each construct. The results represent mean values for experiments done with multiple independent insertion lines. All constructs expressed Dg at elevated levels compared with wild type (Fig. 2).

Dystroglycan Overproduction Disrupts Oocyte Polarity—Dg and Dys are required in the germ line for the early establishment of oocyte polarity (25, 26). To analyze how overproduction of Dg in the germ line affects oocyte polarity, we expressed Dg (FL) in the germ line cells using a germ line-specific driver (*MatTub-Gal4;* Fig. 2*D*, and supplemental Fig. S2*A*) and examined oocyte polarity using Orb as a marker. Orb marks the microtubular organizing center, which is localized at the anterior of the oocyte during stage 1, and then moves to the posterior by stage 3. Between stages 3 and 6, Orb is clearly localized to the posterior of the oocyte, making it an excellent marker to analyze the polarity of the oocyte (Fig. 3*A*–*A*′ and supplemental Fig. S2, *A* and *B*). Absent or mislocalized Orb during these stages indicates a failure to establish early oocyte polarity. When FL is overproduced in the germ line Orb becomes mislocalized (Fig. 3, *B*–*B*′, *C*, and *G*); instead of being localized to the posterior, Orb surrounds the entire oocyte in a circle or accumulates in a clump at one side of the oocyte (48 ± 8%, n = 117). These early polarity defects resulted in abnormalities during the later stages of egg chamber development. In addition to defective appendages and necrosis, abnormal Orb staining was observed in later stage egg chambers. Instead of the smooth Orb staining throughout the oocyte, patchy and dotted staining was observed (80 ± 8%, n = 323; Fig. 3*H*). Therefore, dystroglycan, when expressed at elevated levels in the germ line cells, is sufficient to disrupt oocyte polarity in both early and late stages of oogenesis. Similarly, in vertebrates, overexpression of Dg has been shown to cause defects in neuromuscular junctions (37, 38).

The Region of the Dystroglycan C Terminus Containing WWbsII and SH3bsII Is Sufficient to Disrupt Oocyte Polarity—Because full-length Dg overproduction disturbs oocyte polarity, we analyzed which signaling molecule binding sites in Dg are required for this capacity, in the context of our assays. Each Dg construct (Fig. 1*B*) was expressed in the germ line cells using a *MatTub-Gal4* driver, and the percentage of stage 3–6 egg chambers with abnormal oocyte polarity was quantified (Fig. 3*G*).

Expression of C1 and C2 did not result in as high frequency of Orb mislocalization as the FL construct, suggesting that the C-terminal proline-rich region (absent in C1) is important for full Dg function and the WWbsI Dys-binding site alone is not sufficient to restore the activity of C1 to the wild type level (Figs. 1*B* and 3*G*). Therefore, other sites must act in conjunction with this WW domain binding site to regulate the Dg·Dys complex in the context of oocyte polarity.

Mutation of the conserved tyrosine of WWbsI to proline reduces binding affinity *in vitro* by an order of magnitude (from 7.6 to 172 μM in human and from 3.7 to 47 μM in *Drosophila*) (25). Expression of the 4P construct in *Drosophila* had the same ability to disrupt oocyte polarity as FL (46 ± 3%, n = 194; Fig. 3*G*), suggesting that either the reduced binding observed with the 4P construct is still enough to support functionality of the complex or that WWbsII is able to function in place of WWbsI. To probe this issue further, we expressed DC2, which only contains WWbsII and SH3bsII (Fig. 1*B*). Interestingly, DC2 was also able to disrupt oocyte polarity to the same extent as FL (53 ± 2%, n = 122; Fig. 3*G*), indicating that WWbsII indeed can function and that potential SH3 domain binding sites may play a role in the regulation of the Dg complex.

To test whether the putative SH3 domain binding sites are important for Dg function, we overexpressed the P → A construct, in which both SH3bsI and SH3bsII have been disrupted by proline to alanine substitutions (Fig. 1*B*). Importantly, this construct, in which only the two potential SH3 domain binding

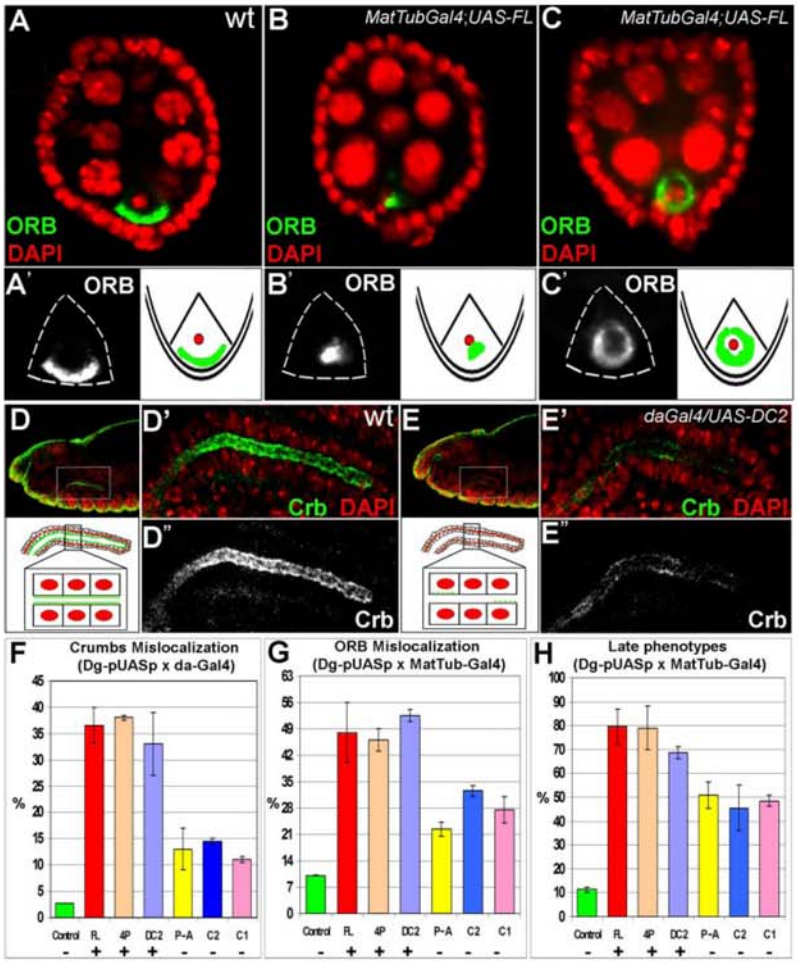


FIGURE 3. **Dg overproduction disrupts cellular polarity.** *A,* wild type (*wt*) stage 4 egg chamber shows Orb (*red*) correctly localized to the posterior of the oocyte. Orb localization is shown magnified in a single channel and schematically drawn below each photo (*A'–C'*). Orb, *green*; DAPI, *red*. *B* and *C*, when Dg is overproduced in the germ line cells, oocyte polarity is disrupted, resulting in mislocalized Orb (*Mat-α4-Tub > Gal4-UP16; pUASp-FL*). *D–D"*, a confocal image of the wild type salivary gland at stage 13 stained with Crb antibody that is localized to the apical side of the salivary gland epithelium. Crb, *green*; DAPI, *red*. *E–E"*, when Dg is overproduced in the embryo (*Daughterless-Gal4/pUASp-DC2*), Crb fails to localize properly to the apical side and has reduced levels in the salivary gland epithelium. Crb, *green*; DAPI, *red*. Schematic drawing of Crb localization in the salivary gland epithelium is shown below each photo (*D* and *E*). *F*, percentage of embryos with Crb mislocalization in salivary gland epithelium when Dg is overproduced using the *Daughterless-Gal4* driver (*Control*, 2.8, $n = 48$; *FL*, 37 ± 4, $n = 46$; *4P*, 38 ± 0.5, $n = 29$; *DC2*, 33 ± 6, $n = 15$; P → A, 13 ± 4, $n = 40$; *C2*,14.5 ± 0.5, $n = 27$; *C1*, 11 ± 0.5, $n = 28$). *G*, percentage of stage 3–6 egg chambers with mislocalized or absent Orb when pUASp-Dg is overproduced using the *MatTub-Gal4* driver (*Control*, 10 ± 0.1, $n = 125$; *FL*, 48 ± 8, $n = 117$; *4P*, 46 ± 3, $n = 194$; *DC2*, 53 ± 2, $n = 122$; P → A, 22 ± 2, $n = 140$; *C2*, 33 ± 2, $n = 135$; *C1*, 28 ± 4, $n = 118$). *H*, percentage of stage 6–10 egg chambers with late stage Orb defects when Dg is overproduced using the *MatTub-Gal4* driver (*Control*, 11 ± 1, $n = 74$; *FL*, 80 ± 8, $n = 323$; *4P*, 79 ± 9, $n = 492$; *DC2*, 69 ± 3, $n = 355$; P → A, 51 ± 6 $n = 330$; *C2*, 46 ± 10, $n = 264$; *C1*, 49 ± 3, $n = 359$). The *error bars* represent differences between different independent insertions lines.

sites had been mutated, has a reduced capacity to affect oocyte polarity, similar to the C1 construct, which lacks all the potential binding sites (Fig. 3*G*). This confirms that the putative SH3 domain binding sites are essential for the full function of the Dg protein in this assay.

We also examined the ability of the various Dg constructs to disrupt oocyte polarity at later stages and observed a similar trend; a higher frequency of phenotypes were observed for all constructs, but C1, C2, and P → A showed less of a capacity to disrupt Orb localization than FL, 4P, and DC2 (Fig. 3*H*). This indicates that presence of at least one pair of WW domain and putative SH3 domain binding sites can disrupt the oocyte polarity during early and late stages of oogenesis and is therefore important for Dg function in the context of this assay.

We also considered whether the amount of disrupted oocyte polarity was simply a result of the level of Dg protein overproduction, rather than the type of Dg construct. We compared the level of Dg production with the degree of Orb mislocalization and found no correlation. For example, different insertion lines with the DC2 construct exhibited a large range in the level of Dg overproduction when induced with the *MatTub-Gal4* driver (supplemental Fig. S1*A*); however, the range in the level of Orb mislocalization was very small (Fig. 3, *G* and *H*). This suggests that the amount of Dg overproduction in the oocyte beyond a 2-fold level is not responsible for the changes in oocyte polarity and, therefore, that the differences in disruption of oocyte polarity are the result of the presence of significant domain binding motifs.

The Region of the Dystroglycan C Terminus Containing WWbsII and SH3bsII Is Sufficient to Disrupt Salivary Gland Apical-Basal Polarity—Previous work has indicated that overproduction of Dg in the salivary gland is sufficient to disrupt epithelial cell apical-basal polarity (26). The salivary gland epithelium has a very clear cell polarity; in wild type embryos, the polarity marker Crumbs localizes to the apical side of the salivary gland (Fig. 3, *D–D"*). However, when Dg is overproduced in the embryo using the *Daughterless-Gal4* driver, Crumbs fails to localize normally in tissue (Fig. 3, *E–E"*, and *F*) (26).

To determine which potential Dg C-terminal signaling molecule binding sites are sufficient to disrupt salivary gland epithelium polarity, we expressed each Dg construct in the embryo using the *Daughterless-Gal4* driver and quantified the percent-

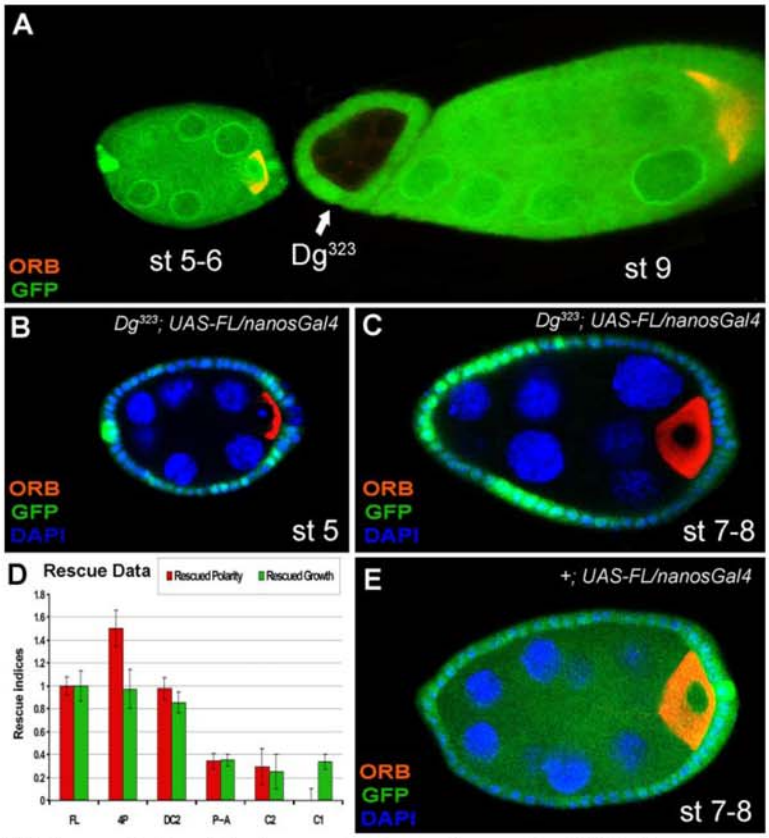


FIGURE 4. **Rescue of Dg loss-of-function germ line clones with expression of pUASp-Dg constructs.** Orb, *red*; GFP, *green*; DAPI, *blue*. *A*, Dg loss-of-function germ line clones (*black, white arrow; hsFLP; FRT42D Dg*^{323}) are arrested prior to stage 6 and have disrupted oocyte polarity (absent or mislocalized Orb). *B*, expression of pUASp-Dg with the *nanos-Gal4* driver in *Dg* clones rescues oocyte polarity in arrested clones stages 3–6 (as indicated by proper localization of Orb to the posterior of the oocyte (*hsFLP; FRT42D Dg*^{323}*; P(w+:nanosGal4:VP-16)Ab-2/pUASp-FL*)). *C*, expression of pUASp-Dg with the *nanos-Gal4* driver in Dg clones rescues oocyte growth to stage 7 or 8 (*hsFLP; FRT42D Dg*^{323}*; P(w+:nanosGal4:VP-16)Ab-2/pUASp-FL*). *D*, FL, 4P, and DC2 are able to rescue Dg loss-of-function phenotypes, whereas P → A, C2, and C1 do not (*red*, rescued polarity index; *green*, rescued growth index). The differences in rescue values between *FL, 4P, DC2, P→A, C2,* and *C1* are significant, and errors are based on independent experiments using independent insertion lines. *E*, although at earlier stages (4–6) the polarity marker Orb is normally localized at the posterior,and at later stages (7–8) Orb uniformly stains the oocyte cytoplasm (*hsFLP; FRT42D-UbiGFP; P(w+:nanosGal4:VP-16)Ab-2/pUASp-FL*).

age of embryos with mislocalized Crumbs staining in the salivary gland (Fig. 3*F*). DC2 and 4P constructs were capable of disrupting salivary gland epithelium polarity as well as FL (Fig. 3, *E–E″* and *F*); however, C1, C2, and P → A constructs did not disrupt polarity to the same extent as FL (Fig. 3*F*). Nevertheless, in all the assays described, some phenotypes above the control level were observed even with the constructs that lack most of the C-terminal domain (*C1*; Fig. 3, *F–H*), indicating that the Dg extracellular domain alone might function in some capacity to regulate cellular polarity (similar to seen in other systems (30, 39).

The Region of the Dystroglycan C Terminus Containing WWbsII and SH3bsII Can Rescue Dg Loss-of-Function Defects in Oocyte—Because Dg is required for oocyte polarity and the overproduction of Dg is sufficient to alter polarity, we analyzed which part of the Dg C-terminal proline-rich region is required for this function. We tested the capacity of our constructs to rescue the polarity of *Dg* germ line mutants. To avoid the polarity defects caused by overexpression of Dg at high levels in the germ line (*MatTub-Gal4*, Fig. 3, *B, C, G,* and *H*), we chose a *nanos-Gal4* driver that induces only low levels of Dg expression at stages 2–6 in oogenesis (supplemental Fig. S2*B*),and causes lower percentage of polarity defects even in the presence of two copies of the *nanos-Gal4* driver (supplemental Fig. S1*B* and supplemental Fig. S2*C*). Dg constructs expressed by only one *nanos-Gal4* copy resulted in even lower levels of Orb mislocalization (data not shown). We analyzed the ability to rescue *Dg* loss-of-function oocyte polarity defects using Dg constructs (Fig. 1) driven by one copy of *nanos-Gal4*. Low levels of FL Dg can partially rescue the *Dg* mutant phenotype, whereas C1 and C2 constructs do not have the same rescue capacity (Fig. 4, *A–D,* and Table 1). As with the overexpression experiments, this indicates that the other C-terminal binding sites, in addition to WWbsI, play a role in the establishment of oocyte polarity. Interestingly, the construct with defective SH3 domain binding sites (P → A) was also unable to rescue polarity to the FL levels (Fig. 4*D,* Table 1) further supporting the idea that SH3 domain binding sites in Dg play an important role in the oocyte polarity. DC2 and 4P, however, rescued oocyte polarity at the same level as the FL (Fig. 4*D* and Table 1), indicating that a single WW domain binding site and a single putative SH3 domain binding site on Dg C terminus are sufficient to partially rescue the establishment of oocyte polarity prior to stage 6, more specifically the anterior to posterior translocation of the microtubular organizing center during stages 1–3.

The Region of the Dystroglycan C Terminus Containing WWbsII and SH3bsII Can Rescue Egg Chamber Growth—In addition to oocyte polarity, Dg is required for egg chamber growth and development; most Dg mutant clones arrest by stage 6 (Fig. 4*A*) (26). To test which proline-rich sites in the C terminus of Dg, if any, are required to rescue egg chamber growth, we expressed the different Dg constructs (Fig. 1*B*) in *Dg* mutant clones using the *nanos-Gal4* driver expressing only one copy of *nanos-Gal4*, as was done for the polarity assay, and we tested how far these egg chambers developed.

TABLE 1
Dg constructs containing putative SH3- and WW-binding sites can rescue Dg loss-of-function phenotypes

Genotype	Egg chambers with growth rescue[a]	Egg chambers with normal Orb localization
Oregon	100%	90 ± 0.1%, n = 125
Dg^{323}[b]	12 ± 2%, n = 54[c,d]	8 ± 5%, n = 58
Dg^{323}; *nanosGal4/pUASp-FL*	47 ± 7%, n = 40	28 ± 2%, n = 116
Dg^{323}; *nanosGal4/pUASp-4P*	46 ± 8%, n = 38	38 ± 4.6%, n = 68
Dg^{323}; *nanosGal4/pUASp-DC2*	42 ± 3%, n = 48	28 ± 3%, n = 68
Dg^{323}; *nanosGal4/pUASp-P → A*	25 ± 3%, n = 49	15 ± 2%, n = 49
Dg^{323}; *nanosGal4/pUASp-C2*	21 ± 7%, n = 46	14 ± 5%, n = 43
Dg^{323}; *nanosGal4/pUASp-C1*	24 ± 3%, n = 11	8 ± 3%, n = 40
Dg^{323}; *nanosGal4/pUASp-AATA*	NA[e]	41 ± 1%, n = 35
Dg^{323}; *nanosGal4/pUASp-ALLP*	NA	16 ± 5%, n = 45

[a] Growth rescue data represent the percentage of mutant clones stage 6 and above from all clones that have reached at least stage 4.
[b] Dg^{323} indicates *hsFLP; FRT42D* Dg^{323}.
[c] ± indicates the average deviation calculated from analysis of independent insertion lines.
[d] n indicates the number of counted egg chambers with the correct genotype.
[e] NA indicates not analyzed.

To determine the degree to which full-length Dg could rescue egg chamber growth using this assay, we first expressed the FL construct in Dg clones and observed that FL was partially able to rescue growth (Fig. 4, *C* and *D*; Table 1). These "rescued" clones were classified as stage 7–8 based on the size of their egg chambers. However, in many cases, the oocyte was smaller than in wild type stage 7–8 oocytes.

Similar to what has been seen with the overexpression and polarity loss-of-function experiments, C1, C2, and P → A constructs were not able to rescue the growth to the FL levels (Fig. 4, *A–D*; Table 1). This indicates that the other C-terminal binding sites, in addition to WwbsI, play a role in the egg chamber growth and that putative SH3 domain binding sites are essential for full Dg function in the context of this assay. Nevertheless, some rescue above the control level was observed even with the construct that lacks most of the C-terminal domain (*C1*, Fig. 4*D*; Table 1), indicating that, as discussed earlier, the Dg extracellular domain alone might function in some capacity to regulate egg chamber growth. This is similar to what is seen with skeletal myotubes (39). DC2 and 4P, however, rescued egg chamber growth at the same level as the FL (Fig. 4*D*; Table 1), indicating that a single WW domain binding site in addition to a single putative SH3 domain binding site are sufficient for the function of Dg proline-rich C terminus for egg chamber growth.

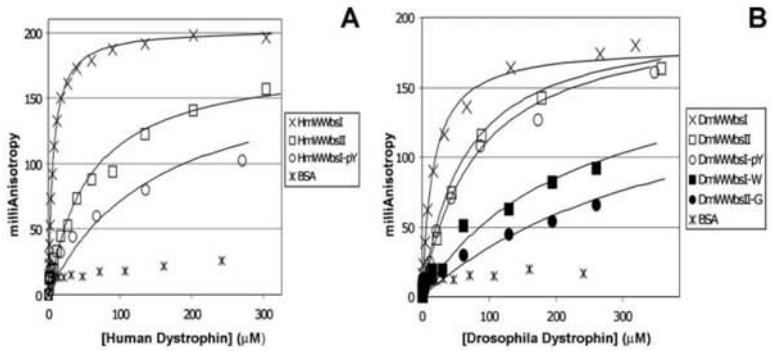
Again, we considered whether the ability to rescue growth and polarity was simply a result of the level of Dg protein overproduction, rather than the type of Dg construct. We compared the level of Dg production with the degree of polarity and growth rescue and found no correlation (supplemental Fig. S1, *C–D*). For example, FL expression varied between 2 and 4 times greater than wild type. However, the level of polarity or growth rescue did not correlate with the level of protein. This suggests that the amount of Dg overproduction in the oocyte is not responsible for differences in the degree of rescue and, therefore, that the differences in ability to rescue polarity and growth are a result of the presence of significant binding motifs.

Drosophila and Human Dystrophin Bind to Dystroglycan WWbsI and WWbsII in Vitro—Both overproduction and rescue experiments indicate that DC2 is able to affect cellular polarity to a similar extent as FL. DC2 includes one putative SH3 domain binding motif (SH3bsII) and one WW domain binding motif (WWbsII). Previous work in our laboratory has established the effectiveness of using a fluorescence polarization assay to measure binding of dystrophin (WW + EF regions) to the first WW domain binding motif (WWbsI) of dystroglycan (25). To assess the ability of dystrophin to bind this second WW domain binding motif (WWbsII), a second *Drosophila* dystroglycan peptide (DmWWbsII) that includes both the SH3bsII and WWbsII domain binding motifs present in DC2 was used. *Drosophila* dystrophin binds to DmWWbsII with a K_d of 46 ± 18 μM (Fig. 5, *A–C*). The affinity of this interaction is lower than that of *Drosophila* dystrophin and WWbsI (16 ± 4 μM), but it is still well within the range of reported dissociation constants for class I WW domains (49). In contrast, mutations predicted to abolish the WW (but not the SH3) binding domain resulted in much lower affinities (DmWWbsI-W, 178 μM and DmWWbsII-G,147 μM; Fig. 5, *B* and *C*). These values are comparable with the K_d value observed with a negative control for the assay (K_d for an unrelated peptide, p53 is 248 μM; Fig. 5*C*).

The second WW domain binding motif is conserved in human Dg, and the unexpected result above prompted us to investigate the same interaction between human dystrophin and human dystroglycan. Again, a second human dystroglycan peptide (HsWWbsII) was assayed for binding to human dystrophin. Human dystrophin binds to HsWWbsII with a K_d of 81 ± 11 μM, demonstrating that this interaction first seen with *Drosophila* peptides can also be seen with the corresponding human peptides (Fig. 5, *A–C*).

To further examine the specificity of the interaction between dystrophin and the second WW domain of dystroglycan, we tested the ability of human dystrophin to bind *Drosophila* dystroglycan peptide and vice versa. Human dystrophin does not bind to DmWWbsII (K_d 282 ± 18 μM); however, *Drosophila* dystrophin does bind to HsWWbsII (59 ± 10 μM; Fig. 5, *A–C*).

The observed importance of the SH3 domain binding sites in Dg (Fig. 1*B*, Fig. 3, *F–H*, and Fig. 4*D*) brings up the possibility that the SH3 domain of a tyrosine kinase could dock on that site, phosphorylate the tyrosine in WW-binding sites, and thereby affect dystrophin WW domain binding to this site. To test on what level tyrosine phosphorylation affects the WW domain binding in this assay, we tested dystrophin binding to Dg peptides that are tyrosine-phosphorylated (DmWWbsI-pY, DmWWbsII-pY, and HmWWbsI-pY). In both *Drosophila* and humans, tyrosine phosphorylation dramatically reduced WW domain binding (86 μM



C **Dissociation Constants (K_d, μM) for Dystrophin-Dystroglycan Interactions in human and *Drosophila***

Dystroglycan WWbs	Sequence	Dystrophin WW + EF Hand	
		Human	Drosophila
HmWWbsI	KNMTPYRSPPPYVSP	7.6 ± 1.6 (×)	3.7 ± 0.3
HmWWbsII	PLILQEEKAPLPPPEYSN	81 ± 11 (□)	59 ± 10
HmWWbsI-pY	KNMTPYRSPPP**pY**VSP	100 (○)	ND
DmWWbsI	GKSPATPYRKPPPYVSP	24 ± 8	16 ± 4 (×)
DmWWbsII	PVILKDEKPPLLPPSYNT	282 ± 18	46 ± 18 (□)
DmWWbsI-pY	GKSPATPYRKPPP**pY**VSP	ND	86 ± 10 (○)
DmWWbsII-pY	PVILKDEKPPLLPPS**pY**NT	ND	112 ± 5
DmWWbsI-W	GKSPATPYRK**W**PPYVSP	ND	178 ± 81 (■)
DmWWbsII-G	PVILKDEKPPLLPPS**G**NT	ND	147 ± 34 (●)
DmSH3bsII-A	PVILKDEKP**A**LLPPSYNT	ND	54 ± 20
p53	SQETFSDLWLLPEN		248

ND - not determined

FIGURE 5. Human and *Drosophila* dystrophin binds both dystroglycan WW-binding sites *in vitro* (*A and B*). Fluorescence polarization analysis of human dystrophin binding to 200 nM HmWWbsI (×), HmWWbsII (□), and HmWWbsI-pY (○) reveals binding to all peptides but shows significantly higher affinity for HmWWbsI and HmWWbsII compared with HmWWbsI-pY. *B, Drosophila* dystrophin binds to both the DmWWbsI (×) and DmWWbsII (□) but shows a significant loss of binding when these sites are mutated in DmWWbsI-W (■) and DmWWbsII-G (●) peptides. Tyrosine phosphorylation of WWbsI (DmWWbsI-pY) (○) results in reduced binding compared with nonphosphorylated DmWWbsI (×). Bovine serum albumin (*BSA*; *) binding by HmWWbsI (*A*) or DmWWbsI (*B*) serves as a nonspecific protein binding control. *C, table* that represents the dissociation constants (K_d, μM) for dystrophin-dystroglycan interactions in human and *Drosophila*.

compared to 16 μM, 112 μM to 46 μM and 100 μM to 7.6 μM, respectively; Fig. 5, *A–C*).

A Putative SH3 Domain Binding Motif Is Critical for Dg Function in Oocyte Polarity—Both overexpression and loss-of-function experiments revealed that SH3 domain binding sites in the Dg C terminus are essential for its function in cellular polarity. To further dissect which of the two SH3 domain binding sites is critical, we disrupted each site independently by proline to alanine substitutions and tested the capacity of the mutant proteins to affect oocyte polarity in both loss-of-function and overexpression analyses. Two proline to alanine substitutions in the SH3bsI caused no reduction in the activity of the wild type protein in overexpression and loss-of-function experiments (Fig. 6, *B–D, AATA*; Table 1). In sharp contrast, a single proline to alanine substitution in SH3bsII is functionally equivalent to deletion of the whole proline-rich region (Fig. 6, *B–D, ALLP*; Table 1). Thus, SH3bsII but not SH3bsI appears to be required for Dg function in oocyte polarity. Furthermore, the mutation apparently interferes specifically with SH3 domain binding and not WW domain binding because dystrophin WW domain still binds to the Dg peptide with this mutation *in vitro* (DmSH3bsII-A, K_d = 54 μM; Fig. 5*C*).

DISCUSSION

Unexpectedly, we find that the WW domain binding site at the very C terminus of dystroglycan, which has been previously implicated in dystrophin binding, is not essential for the function of the Dg·Dys complex in cellular polarity in *Drosophila*. Instead, an internal region of the dystroglycan C terminus containing a second WW domain binding site and a putative SH3 domain binding site appears to be sufficient for function in this context. We also find that mutating a single proline to alanine within this conserved, putative SH3 domain binding site dramatically reduces the functionality of this protein when compared with full-length Dg in these assays (Fig. 6, *B–D, ALLP*; Table 1). Finally, we show that dystrophin binds both the C-terminal and the internal WW domain binding site *in vitro* and that these interactions are conserved between humans and flies (Fig. 6*A*). Taken together, these results suggest that the internal WW domain binding site can mediate interactions with dystrophin, and presently unidentified SH3 domain containing protein(s) may functionally interact with a conserved region of the dystroglycan C terminus.

Previous studies (16–18) have indicated that dystrophin primarily binds to the first but not the second PPXY motif of dystroglycan. In contrast, here we show that dystrophin can indeed bind this second WW domain binding motif (WWbsII), and we suggest that from the C-terminal proline-rich region this site in combination with an SH3 domain binding site is sufficient for Dg C-terminal function in the establishment of cellular polarity in *Drosophila*. *In vitro*, dystrophin binds WWbsII with lower affinity than WWbsI (46 μM compared with 16 μM). It appears that WWbsII functions just as well as WWbsI in our *in vivo* assays, because the DC2 phenotype is similar to the FL phenotype in both overproduction and loss-of-function experiments.

The *in vitro* interaction between dystrophin and the Dg WWbsII is conserved in humans. This is interesting in light of the fact that mutations in Dg are not observed clinically in patients with MD; instead, mutations in Dys, Dg-modifying enzymes, or extracellular matrix proteins result in MD (1). Because Dg knockouts die during embryonic development in mice and as an oocyte in *Drosophila*, it was assumed that the

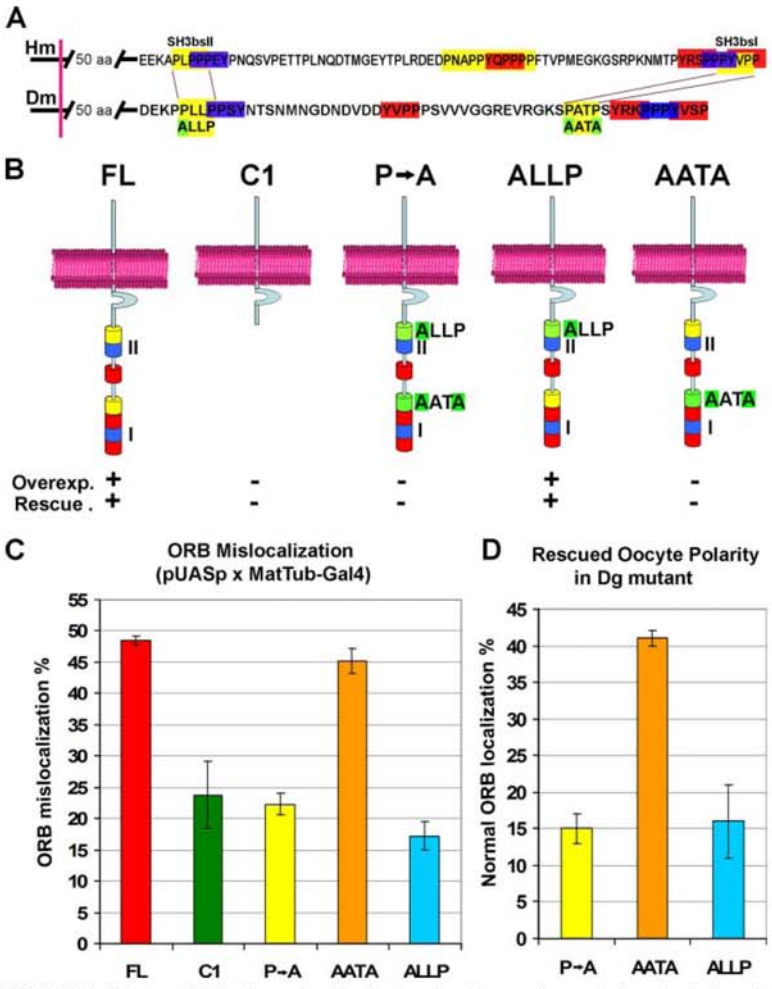


FIGURE 6. **SH3bsII but not SH3bsI is required for Dg function.** Two proline to alanine substitutions in the SH3bsI (*AATA*; *A* and *B*) cause no reduction in the activity of the wild type protein in overexpression (*C*, *Mat-α4-Tub > Gal4-UP16; pUASp-Dg*) or loss-of-function rescue (*D*, *hsFLP; FRT42D Dg*^{323}*; P(w+:nanosGal4:VP-16)Ab-2/p-pDg*) assays. In sharp contrast, a single proline to alanine substitution in SH3bsII (*ALLP*, *A* and *B*) completely eliminates the activity and is functionally equivalent to deletion of the whole proline-rich region (*C* and *D*). SH3bsII is conserved between human (*Hm*) and *Drosophila* (*Dm*) proline-rich C terminus of Dg (SH3bsII, *A*). SH3bs, *yellow*; SH3bs, *red*; WWbs, *blue*.

lack of MD patients with any mutations in Dg could be explained by its lethality. However, the results presented suggest a potentially new explanation; perhaps WWbsI and WWbsII are redundant. Perhaps humans with a mutation in WWbsI exist, but they do not show any MD phenotypes because WWbsII can substitute in place of the mutant WW domain binding site.

As discussed previously, *in vitro* work suggests that when the tyrosine of the PPPY motif (WWbsI) is phosphorylated, the binding between Dg and Dys is abolished (19), signifying that the Dg·Dys complex may be regulated in a tyrosine phosphorylation-dependent manner. In this study, we show using a quantitative assay that tyrosine phosphorylation of either of the two WW domain binding sites, PPPY motif or PPSY motif, does reduce the binding affinity (Fig. 5, *A–C*), suggestive of fine regulation. In addition, our *in vivo* work indicates that a putative SH3 domain binding site in Dg is required for proper function of the protein. These data suggest a more specific mechanism of regulation. One possibility is that an SH3 domain containing tyrosine kinase may dock to the SH3 domain binding site in Dg. This may result in a kinase activation and phosphorylation of the WW domain binding site in Dg thereby reducing dystrophin binding to this site.

The evidence thus far regarding the regulation of the Dg-Dys interaction depicts a model that strikingly resembles what we know about integrin-talin interactions (40–42). Integrins are heterodimeric, transmembrane proteins that like dystroglycan link the extracellular matrix to the intracellular cytoskeleton. The NP*X*Y motif on the integrin β subunit interacts with talin, an actin-binding protein, via the F3 subdomain within the FERM domain of talin, a PTB-like domain (43). Talin plays a role analogous to dystrophin by binding the NP*X*Y motif on integrin β cytoplasmic tails and linking integrins to the actin cytoskeleton. Binding of Talin to the NP*X*Y motif is required for energy-dependent activation of integrins (44). In addition to performing analogous structural roles, a similar regulatory mechanism may exist. It is known that integrin-talin interaction is mediated in a phosphorylation-dependent manner. When the tyrosine of the NP*X*Y motif is phosphorylated, binding of the integrin to talin is abolished (43, 45–47). Focal adhesion kinase and integrin-linked kinase bind to integrins *in vitro* and may regulate integrin-talin interaction, although this remains to be demonstrated *in vivo*. Furthermore, several other proteins, including platelet myosin, SHC, and Grb2, have been shown to bind integrins in their phosphorylated state *in vitro* (48). This study provides evidence that a similar mechanism may act to regulate Dg-Dys interaction. We have shown that a P*XX*P motif, which may be an SH3 domain binding site, is important for Dg function, opening up the possibility that an SH3 domain containing kinase may bind to Dg and phosphorylate the tyrosine on the WW domain binding site. Other signaling molecules may then interact with Dg in a phosphorylation-dependent manner.

We have identified two putative signaling molecule binding sites, the second WW domain binding site and a putative SH3

domain binding site, that are important for the regulation of the Dg complex. The key question now is to identify the signaling molecules that bind to these sites. Previous work has revealed SH3 domain-mediated interaction between Dg and Grb2 (21). However, we have not been able to observe direct binding between Dg and Drk, a *Drosophila* homologue of Grb2 (K_d = 480 μM), suggesting a different candidate for the SH3 domain interaction in *Drosophila*. Identification of the critical molecule that will associate with the putative SH3 domain binding site in Dg will further our understanding of the role Dg plays in signaling and may provide new insights into the pathogenesis of muscular dystrophy.

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REFERENCES

1. Davies, K. E., and Nowak, K. J. (2006) *Nat. Rev. Mol. Cell Biol.* **7,** 762–773
2. Barresi, R., and Campbell, K. P. (2006) *J. Cell Sci.* **119,** 199–207
3. Ervasti, J. M., Ohlendieck, K., Kahl, S. D., Gaver, M. G., and Campbell, K. P. (1990) *Nature* **345,** 315–319
4. Lapidos, K. A., Kakkar, R., and McNally, E. M. (2004) *Circ. Res.* **94,** 1023–1031
5. Langenbach, K. J., and Rando, T. A. (2002) *Muscle Nerve* **26,** 644–653
6. Cohn, R. D., Henry, M. D., Michele, D. E., Barresi, R., Saito, F., Moore, S. A., Flanagan, J. D., Skwarchuk, M. W., Robbins, M. E., Mendell, J. R., Williamson, R. A., and Campbell, K. P. (2002) *Cell* **110,** 639–648
7. Moore, S. A., Saito, F., Chen, J., Michele, D. E., Henry, M. D., Messing, A., Cohn, R. D., Ross-Barta, S. E., Westra, S., Williamson, R. A., Hoshi, T., and Campbell, K. P. (2002) *Nature* **418,** 422–425
8. Cohn, R. D. (2005) *Neuromuscul. Disord.* **15,** 207–217
9. Martin-Rendon, E., and Blake, D. J. (2003) *Trends Pharmacol. Sci.* **24,** 178–183
10. Martin, P. T. (2003) *J. Neurocytol.* **32,** 915–929
11. Martin, P. T., and Freeze, H. H. (2003) *Glycobiology* **13,** R67–R75
12. Muntoni, F., Brockington, M., Torelli, S., and Brown, S. C. (2004) *Curr. Opin. Neurol.* **17,** 205–209
13. Sgambato, A., and Brancaccio, A. (2005) *J. Cell. Physiol.* **205,** 163–169
14. Henry, M. D., Cohen, M. B., and Campbell, K. P. (2001) *Hum. Pathol.* **32,** 791–795
15. Muschler, J., Levy, D., Boudreau, R., Henry, M., Campbell, K., and Bissell, M. J. (2002) *Cancer Res.* **62,** 7102–7109
16. Jung, D., Yang, B., Meyer, J., Chamberlain, J. S., and Campbell, K. P. (1995) *J. Biol. Chem.* **270,** 27305–27310
17. Rentschler, S., Linn, H., Deininger, K., Bedford, M. T., Espanel, X., and Sudol, M. (1999) *Biol. Chem.* **380,** 431–442
18. Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M., and Eck, M. J. (2000) *Nat. Struct. Biol.* **7,** 634–638
19. Sotgia, F., Lee, H., Bedford, M. T., Petrucci, T., Sudol, M., and Lisanti, M. P. (2001) *Biochemistry* **40,** 14585–14592
20. Ilsley, J. L., Sudol, M., and Winder, S. J. (2002) *Cell. Signal.* **14,** 183–189
21. Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K. P. (1995) *J. Biol. Chem.* **270,** 11711–11714
22. Spence, H. J., Dhillon, A. S., James, M., and Winder, S. J. (2004) *EMBO Rep.* **5,** 484–489
23. Zhou, Y. W., Thomason, D. B., Gullberg, D., and Jarrett, H. W. (2006) *Biochemistry* **45,** 2042–2052
24. Kay, B. K., Williamson, M. P., and Sudol, M. (2000) *FASEB J.* **14,** 231–241
25. Shcherbata, H. R., Yatsenko, A. S., Patterson, L., Sood, V. D., Nudel, U., Yaffe, D., Baker, D., and Hannele Ruohola-Baker, D. (2007) *EMBO J.* **26,** 481–493
26. Deng, W. M., Schneider, M., Frock, R., Castillejo-Lopez, C., Gaman, E. A., Baumgartner, S., and Ruohola-Baker, H. (2003) *Development (Camb.)* **130,** 173–184
27. Schneider, M., Khalil, A. A., Poulton, J., Castillejo-Lopez, C., Egger-Adam, D., Wodarz, A., Deng, W. M., and Baumgartner, S. (2006) *Development (Camb.)* **133,** 3805–3815
28. Poulton, J. S., and Deng, W. M. (2006) *Proc. Natl. Acad. Sci. U. S. A.* **103,** 12775–12780
29. van der Plas, M. C., Pilgram, G. S., Plomp, J. J., de Jong, A., Fradkin, L. G., and Noordermeer, J. N. (2006) *J. Neurosci.* **26,** 333–344
30. Weir, M. L., Oppizzi, M. L., Henry, M. D., Onishi, A., Campbell, K. P., Bissell, M. J., and Muschler, J. L. (2006) *J. Cell Sci.* **119,** 4047–4058
31. Tracey, W. D., Jr., Ning, X., Klingler, M., Kramer, S. G., and Gergen, J. P. (2000) *Genetics* **154,** 273–284
32. Van Doren, M., Williamson, A. L., and Lehmann, R. (1998) *Curr. Biol.* **8,** 243–246
33. Hacker, U., and Perrimon, N. (1998) *Genes Dev.* **12,** 274–284
34. Wodarz, A., Hinz, U., Engelbert, M., and Knust, E. (1995) *Cell* **82,** 67–76
35. Pignoni, F., and Zipursky, S. L. (1997) *Development (Camb.)* **124,** 271–278
36. Rorth, P. (1998) *Mech. Dev.* **78,** 113–118
37. Kahl, J., and Campanelli, J. T. (2003) *J. Neurosci.* **23,** 392–402
38. Heathcote, R. D., Ekman, J. M., Campbell, K. P., and Godfrey, E. W. (2000) *Dev. Biol.* **227,** 595–605
39. Tremblay, M. R., and Carbonetto, S. (2006) *J. Biol. Chem.* **281,** 13365–13373
40. Brown, N. H., Gregory, S. L., Rickoll, W. L., Fessler, L. I., Prout, M., White, R. A., and Fristrom, J. W. (2002) *Dev. Cell* **3,** 569–579
41. Carman, C. V., and Springer, T. A. (2003) *Curr. Opin. Cell Biol.* **15,** 547–556
42. Kinashi, T. (2005) *Nat. Rev. Immunol.* **5,** 546–559
43. Campbell, I. D., and Ginsberg, M. H. (2004) *Trends Biochem. Sci.* **29,** 429–435
44. Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003) *Science* **302,** 103–106
45. Tapley, P., Horwitz, A., Buck, C., Duggan, K., and Rohrschneider, L. (1989) *Oncogene* **4,** 325–333
46. Ling, K., Doughman, R. L., Iyer, V. V., Firestone, A. J., Bairstow, S. F., Mosher, D. F., Schaller, M. D., and Anderson, R. A. (2003) *J. Cell Biol.* **163,** 1339–1349
47. Calderwood, D. A. (2004) *Biochem. Soc. Trans.* **32,** 434–437
48. Liu, S., Calderwood, D. A., and Ginsberg, M. H. (2000) *J. Cell Sci.* **113,** 3563–3571
49. Kato, Y., Ito, M., Kawai, K., Nagata, K., and Tanokura, M. (2002) *J. Biol. Chem.* **277,** 10173–10177

Supplementary Information

Figure S1

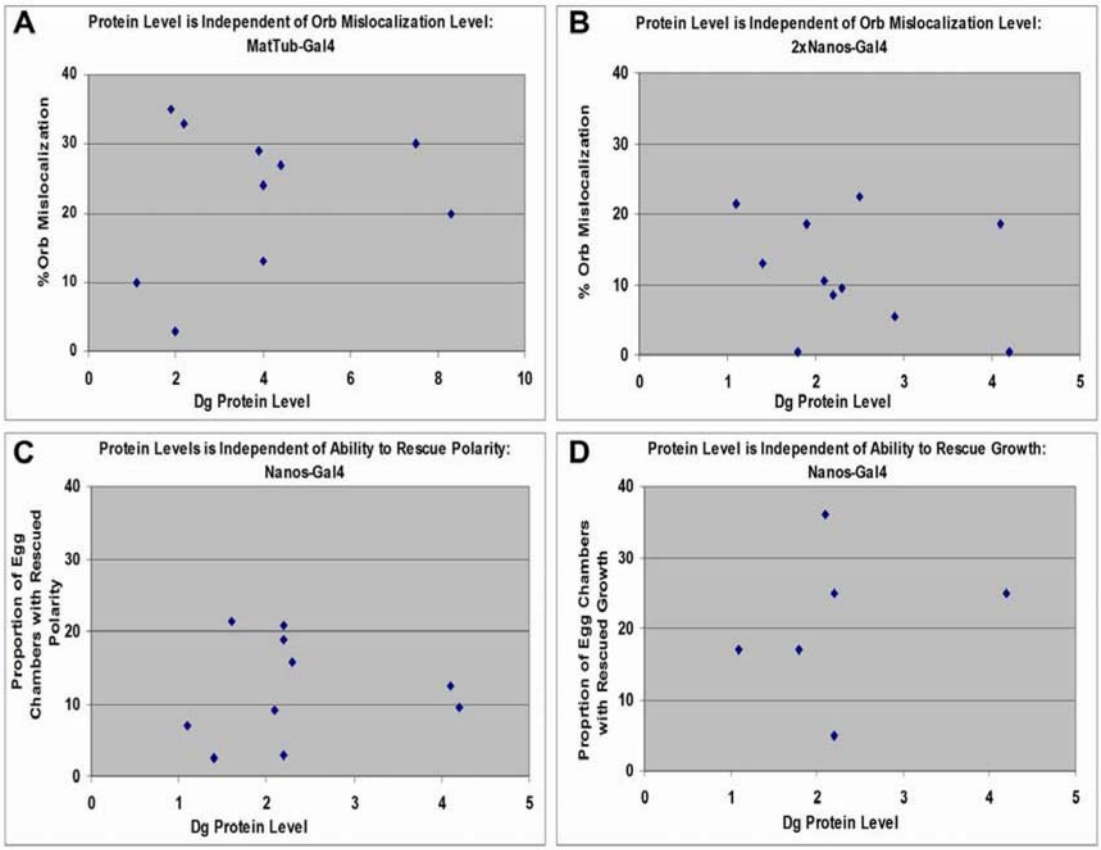

Fig. S1. Levels of Dystroglycan protein expression do not correlate with the frequency of observed phenotypes or its ability to rescue. *A.* Frequency of Orb mislocalization with varying levels of pUASp-Dg expression driven by *MatTub-Gal4. B.* Frequency of Orb mislocalization with varying levels of pUASp-Dg expression driven by two copies of *nanos-Gal4*. *C.* Levels of Dg expression do not correlate with ability to rescue oocyte polarity. *D.* Levels of Dg expression no not correlate with ability to rescue oocyte growth.

Figure S2

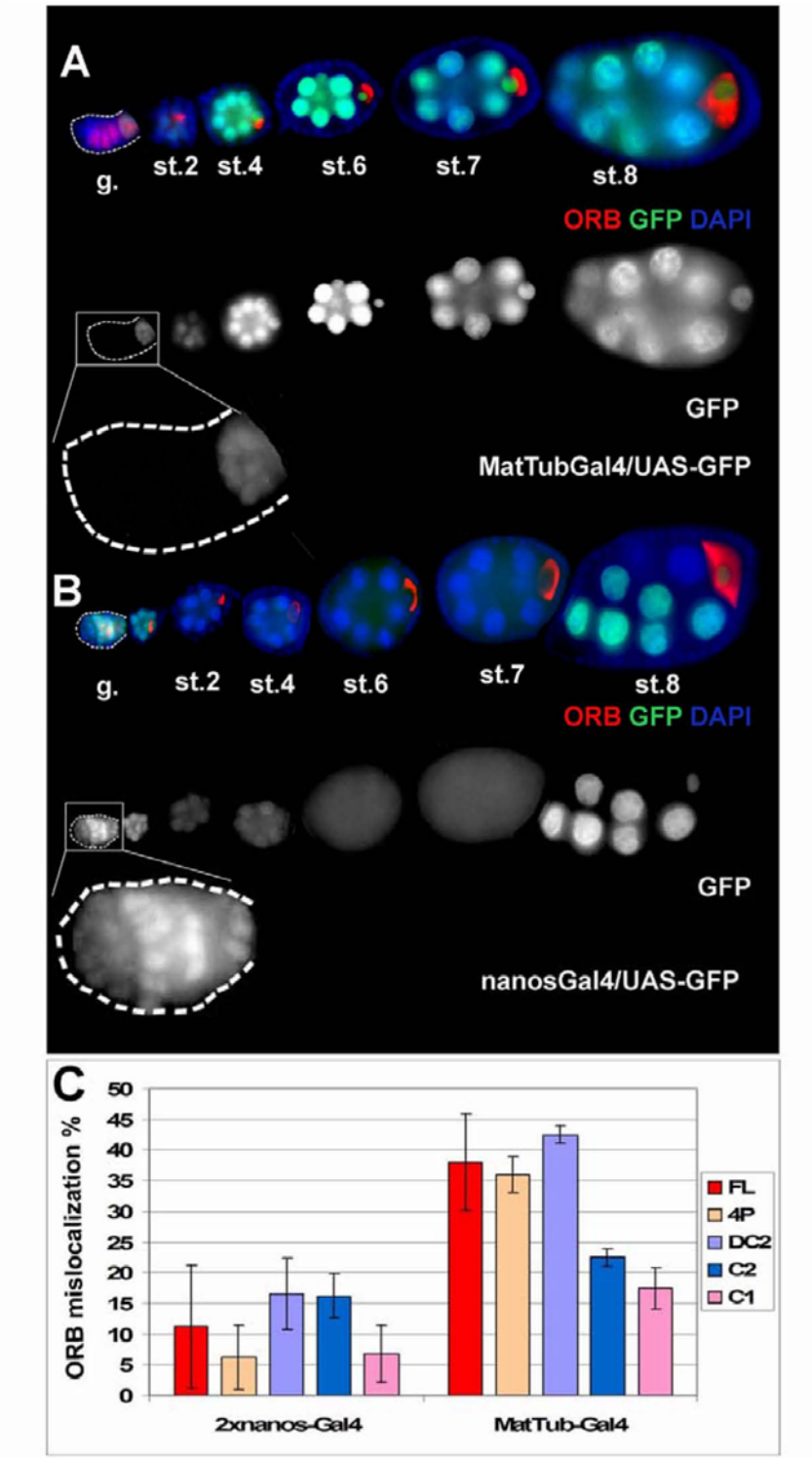

Fig. S2. Expression patterns of *MatTub-Gal4* and *nanos-Gal4 drivers.* Orb (Red)*;* GFP (Green); DAPI (Blue) *A.* GFP expression using *MatTub-Gal4* driver. High levels of GFP can be observed at all stages of egg development, except early germarium (white box, dashed line). *B.* GFP expression using two copies of *nanos-Gal4* driver. High level of GFP is present in germarium (white box, dashed line), low level at st.2-7 and high level thereafter. *C.* Dg constructs driven by a Gal4-line that contained two copies of the *nanosGal4* show lower levels of overexpression phenotype in comparison to *MatTubGal4* driver.

3.2. The conserved WW-domain binding sites in Dystroglycan C-terminus are essential but partially redundant for Dystroglycan function

My previous studies have shown that both PPxY motifs on the *Drosophila* C-terminal cytoplasmic domain of Dystroglycan can bind Dystrophin protein *in vitro* (Yatsenko et al., 2007), indicating the potential importance of the WWbsII as well as WWbsI for Dystroglycan function. Additionally, the *in vivo* structure-functional analyses have revealed that a specific set of C-terminal domains are critical for the function of Dystroglycan. It was found that a putative SH3 binding motif but, surprisingly, not the most C-terminal Dystrophin WW domain binding motif is required for Dystroglycan function in cellular polarity in *Drosophila* (Yatsenko et al., 2007**)**. However, since two potential WW binding sites exist near the Dystroglycan C-ternimus, it is possible that the second WW binding site can bind Dystrophin *in vivo* in order to secure functionality of the complex. In this study I dissected the role of two WW binding sites in the *Drosophila* Dystroglycan C-terminus *in vivo.* For this purpose, the fly strains carrying the transgenic constructs, which encode the forms of Dystroglycan with changes in the C-terminal domain, were generated. Flies expressing full length Dystroglycan (*FL*), with missing the entire C-terminal domain (*C1*), with mutations in both WW binding sites (*2WW*) and two constructs with each of WW binding sites mutated (*PPSG* and *4P* (Yatsenko et al., 2007)) were created. Gain-of-function and rescue analysis showed that each of two PPxY motifs on the C-terminal end of Dystroglycan are required for protein function. In addition, the sequence comparison of WW binding sites in 12 species of *Drosophila*, as well as in humans, reveals that both sites are highly conserved and do not show any variations in analyzed human populations. These findings suggest that both PPxY motifs on the Dystroglycan C-terminus are essential and their functions are partially overlapping.

The conserved WW-domain binding sites in Dystroglycan C-terminus are essential but partially redundant for Dystroglycan function

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*equal contribution

Author contribution to the practical work:

MM Kucherenko and *AS Yatsenko*	All experiments except [1] and [2]
M Pantoja and *KA Fischer*	[1] Western blot analysis of expression of transgenic constructs
J Madeou and *J Akey*	[2] Sequencing and analysis of humans Dystroglycan C-terminus

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The conserved WW-domain binding sites in Dystroglycan C-terminus are essential but partially redundant for Dystroglycan function

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Abstract

Background: Dystroglycan (Dg) is a transmembrane protein that is a part of the Dystrophin Glycoprotein Complex (DGC) which connects the extracellular matrix to the actin cytoskeleton. The C-terminal end of Dg contains a number of putative SH3, SH2 and WW domain binding sites. The most C-terminal PPXY motif has been established as a binding site for Dystrophin (Dys) WW-domain. However, our previous studies indicate that both Dystroglycan PPXY motives, WWbsI and WWbsII can bind Dystrophin protein *in vitro*.

Results: We now find that both WW binding sites are important for maintaining full Dg function in the establishment of oocyte polarity in *Drosophila*. If either WW binding site is mutated, the Dg protein can still be active. However, simultaneous mutations in both WW binding sites abolish the Dg activities in both overexpression and loss-of-function oocyte polarity assays *in vivo*. Additionally, sequence comparisons of WW binding sites in 12 species of *Drosophila*, as well as in humans, reveal a high level of conservation. This preservation throughout evolution supports the idea that both WW binding sites are functionally required.

Conclusion: Based on the obtained results we propose that the presence of the two WW binding sites in Dystroglycan secures the essential interaction between Dg and Dys and might further provide additional regulation for the cytoskeletal interactions of this complex.

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Background

The Dystroglycan-Dystrophin (Dg-Dys) complex has been shown to provide cells with structural integrity by forming a conduit between the extracellular matrix and the cytoskeletal network and there are lines of evidence that implicate an additional signaling role for the complex [1,2] Dystroglycan binds to extracellular matrix components, including Laminin at its N-terminus and the actin cytoskeleton via Dystrophin at its C-terminus [3,4] Defects in these interactions can result in muscular dystrophies (MD) and various epithelial cancers [5]

The characterization of the Dystrophin Glycoprotein Complex (DGC) in *Drosophila* has revealed that it possesses similar roles in muscle integrity and neuronal migration in flies as it does in humans [6] These abnormalities include age dependent muscle degeneration, reduced mobility, defects in eye development as manifested by altered photoreceptor axon path finding and photoreceptor morphology. Additionally, mutations in Dys and Dg affect cell polarity in *Drosophila* [6-8] Interestingly, some of these phenotypes are affected by the nutrition or energy metabolism in the animals [9] Recently, a reduced lifespan, as well as heart and muscle abnormalities, have been reported in *Drosophila* mutants of another component of the DGC, -sarcoglycan [10] and heart and further eye phenotypes have been observed in *Drosophila* Dys and Dg mutants [11,12]

Analogous defects observed when the Dg-Dys complex is disturbed in both flies and humans make *Drosophila* an attractive model for further studies on clarifying the cellular function of the DGC. Recent biochemical and *in vivo* structure-function analyses have revealed that a specific set of C-terminal domains are critical for the function of Dystroglycan. We have found that a putative SH3 domain binding motif but, surprisingly, not the most C-terminal Dystrophin WW domain binding motif is required for Dg function in cellular polarity in *Drosophila* [13]. However, since two potential WW binding sites exist near the Dg C-terminus it is possible that the second WW binding site can also bind Dystrophin *in vivo*, as has been shown *in vitro* [13]. In this study we dissect the roles of the two WW binding sites in the *Drosophila* Dystroglycan C-terminus *in vivo* and, interestingly, find that the sites are essential and their functions are partially overlapping.

Results

In order to understand the regulation of Dg and its role in signaling, we have analyzed the binding motifs that are required for the function of the Dg-Dys complex in cellular polarity in *Drosophila*. The proline-rich C-terminus of Dg has several potential protein binding motifs, which suggests that it may be involved in regulating the complex and potentially may have signaling role(s). Proline-rich sequences have been shown to be the targets of several protein interaction domains involved in signal transduction. The most C-terminal PPxY motif has been established as a binding site for the WW domain of Dystrophin in humans [14-16] and in *Drosophila* by *in vitro* binding studies [6]. However, this WW domain binding site at the very C-terminus of Dystroglycan, is not essential for the function of the Dg-Dys complex in cellular polarity in *Drosophila*. An internal region of the Dystroglycan C-terminus containing a second WW domain binding site and a putative SH3 domain binding site appear to be sufficient for function in this context. We have also shown that Dystrophin can bind both the C-terminal and the internal WW domain binding sites *in vitro* [13]. We now test whether the internal WW domain binding site is essential, whether the two WW domain binding sites are redundant or whether neither is required for Dg function in *Drosophila*. To distinguish between these possibilities we used both overexpression and loss-of-function rescue analyses.

Generation of transgenic lines expressing biochemically verified WWbs mutations

Previous results show that two mutations designed from computer predictions resulted in dramatic alterations in the affinity between Dg and Dys *in vitro* [13] These two mutations, predicted to abolish the WW but not the SH3 binding domain, resulted in very low binding affinities between the Dystroglycan C-terminal peptide and the Dystrophin WW domain with EF-hand region (DmWWbsI-W: Kd = 178 μM and DmWWbsII-G: Kd = 147 μM), as compared to wild type peptides (DmWWbsI: Kd = 16 μM and DmWWbsII: Kd = 46 μM). These values are comparable to the dissociation constant observed with a negative control for the assay (p53: Kd = 248 μM), suggesting that specific binding is abolished. We therefore generated transgenic lines expressing the following representative mutations: PPSG, which has a mismatch in WWbsII (PPSY → PPSG) and 2WW, which has mutations in both WW binding sites (WWbsI, PPPY → WAPY and WWbsII, PPSY → PPSG) (Figure 1). At least two independent transgenic *Drosophila* lines for each construct were obtained and analyzed. Similar results with two independent transgenic lines confirmed that the phenotype was due to the Dg mutation and not due to positional effects of the transgene inserts.

We first tested the ability of the transgenic constructs to produce functional forms of the Dg protein using the *Gal4/UAS* system. In order to overexpress the transgenic constructs in follicle cells we used the *hsFlp; actin-FRT<CD2>FRTGal4/UAS* system in which clonal cells that overexpress the gene of interest were marked with GFP. Dg, in the wild type follicular epithelium, is located at the basal membrane (Figure 2C; WT). Overexpression of the transgenes resulted in Dg localizing to both the apical and

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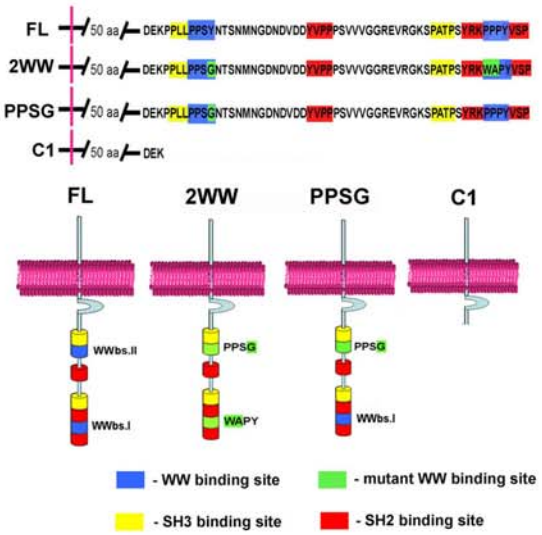

Figure 1
Transgenic constructs with mutations of WW binding sites at the Dystroglycan C-terminal end. Schematic drawing of pUASp constructs with mutations in different WW binding sites. FL – construct which encodes full length Dg, 2WW – constructs with mutations in both WW binding sites, PPSG – mutation in the N-terminal WW binding motif WWbsII PPSY → PPSG. C1 – deletion of the proline-rich C-terminus.

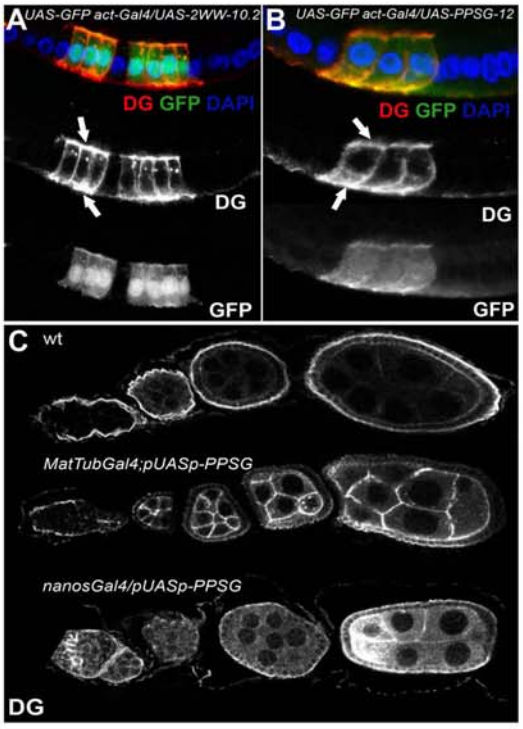

Figure 2
Overexpression of Dg constructs with mutation in WW binding sites in follicle and germline cells. A, B. Overexpression of 2WW (A) and PPSG (B) constructs in follicle cells marked by GFP. Dg in the wild type cells is expressed at the apical side of the follicle cell epithelium, in contrast to overexpression where Dg is localized in both apical and basal sides (indicated by arrows). C. Overexpression of the constructs in the germline cells.wt – Dg expression in wild type germline cells, MatTubGal4;pUASp-PPSG, nanosGal4/pUASp-PPSG – overexpression of transgenic constructs in germline cells. Both MatTub- and nanosGal4 have distinct expression patterns.

basal sides of the follicle cells (Figure 2A, B). We also tested the expression of the constructs in germline cells using the *MatTubGal4* and *nanosGal4* drivers. During oogenesis, Dg is expressed at low levels in the germline (Figure 2C; WT). At stage 2–3 of oogenesis overexpression with *MatTubGal4* shows Dg levels are substantially increased in germline cells (Figure 2C). Increased protein levels were also observed using the *nanosGAl4* driver which showed a distinct pattern starting with high levels in the germarium, lower levels during stages 3–6 and with higher levels during later stages (Figure 2C). Similar patterns and levels of the Dg constructs were observed with all the transgenic lines analyzed in these experiments (Figure 2, Additional Figure 1, Additional Figure 4).

WW binding site function as assayed by oocyte polarity

To analyze whether the Dg mutant forms are functional in oocyte polarity, we expressed mutant and wild type Dg constructs in germline cells using a germline specific driver (*MatTubGal4*), and examined oocyte polarity using Orb protein as a marker. Orb is a member of the cytoplasmic polyadenylation element binding (CPEB) family of RNA-binding proteins that are implicated in local protein synthesis [17]. In *Drosophila* oogenesis Orb co-localizes with the microtubule organizing center (MTOC), which is localized to the anterior of the oocyte during stage 1, and then moves to the posterior by stage 3. Between stages 3 and 6, Orb is clearly localized to the posterior of the oocyte, making it an excellent marker to analyze the polarity of the oocyte (Figure 3A, 4A). Absent or mislocalized Orb during these stages indicates a failure to establish early oocyte polarity.

We have previously shown that overexpression of the wild type form of *Drosophila* Dystroglycan (FL = full length) is sufficient to generate oocyte polarity defects [13] (Figure 3B). When FL is overexpressed in the germline, Orb

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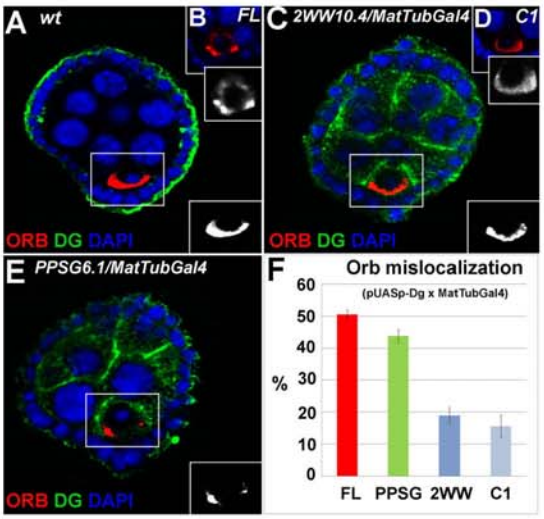

Figure 3
Overexpression of pUASp with MatTubGal4 in germline disrupts the polarity marker Orb. (Orb-red, Dg-green, DAPI – blue, separate channels for Orb are shown on the side of each corresponding picture). A. wild type (wt) stage 4 egg chamber shows normal Orb (red) localization at the posterior side of the oocyte. B. Overexpression of the pUASp-FL transgenic construct disrupts the normal Orb localization. C. Overexpression of the pUASp-2WW does not disrupt normal Orb (red) localization. Similar phenotype is seen with C1-construct that lacks the entire C-terminal region of Dg (D; Fig. 1). E. Overexpression of pUASp-PPSG constructs disrupts oocyte polarity indicated by mislocalization of Orb which has an abnormal side location, F. Percentage of Orb mislocalization as the result of overexpression of different pUASp-Dg constructs. (FL, 49 ± 2, PPSG 44 ± 2, 2WW 19 ± 2, C1 16 ± 3).

becomes mislocalized, surrounds the entire oocyte nucleus, or accumulates in a clump to one side of the oocyte instead of localizing to the posterior. Therefore, Dystroglycan, when expressed at elevated levels in germline cells, is sufficient to disrupt oocyte polarity. Overexpression of the full length form of Dg with the *tubGal4* driver causes semi-lethality (data not shown). Similarly, in vertebrates overexpression of Dg has been shown to cause defects in neuromuscular junctions [18,19]. We used the overexpression oocyte polarity assays to test whether either of the WW domain binding sites is essential for Dystroglycan function.

To test the function of WWbsI *in vivo* we overexpressed the PPSG mutant protein in germline cells using the *MatTubGal4* driver and determined the localization of the early oocyte polarity marker Orb. As discussed, in wild

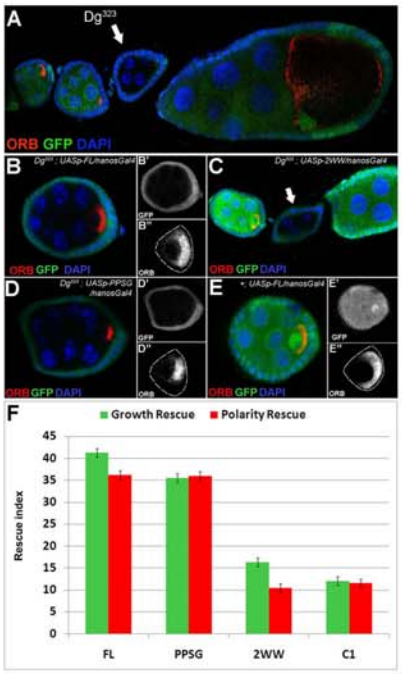

Figure 4
Rescue of Dg loss-of-function germline clones with expression of pUASp-Dg constructs. Orb (Red); GFP (Green), DAPI (Blue) A', B', D' GFP of the corresponding stages shown in a separate channel; A", B", D" Orb staining of the corresponding stages shown in a separate channel with dotted lines which indicates the border of the oocyte. A. Dg loss-of-function germline clones (black, white arrow; *hsFLP; FRT42D Dg*^{323}) are arrested prior to stage 6 and have disrupted oocyte polarity (absent or mislocalized Orb). B. Expression of *pUASp-FL* with the nanos-Gal4 driver in Dg clones partially rescues oocyte polarity in arrested clones stages 3–6 as indicated by proper localization of Orb to the posterior of 36% of the oocytes; (*hsFLP; FRT42D Dg*^{323}; *P(w+:nanosGal4:VP-16)Ab-2/pUASp-FL) C*. Expression of pUASp-2WW with the *nanos-Gal4* driver in *Dg* clones does not rescues oocyte polarity in arrested clones stages 3–6 (arrow) [as indicated by development arrest and absent Orb marker; (*hsFLP; FRT42D Dg*^{323}; *P(w+:nanosGal4:VP-16)Ab-2/pUASp-2WW)*] *D*. Expression of *pUASp-PPSG* with the *nanos-Gal4* driver in *Dg* clones rescues oocyte polarity in arrested clones stages 3–6 [as indicated by proper localization of Orb to the posterior of the oocyte; (*hsFLP; FRT42D Dg*^{323}; *P(w+:nanosGal4:VP-16)Ab-2/pUASp-PPSG)*]*E*. Wild type egg chamber with posterior Orb localization (+/+; *P(w+:nanosGal4:VP-16)Ab-2/pUASp-FL) F*. FL, PPSG are able to rescue Dg loss-of-function phenotypes, while 2WW and C1 do not (Red: rescued polarity index, Green: rescued growth index).

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type cells Orb marks the localization of the microtubule organizing center and is localized to the posterior side of the oocyte during stages 3–6 (Figure 3A). Overproduction of the PPSG protein results in the mislocalization of the usually posterior Orb marker. In mutants Orb surrounds the oocyte nucleus or localizes to the sides of the oocyte nucleus in 44 ± 2% of 3–6 stage oocytes (n = 147, Figure 3E–F). The level of this defect is similar to the one observed with the FL construct [6,13], which contains both WW binding sites (Figure 1; Figure 3B, D; 49 ± 2%, n = 80). These data suggest that disturbing the second WW binding site at the Dg C-terminus does not dramatically affect the functionality of the protein; similar to FL construct, when overexpressed it still is sufficient to disturb the oocyte polarity.

In contrast to the FL and PPSG constructs, overexpression of a 2WW mutant construct did not result in a high percentage of Orb mislocalization (Figure 3C, F, 19 ± 3%, n = 123). With 2WW overexpression, Orb, in most cases, was localized to the posterior of the oocyte (Figure 3C). The frequency of mislocalization with the 2WW construct, in which both WW binding sites were mutated was similar to that of the C1 construct which lacked all the C-terminal binding sites (Figure 1, Figure 3D, F, 16 ± 2%, n = 86).

These data, in combination with our previous data [13] show that a single mutation in WWbsII or the lack of WWbsI does not result in dramatic defects in Dg activity in this sufficiency assay measuring the oocyte polarity. However, simultaneous mutations in both WW binding sites dramatically reduce the function of Dystroglycan in this assay.

One WW binding site is required for Dystroglycan function

We also tested the function of the WW binding site mutants in rescue experiments by expressing the transgenes in a Dg loss-of-function background. Dg^{323} germline mutant clones are arrested prior to stage 3–4 and have mislocalized or missing Orb protein (Figure 4A). We have previously shown that these defects are partially rescued by wild type (full-length) Dg expression [13] (Figure 4, 36–40% rescue). Full rescue is not expected since the Dg^{323} deletion also affects a newly described neighboring gene mRpl34 (Additional Figure 3) and recent data implies that the level of nutrients and energy metabolism in the animal may affect cellular polarity [9]. To test if our mutant constructs were capable of rescuing the developmental arrest and the defects in oocyte polarity on the same level as the Dg full-length construct, we expressed them using the germline driver *nanosGal4* and calculated the percentage of loss-of-function clones with rescued growth and polarity. Using this assay we tested whether the Dg WWbs mutations were capable of a similar level of rescue as full-length Dg. If the Dg mutant with both WW binding sites mutated (2WW, Figure 1) could rescue the Dg^{323} phenotype in oocyte polarity at the same level as wild type Dg, we conclude that neither of the WW binding sites in *Drosophila* is required for Dg activity. On the other hand, if Dg with two WWbs mutations cannot rescue, we conclude that both or just the internal WW binding site is essential for Dg activity (we have already shown that the C-terminal WWbs is not essential [13]). As discussed above, to distinguish between these possibilities, we have generated a single mutation in WWbsII (PPSG, Figure 1) and will test whether this mutant still has the full length Dg activity in the loss-of-function rescue assays.

Similar to the full length Dg (FL, Figure 4B), the PPSG mutant constructs were capable of partially rescuing the Dg mutant phenotype (Figure 4D). Loss-of-function clones with expression of FL (Figure 4B-B") and PPSG (Figure 4D-D") had similar levels of posterior localization of the polarity marker, Orb (Figure 4F, FL: 36 ± 0.5% n = 52; PPSG: 41% n = 22). These mutants were also capable of restoring the developmental arrest phenotype by showing a higher percentage of loss-of-function clones that were older than stage 4–6 (Figure 4F, FL: 47 ± 8% n = 55; PPSG 38% n = 21). In contrast, 2WW was unable to rescue (2WW rescued at the level of the C1 mutant that lacks most of the Dg C-terminus; [13]; Figure 1.). Dg loss-of-function clones with expression of 2WW and C1 showed lower percentages of normal polarity (Figure 4F; 2WW: 12 ± 0.6% n = 66; C1: 9% n = 22) and growth rescue (Figure 4F; 2WW: 19 ± 2% n = 66; C1: 13%) than FL or PPSG constructs (Figure 4F). This result indicates that at least one WW binding site is required for normal function of Dg but a mutation in only one of the sites does not alter the functionality of Dg protein dramatically.

Since a single WWbsII mutation or a WWbsI deletion does not cause a severe loss of Dg activity but the double mutant does, we conclude that the two binding sites act, at least partially in a redundant manner in oocyte polarity and growth assays.

WW binding sites are highly conserved

Since both WW binding sites proved to be important in our *in vivo* experiments we wanted to know if the importance of these sites has been preserved among the interspecies population. To analyze the conservation of WW binding sites, we tested for variability in the sequence of those sites among all *Drosophila* species. For this purpose, using the ClustalW program, we aligned the *Dystroglycan* sequences of the 12 species of *Drosophila* obtained from the GBrowse database. The alignment analysis indicates that the two WW binding sites are fully conserved among all 12 *Drosophila* species (Figure 5A). Some variation in the nucleotide sequences of the WW binding sites were observed between the species, however these changes did

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not lead to amino acid sequence changes (Additional Figure 2). Furthermore, both Dg WW binding sites were also conserved between *Drosophila* and humans (Figure 5B).

In order to better understand patterns of polymorphisms in human Dystroglycan (*DAG*), and, in particular, the WW domains, we sequenced a 348 bp fragment spanning the region of interest in 88 samples from six geographically diverse human populations. In total, only one segregating site was identified among the 176 chromosomes sequenced (table 1) and none were identified in either of the WW domains. The estimated nucleotide diversity (defined as the average number of pairwise differences between two randomly selected chromosomes per nucleotide) in the combined sample is 3.24×10^{-5}. In contrast, the average nucleotide diversity of 322 genetic regions that were sequenced in a panel of 23 European-Americans and 22 African-Americans is 8.53×10^{-4}, suggesting that the sequenced region of *DAG* is under significant functional constraint. These data suggest that during evolution both WW binding sites have been important and therefore are preserved among species.

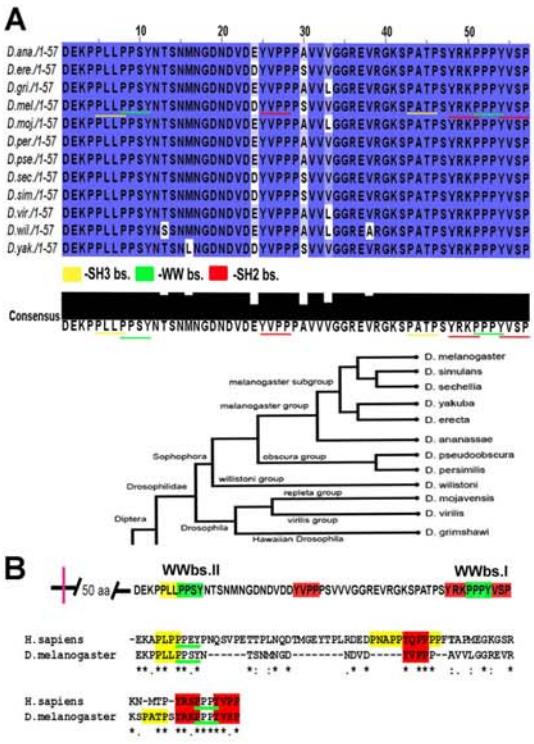

Figure 5
Both WW binding sites are conserved in all 12 species of *Drosophila*. A. Amino acid sequence alignment of the C-terminal end from 12 species of *Drosophila* using the computer program ClustalW shows absolutely no variation between both WW binding sites. B. Both WW binding sites are highly conserved between humans and *Drosophila*.

Table 1: Summary statistics of sequencing data.

Population	N[a]	S[b]	θ_W[c]	π[d]
CEPH	40	0	0	0
Han Chinese	40	0	0	0
Middle East	20	0	0	0
Pygmy	20	0	0	0
South Africa	16	0	0	0
South America	20	1	5.26×10^{-3}	2.73×10^{-4}
South East Asia	20	0	0	0
Total	176	1	6.49×10^{-5}	3.24×10^{-5}

[a] Number of chromosomes
[b] Number of segregating sites
[c] Watterston's theta per bp
[d] Nucleotide diversity per bp

Discussion

The functional redundancy of the WW binding sites poses interesting questions: have both binding sites survived through evolution to protect organisms from the mutations in an essential complex or does each binding site have a specific function in different tissues and/or developmental stages. Mutations in the DGC cause muscular dystrophies; however only mutations in Dystrophin, but not Dystroglycan per se, are associated with known types of muscular dystrophies in vertebrates. In mice, mutations in Dystroglycan are embryonic lethal, which suggests that Dg is an essential gene and, perhaps the redundant Dystrophin binding sites in Dystroglycan provide an additional means for DGC regulation.

The comparative sequence analysis of *Drosophila* and human WW binding motifs revealed very high conservation. However, each WWbs resides in a specific protein micro-environment, which may suggest that each site has specific binding partners. The previously performed genetic screens for modifiers [20] of Dg and Dys showed that the Dg-Dys complex interacts with components of different signaling pathways and components involved in cell/neuronal migration, cytoskeletal rearrangement and muscle development. This suggests that the Dg-Dys complex might be a major hub that regulates transfer of extracellular information to the cytoskeleton. Therefore it will be important in the future to test if WW binding sites have specific and independent biological functions in different tissues. This kind of analysis is likely to provide insights into the specific functions of the Dg-Dys complex and

serve as a basis for the development of novel therapeutic approaches for the treatment of muscular dystrophy.

Conclusion

We have investigated the role of the WW binding sites at the C-terminus of Dystroglycan protein and found that both sites may bind to the WW+EF hand domain of Dystrophin. Our previous studies [6,13], indicate that WWbsI and WWbsII both can bind Dystrophin protein *in vitro*. To test whether both WW binding sites can function and are required *in vivo* we generated two transgenic mutants: 2WW, which has mutations in both WW binding sites (WWbsI, PPPY → WAPY and WWbsII, PPSY → PPSG), and PPSG, which has a mismatch in WWbsII (PPSY → PPSG). We used the establishment of early oocyte polarity as an assay to verify the functionality of WWbsI and WWbsII. Importantly, the data show that while each WW binding site mutation yields to close to normal Dg function, the double WWbs mutation has lost Dg C-terminal activity. These data suggest that at least one WWbs is required for full Dg function *in vivo* and that the two sites may be partially redundant.

Methods

Fly Stocks

Drosophila melanogaster stocks were raised on standard cornmeal/yeast/agar medium at 25°C. For overproduction of pUASp-Dg in the germline, we used the following: *NGT40; P{w+:nanosGal4:VP-16)Ab-2* [21,22] and *Mat-α4-TubGal4-VP16/CyO* [23]. For overproduction of pUASp-Dg in the follicle cells, we used *hsFlp; act < FRT-CD2-FRT < Gal4; UAS-GFP*[24]. For generation of *Dystroglycan* clones, we used *FRT42D-Dg^{323}/CyO* (*Dg^{323}* is a *Dystroglycan* loss-of-function mutant with a 3324 bp deletion between bp 32,345 and 35,669 of DS03910 [7] disrupting the Dg 5' region and the adjacent mRPL34 gene; Additional Figure 3) and *hsFLP;FRT42D Ubi-GFP/CyO*. For overproduction of pUASp-Dg in a *Dystroglycan* mutant background, we used *FRT42D-Dg323/CyO; P{w nos-Gal4:VP16)A4-2 III*, and *hsFLP; FRT42D Ubi-GFP/CyO; pUASp-Dg/TM3* (pUASp-Dg refers to all *Dystroglycan* constructs: FL, C1, 2WW, PPSG). Two deletions in the *Dystroglycan* region exist; *Dg^{248}* (11985709:11986494) whose breakpoints are 333 bp downstream of the *Dg* transcription start site (11986042) and 3 bp upstream of the mRpL34 start codon (11986498) and *Dg^{323}* (11983340:11986664) whose breakpoints are 2.7 kb downstream of the *Dg* transcription start site and 166 bp downstream of the mRpL34 start codon (Additional Figure 3). We also used: dg043 [25].

Generation of pUASp-Dg Transgenic Animal

Full length and modified *Dystroglycan* PCR products that can be expressed in the germline were synthesized from the template LD11619. pUASp-FL and pUASp-C1 constructs used in this work have been described previously [13]. To generate a construct with mutated WWbsII (pUASp-PPSG) LD11619 was used as a template with the following primers: 5'-GGGGTACCAACATGAGATTC-CAGTGGTTCT-3' 5'-GCTCTAGATTATGGCGACACA-CATA-TGGCGGT-3'. The PCR products were digested with KpnI and XbaI and cloned into the pUASp vector [26]. The constructs were injected into embryos to obtain at least two independent stable transformant lines. Injections were done by Rainbow Transgenic Flies, Inc. (California, USA).

Overproduction of Dystroglycan in the Germline and Follicle Cell

For overproduction in germline cells, balanced *pUASp-Dg/Mat-α4-TubGal4-UP16/CyO or P{w+: nanosGal4:VP-16)Ab-2* animals were raised in yeasted vials at 25°C for 3 days before dissection and analysis. For overproduction in the follicle cells, *hsFlp; UAS-GFP act <FRTCD2FRT <Gal4/pUASp-Dg* animals were heat-shocked at 37°C for 1 h, raised in yeasted vials at 25°C for 3 days before dissection and analysis. All pUASp-Dg constructs used were crossed to these three Gal4 drivers to test for proper overproduction of protein and correct localization of protein to the membrane in the germline and somatic cells. The following pUASp-Dg lines were used for germline analysis: FL-1, 5; C1-1, -2; 2WW-10.2, -5.6, -13, 15.4; PPSG-11.1, -12.5, -6.3, -13.4. For the rescue experiments the following lines were used: FL-1, -2, -5; C1-1, -2; 2WW-10.4, -13, -15.6; PPSG-11.4, 11.1.

Antibody Staining Procedures

Drosophila ovaries were dissected rapidly in PBS and fixed in 4% paraformaldehyde for 10 minutes. The antibody staining procedure was the same as described previously [13]. The following primary antibodies were used at the following designated dilutions: rabbit anti-Dystroglycan (1:3000 [7]), mouse anti-Orb (1:20; Developmental Studies Hybridoma Bank), the following secondary antibodies were used at the designated dilutions: Alexa 488 anti-rabbit and Alexa 568 anti-mouse (1:500; Molecular Probes).

Western Blot and densitometry analyses

Sample preparation and SDS-PAGE have been described previously [13]. Bio-Rad ready-made 4–20% polyacrylamide gels were used for protein separation. Proteins were transferred to polyvinylidene difluoride (PDVF) membranes (Immobilon) using a semi-dry transfer apparatus (Bio-Rad). Primary affinity purified anti-Dg antibodies were used at 1:30,000 dilutions. Goat anti-rabbit HRP conjugated antibodies (Bio-Rad) were used as detection reagents at 1:10,000 dilutions. Proteins were visualized via enhanced chemiluminescence (Millipore). Densitometry analysis was performed with the public domain NIH IMAGEJ program (developed at the US National Institutes

of Health and available on the Web at http://rsb.info.nih.gov/ij/). Scans of immunoblots determined to be in the linear range (i.e. twice the amount of protein correlated with twice the signal seen on photographic film) were used as sources for analysis.

Sequence alignment

Sequences of 12 species of *Drosophila* were obtained from the FlyBase genome database. Sequence alignment was done using software ClustalW designed by the European Bioinformatics Institute http://www.ebi.ac.uk/Tools/clustalw/index.html.

DNA samples used for sequencing

We sequenced a 348 bp fragment of *DAG* that includes both WW domains in DNA samples from 88 humans representing six populations. Samples were obtained from the Coriell Institute for Medical Research Cell Repositories (Camden, NJ, USA). Coriell repository numbers for these samples are as follows: CEPH European-American (NA06990, NA07019, NA10830, NA10831, NA07348, NA07349, NA10842, NA10843, NA10844, NA10845, NA10848, NA10850, NA10851, NA10852, NA10853, NA10854, NA10857, NA10858, NA10860, NA10861, NA17201) Han Chinese of L.A. (NA17733 – NA17747, NA17749, NA17752 – NA17757, NA17759, and NA17761), Middle East (NA17041 – NA17050), Pygmy (NA10469 – NA10473, NA10492 – NA10496), South Africa (NA17319, NA17341 – NA17348), South America (NA17301 – NA17310) and South East Asia (NA17081 – NA17090). We compared patterns of polymorphism to 322 genes that were sequenced as part of the SeattleSNPs project [27].

DNA sequencing and statistical analysis

Sequencing primers were designed with primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi; primer sequences available upon request). We used standard PCR-based sequencing reactions using Applied Biosystem's Big Dye sequencing protocol on an ABI 3130xl. Sequence data was assembled using Phred/Phrap [28,29] and the alignments were inspected for accuracy with Consed [30,31]. Polymorphisms were identified with PolyPhred 4.0 [32]. All polymorphic sites were manually verified and confirmed by sequencing the opposite strand. Standard measures of nucleotide diversity, including θ_W and π were calculated as previously described [27].

Authors' contributions

ASY conception, design, acquisition, analysis and interpretation of the data, drafting the manuscript. MMK conception, design, acquisition, analysis and interpretation of the data. HRS drafting the manuscript. MP conception, design, acquisition, analysis and interpretation of the data, revising the manuscript. KAF acquisition, analysis and interpretation of the data. JM acquisition, analysis and interpretation of the data. WMD conception and interpretation of the data. MS conception and interpretation of the data. SB conception and interpretation of the data. JA conception, design and interpretation of the data, drafting the manuscript. HRB conception, design and interpretation of the data, drafting the manuscript. All the authors have read the article and accepted the final manuscript.

Additional material

Additional file 1

Figure 1. Overexpression of Dg constructs with mutation in WW binding sites in follicle and germline cells. A, B. Overexpression of 2WW (A) and PPSG (B) constructs in follicle cells marked by GFP. Dg in the wild type cells is expressed at the apical side of the follicle cell epithelium, in contrast to overexpression where Dg is localized in both apical and basal sides (indicated by arrows). To compare the expression levels of different constructs and insertions the intensities of Dg expression was compared to the intensity of the GFP signal in the same cell. The observed mean intensity ratios are similar in the two constructs (2WW = 1.2, PPSG = 1.1), suggesting that the differences observed between these two conatructs in oocyte polarity assay are not due to dramatically different levels of expression. C. Overexpression of the constructs in the germline cells.wt – Dg expression in wild type germline cells, MatTubGal4; pUASp-WW, nanosGal4/pUASp-WW – overexpression of transgenic constructs in germline cells. Both MatTub- and nanosGal4 have distinct expression patterns.

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Additional file 2

Figure 2. Comparative analysis of Dg C-terminus nucleic acid sequences in 12 species of Drosophila.

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Additional file 3

Figure 3. The genomic region of the Dystroglycan *gene. The genomic regions that are deleted in the* Dystroglycan *mutant alleles* Dg^{323} *and* Dg^{248} *are indicated as black bars.*

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Additional file 4

Figure 4. Western blot analysis of Dg protein in wild type, DgO43, 2WW and PPSG ovaries and whole animals show the following Dg intensities compared to OregonR (WT): DgO43 [25] = 0.4, 2WW = 1.3, PPSG = 1.2. The specific bands that correspond to different Dg forms can be seen at ~180 (two bands), 110 and faintly at 70 kD. A presumable degradation product can be seen below 25 kD. Increased band intensities can be seen with the 110 kD band and most notably with the higher 180 kD species. Band intensities were normalized to actin and samples were run on a gradient 4–20% gel.

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References

1. Russo K, Di Stasio E, Macchia G, Rosa G, Brancaccio A, Petrucci TC: **Characterization of the beta-dystroglycan-growth factor receptor 2 (Grb2) interaction.** *Biochem Biophys Res Commun* 2000, **274**:93-8.
2. Zhou YW, Thomason DB, Gullberg D, Jarrett HW: **Binding of laminin alpha1-chain LG4-5 domain to alpha-dystroglycan causes tyrosine phosphorylation of syntrophin to initiate Rac1 signaling.** *Biochemistry* 2006, **45**:2042-52.
3. Davies KE, Nowak KJ: **Molecular mechanisms of muscular dystrophies: old and new players.** *Nat Rev Mol Cell Biol* 2006, **7**:762-73.
4. Haenggi T, Fritschy JM: **Role of dystrophin and utrophin for assembly and function of the dystrophin glycoprotein complex in non-muscle tissue.** *Cell Mol Life Sci* 2006, **63**:1614-31.
5. Barresi R, Campbell KP: **Dystroglycan: from biosynthesis to pathogenesis of human disease.** *J Cell Sci* 2006, **119**:199-207.
6. Shcherbata HR, Yatsenko AS, Patterson L, Sood VD, Nudel U, Yaffe D, Baker D, Ruohola-Baker H: **Dissecting muscle and neuronal disorders in a Drosophila model of muscular dystrophy.** *Embo J* 2007, **26**:481-93.
7. Deng WM, Schneider M, Frock R, Castillejo-Lopez C, Gaman EA, Baumgartner S, Ruohola-Baker H: **Dystroglycan is required for polarizing the epithelial cells and the oocyte in Drosophila.** *Development* 2003, **130**:173-84.
8. Poulton JS, Deng WM: **Cell-cell communication and axis specification in the Drosophila oocyte.** *Dev Biol* 2007, **311**:1-10.
9. Mirouse V, Christoforou CP, Fritsch C, St Johnston D, Ray RP: **Dystroglycan and perlecan provide a basal cue required for epithelial polarity during energetic stress.** *Dev Cell* 2009, **16**:83-92.
10. Allikian MJ, Bhabha G, Dospoy P, Heydemann A, Ryder P, Earley JU, Wolf MJ, Rockman HA, McNally EM: **Reduced life span with heart and muscle dysfunction in Drosophila sarcoglycan mutants.** *Hum Mol Genet* 2007, **16**:2933-43.
11. Taghli-Lamallem O, Akasaka T, Hogg G, Nudel U, Yaffe D, Chamberlain JS, Ocorr K, Bodmer R: **Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype.** *Aging Cell* 2008, **7**:237-49.
12. Zhan M, Yamaza H, Sun Y, Sinclair J, Li H, Zou S: **Temporal and spatial transcriptional profiles of aging in Drosophila melanogaster.** *Genome Res* 2007, **17**:1236-43.
13. Yatsenko AS, Gray EE, Shcherbata HR, Patterson LB, Sood VD, Kucherenko MM, Baker D, Ruohola-Baker H: **A putative Src homology 3 domain binding motif but not the C-terminal dystrophin WW domain binding motif is required for dystroglycan function in cellular polarity in Drosophila.** *J Biol Chem* 2007, **282**:15159-69.
14. Jung D, Yang B, Meyer J, Chamberlain JS, Campbell KP: **Identification and characterization of the dystrophin anchoring site on beta-dystroglycan.** *J Biol Chem* 1995, **270**:27305-10.
15. Rentschler S, Linn H, Deininger K, Bedford MT, Espanel X, Sudol M: **The WW domain of dystrophin requires EF-hands region to interact with beta-dystroglycan.** *Biol Chem* 1999, **380**:431-42.
16. Huang X, Poy F, Zhang R, Joachimiak A, Sudol M, Eck MJ: **Structure of a WW domain containing fragment of dystrophin in complex with beta-dystroglycan.** *Nat Struct Biol* 2000, **7**:634-8.
17. Keleman K, Kruttner S, Alenius M, Dickson BJ: **Function of the Drosophila CPEB protein Orb2 in long-term courtship memory.** *Nat Neurosci* 2007, **10**:1587-93.
18. Heathcote RD, Ekman JM, Campbell KP, Godfrey EW: **Dystroglycan overexpression in vivo alters acetylcholine receptor aggregation at the neuromuscular junction.** *Dev Biol* 2000, **227**:595-605.
19. Kahl J, Campanelli JT: **A role for the juxtamembrane domain of beta-dystroglycan in agrin-induced acetylcholine receptor clustering.** *J Neurosci* 2003, **23**:392-402.
20. Kucherenko MM, Pantoja M, Yatsenko AS, Shcherbata HR, Fischer KA, Maksymiv DV, Chernyk YI, Ruohola-Baker H: **Genetic modifier screens reveal new components that interact with the Drosophila dystroglycan-dystrophin complex.** *PLoS ONE* 2008, **3**:e2418.
21. Jr WD Tracey, Ning X, Klingler M, Kramer SG, Gergen JP: **Quantitative analysis of gene function in the Drosophila embryo.** *Genetics* 2000, **154**:273-84.
22. Doren M Van, Williamson AL, Lehmann R: **Regulation of zygotic gene expression in Drosophila primordial germ cells.** *Curr Biol* 1998, **8**:243-6.
23. Hacker U, Perrimon N: **DRhoGEF2 encodes a member of the Dbl family of oncogenes and controls cell shape changes during gastrulation in Drosophila.** *Genes Dev* 1998, **12**:274-84.
24. Pignoni F, Zipursky SL: **Induction of Drosophila eye development by decapentaplegic.** *Development* 1997, **124**:271-8.
25. Christoforou CP, Greer CE, Challoner BR, Charizanos D, Ray RP: **The detached locus encodes Drosophila Dystrophin, which acts with other components of the Dystrophin Associated Protein Complex to influence intercellular signalling in developing wing veins.** *Dev Biol* 2008, **313**:519-32.
26. Rorth P: **Gal4 in the Drosophila female germline.** *Mech Dev* 1998, **78**:113-8.
27. Akey JM, Eberle MA, Rieder MJ, Carlson CS, Shriver MD, Nickerson DA, Kruglyak L: **Population history and natural selection shape patterns of genetic variation in 132 genes.** *PLoS Biol* 2004, **2**:e286.
28. Ewing B, Hillier L, Wendl MC, Green P: **Base-calling of automated sequencer traces using phred. I. Accuracy assessment.** *Genome Res* 1998, **8**:175-85.
29. Ewing B, Green P: **Base-calling of automated sequencer traces using phred. II. Error probabilities.** *Genome Res* 1998, **8**:186-94.
30. Gordon D, Abajian C, Green P: **Consed: a graphical tool for sequence finishing.** *Genome Res* 1998, **8**:195-202.
31. Gordon D, Desmarais C, Green P: **Automated finishing with autofinish.** *Genome Res* 2001, **11**:614-25.
32. Bhangale TR, Stephens M, Nickerson DA: **Automating resequencing-based detection of insertion-deletion polymorphisms.** *Nat Genet* 2006, **38**:1457-62.

Supplementary Information

Figure S1

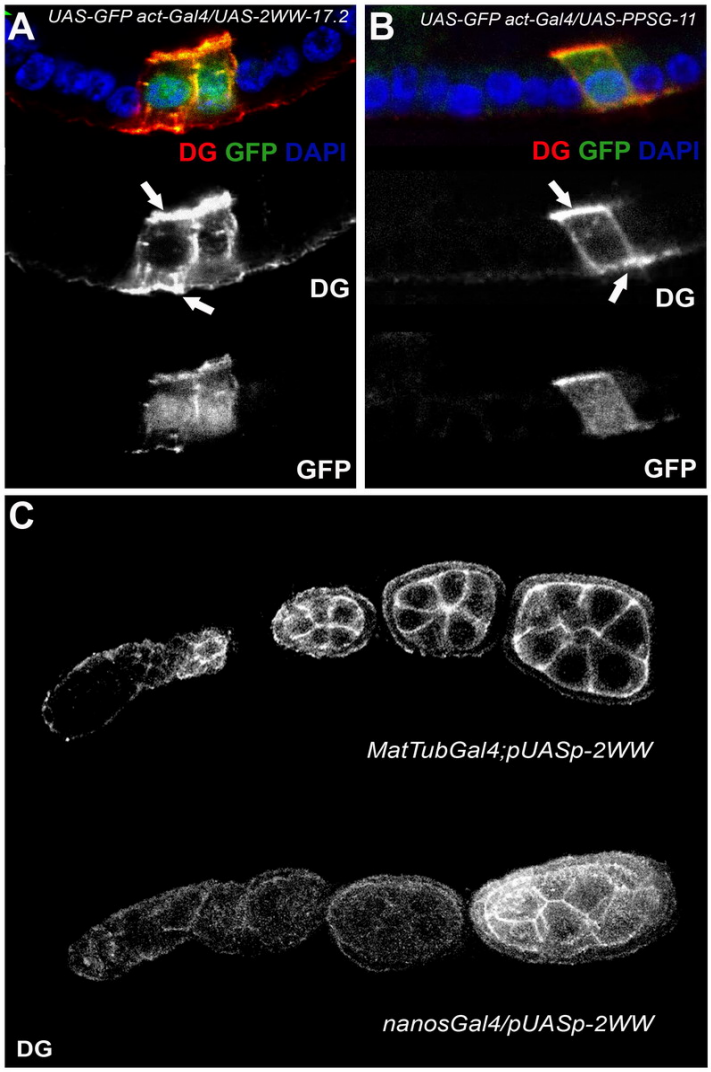

Fig. S1. Overexpression of Dg constructs with mutation in WW binding sites in follicle and germline cells. A, B. Overexpression of 2WW (A) and PPSG (B) constructs in follicle cells marked by GFP. Dg in the wild type cells is expressed at the apical side of the follicle cell epithelium, in contrast to overexpression where Dg is localized in both apical and basal sides (indicated by arrows). To compare the expression levels of different constructs and insertions the intensities of Dg expression was compared to the intensity of the GFP signal in the same cell. The observed mean intensity ratios are similar in the two constructs (2WW = 1.2, PPSG = 1.1), suggesting that the differences observed between these two conatructs in oocyte polarity assay are not due to dramatically different levels of expression. C. Overexpression of the constructs in the germline cells.wt – Dg expression in wild type germline cells, MatTubGal4; pUASp-WW, nanosGal4/pUASp-WW – overexpression of transgenic constructs in germline cells. Both MatTub- and nanosGal4 have distinct expression patterns.

Figure S2

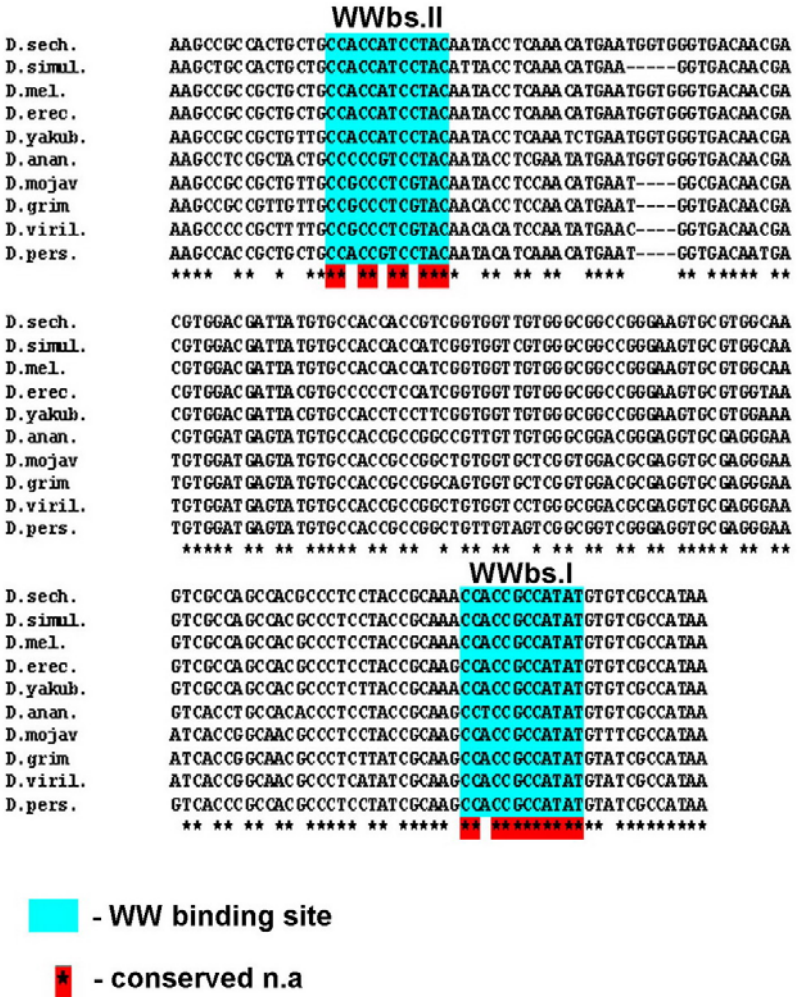

Fig. S2. Comparative analysis of Dg C-terminus nucleic acid sequences in 12 species of *Drosophila*.

Figure S3

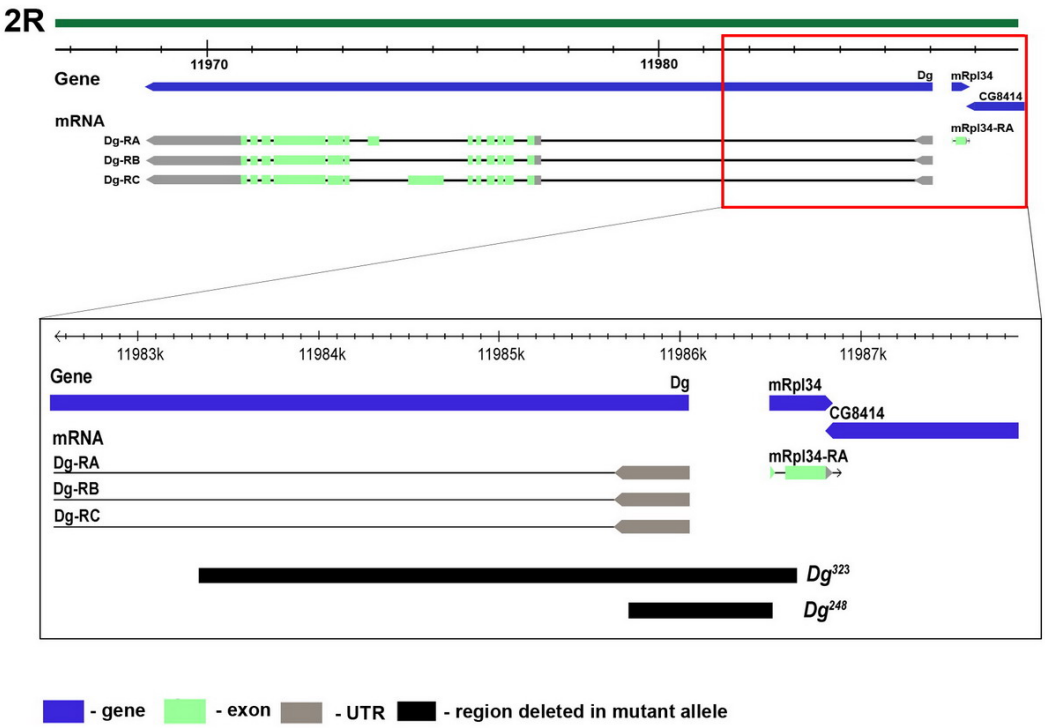

Fig. S3. The genomic region of the *Dystroglycan* gene. The genomic regions that are deleted in the *Dystroglycan* mutant alleles Dg^{323} and Dg^{248} are indicated as black bars.

Figure S4

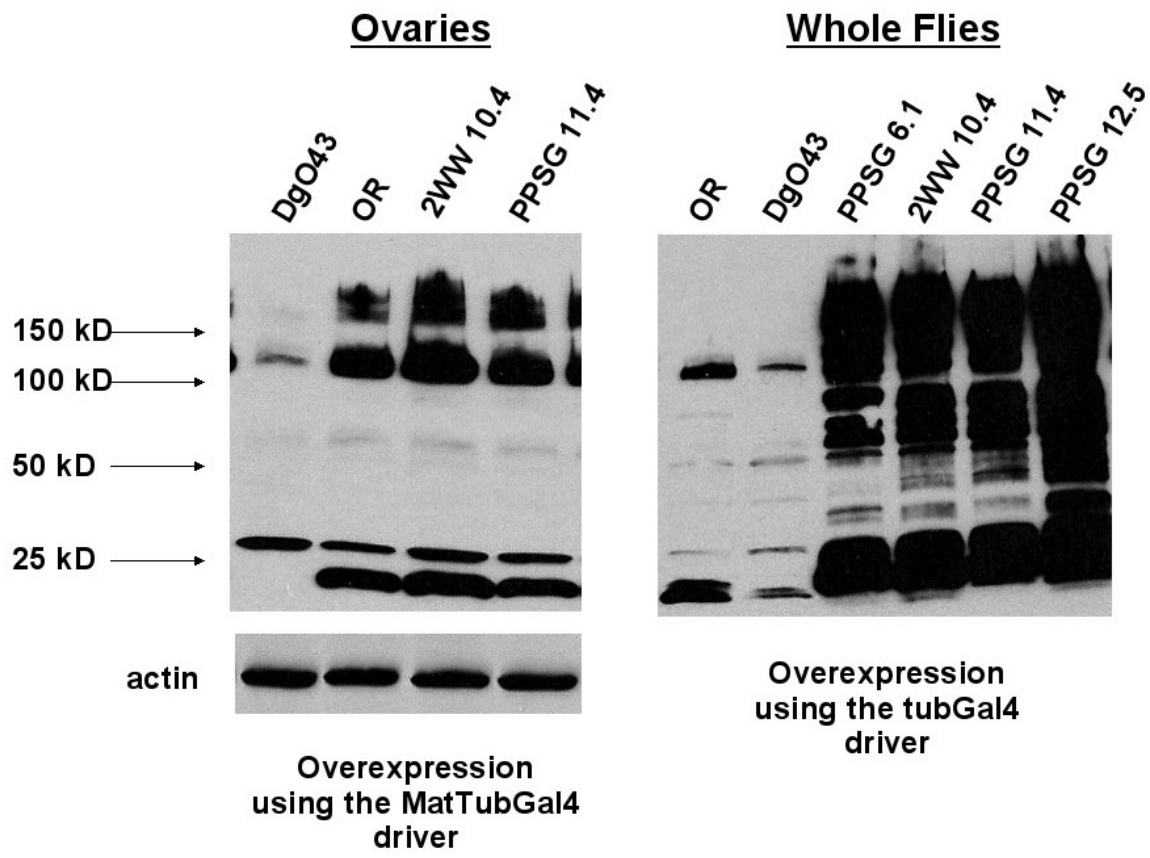

Fig. S4. Western blot analysis of Dg protein in wild type, DgO43, 2WW and PPSG ovaries and whole animals show the following Dg intensities compared to OregonR (WT): DgO43 = 0.4, 2WW = 1.3, PPSG = 1.2. The specific bands that correspond to different Dg forms can be seen at ~180 (two bands), 110 and faintly at 70 kD. A presumable degradation product can be seen below 25 kD. Increased band intensities can be seen with the 110 kD band and most notably with the higher 180 kD species. Band intensities were normalized to actin and samples were run on a gradient 4–20% gel.

3.3. Genetic modifier screen reveal new components that interact with the Drosophila Dystroglycan-Dystrophin complex

Previously, I demonstrated that phosphorylation of each of the Dystroglycan PPxY motifs reduces Dystrophin-Dystroglycan binding affinity (Yatsenko et al., 2007). Moreover, it has been shown that the Dystroglycan SH3 binding site is required for *in vivo* protein function (Yatsenko et al., 2007). These findings suggest a potential mechanism to regulate the Dg and Dys interaction, in which signaling proteins containing SH3 domains may bind to Dg in a tyrosine phosphorylation-dependent manner. In addition, my *in vitro* and *in vivo* analyses have shown the importance of both Dystroglycan WW binding sites for interaction with Dystrophin and for maintaining the Dg function in the establishment of oocyte polarity in *Drosophila* (Yatsenko et al., 2007; Yatsenko et al., 2009), suggesting that the presence of the two WW binding sites in Dg secure the essential interaction between Dg and Dys and might further provide additional regulation for the cytoskeletal interactions of the complex. In this present work I have used a previously developed *Drosophila* model for DGC-dependent muscular dystrophy (Shcherbata et al., 2007) to search for novel components of the DGC as well as components that may be involved in its signaling and regulation. I have performed a genetic screen to find dominant modifiers of an easily score-able phenotype caused by reduction of Dys and Dg, an alteration of the posterior cross wing vein. Three different screening strategies were used to identify the cross-vein phenotype suppressors or enhancers: the EMS-induced mutagenesis screen, the “Deficiency screen” and the “P-element lethal” screen. As a result, I have found modifiers that belong to different functional groups: genes involved in muscle function, neuronal/cell migration and motor function as well as cytoskeletal components and components of the TGF-beta, EGFR and Notch signaling pathways. Further functional analysis of found components showed that a number of modifiers are required in the germline for the proper oocyte polarity establishment, similar to what has been shown for Dg and Dys (Deng et al., 2003; Shcherbata et al., 2007). Furthermore, to initiate the secondary screen in muscle tissue I demonstrated that the strong modifier of *Dys* phenotype in wing vein - *mbl* also strongly enhances muscle degeneration phenotype caused by reduction of Dys. These results show that the screen performed in wing vein successfully identified genes that interact with Dystrophin to establish normal muscle function.

Genetic Modifier Screens Reveal New Components that Interact with the *Drosophila* Dystroglycan-Dystrophin Complex

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Author contribution to the practical work:

Mariya M. Kucherenko and *Mario Pantoja*	All the experiments
Andriy S. Yatsenko, Karin A. Fischer and *Yaroslava I. Chernyk*	EMS-induced mutagenesis screen
Dariya V. Maksymiv	"P-element lethal screen" and the experiments for the oocyte polarity analysis
Halyna R. Shcherbata	The oocyte polarity analysis

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Genetic Modifier Screens Reveal New Components that Interact with the *Drosophila* Dystroglycan-Dystrophin Complex

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Abstract

The Dystroglycan-Dystrophin (Dg-Dys) complex has a capacity to transmit information from the extracellular matrix to the cytoskeleton inside the cell. It is proposed that this interaction is under tight regulation; however the signaling/regulatory components of Dg-Dys complex remain elusive. Understanding the regulation of the complex is critical since defects in this complex cause muscular dystrophy in humans. To reveal new regulators of the Dg-Dys complex, we used a model organism *Drosophila melanogaster* and performed genetic interaction screens to identify modifiers of Dg and Dys mutants in *Drosophila* wing veins. These mutant screens revealed that the Dg-Dys complex interacts with genes involved in muscle function and components of Notch, TGF-β and EGFR signaling pathways. In addition, components of pathways that are required for cellular and/or axonal migration through cytoskeletal regulation, such as Semaphorin-Plexin, Frazzled-Netrin and Slit-Robo pathways show interactions with Dys and/or Dg. These data suggest that the Dg-Dys complex and the other pathways regulating extracellular information transfer to the cytoskeletal dynamics are more intercalated than previously thought.

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Introduction

Muscular dystrophies are a group of inherited neuromuscular disorders that share the same basic phenotype of progressive loss of muscle integrity. Many muscular dystrophies are caused by defects in a specialized cell adhesion complex called the Dystrophin Glycoprotein Complex (DGC). It has become evident that this complex plays a central role in muscle integrity and forms a mechanical link from the actin cytoskeleton to the extracellular matrix (ECM). The core DGC is composed of a transmembrane component, Dystroglycan (Dg), which associates with the ECM protein, Laminin and the cytoplasmic protein Dystrophin which binds Actin (reviewed in [1,2]).

Many lines of evidence confirm that maintaining the structural link from the extracellular matrix to the actin cytoskeleton is crucial in preventing many forms of muscular dystrophy. Mutations that disrupt any component of this structural link results in a variety of muscular dystrophies like Duchenne's, Becker's, Muscle-eye-brain disease, Walker-Warburg syndrome, congenital muscular dystrophies 1C and 1D as well as limb girdle muscular dystrophy 2I. These diseases share the common symptoms of skeletal muscle degeneration, cardiomyopathy, as well as a reduced life span for afflicted individuals [3].

Additionally, alterations which reduce the affinity of components of the DGC lead to congenital muscular dystrophies like Fukuyama's which, aside from muscular defects, also are associated with aberrant neuronal migrations that lead to mental retardation, epilepsy, as well as abnormal eye development. The use of animal model systems has led to the clarification of the roles of specific gene products in maintaining muscle integrity and function (reviewed in [4]), however, the regulation of this complex is largely unknown.

Initial characterization of the DGC in *Drosophila* has determined that components studied so far possess similar roles in muscle integrity and neuronal migration in flies as in humans (Figure 1, [5,6,7,8]). These abnormalities include age dependent muscle degeneration, reduced mobility, defects in eye development as manifested by altered photoreceptor axon pathfinding, and a shorter life span. Additionally, mutations in *Dys* and *Dg* affect cell polarity in the *Drosophila* germ line as both the follicular cell epithelium and the oocyte are disrupted [6,9,10]. Recently, a reduced lifespan in *Drosophila*, as well as heart and muscle abnormalities, have been reported in mutants of another component of the DGC, δ-sarcoglycan [5]. In addition, heart and eye phenotypes have been observed in *Drosophila Dys* and *Dg* mutants [11,12].

The similar defects in both flies and humans make *Drosophila* an attractive model for further studies on clarifying the role of the DGC. Such studies may reveal novel components that may likely have counterparts in humans. Additionally, since very little is

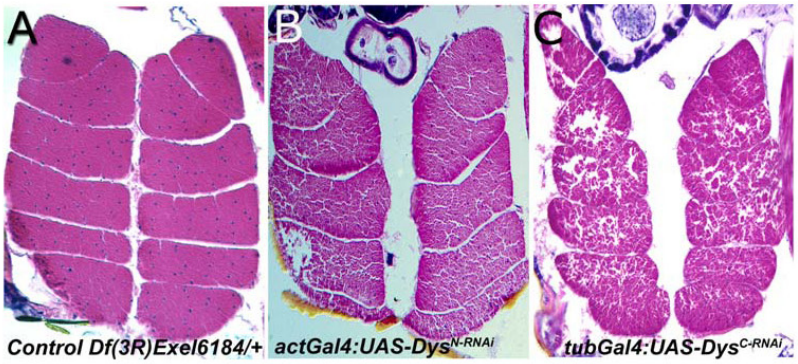

Figure 1. Muscle Phenotypes of *Dys* Mutants. Transverse histological sections of indirect flight muscles showing age dependent muscle degeneration. Control muscle section from *Df(3R)Exel6184/+* flies at 18 days of age do not show any type of abnormalities (A). While lower penetrance and milder muscle degeneration phenotype is observed from RNAi knockdown of long forms of *dystrophin* (*act-Gal4:UAS-Dys$^{N\ RNAi}$/+* flies at 18 days of age (B), *KX43/Df(3R)Exel6184* and *Dys8-2/KX43*), a strong muscle degeneration phenotype is seen when all forms of *dystrophin* are reduced (C; *tub-Gal4:UAS-Dys$^{C\ RNAi}$/+* flies at 12 days of age).
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known about how the DGC is regulated insights may be gained on this heretofore unknown process. More recently, transmembrane signaling has been implicated in the function of the DGC. The C-terminus of Dystroglycan, in addition to having, EF and WW domain binding sites, also possesses SH2 and SH3 domain binding sites. These known protein-protein interaction motifs support the idea that Dystroglycan is a signaling receptor in addition to its known role as a conduit between the ECM and the cytoskeleton. Changes in MAPK kinase and GTPase signaling have also been observed when the DGC is perturbed [2,13]. Recent work has shown that specific sets of domains are critical in the function of *Drosophila* Dystroglycan [8].

In the present work, we have used the genetic tractability of *Drosophila* to search for novel components of the DGC, as well as components that may be involved in its signaling and regulation. Such a search is straightforward because in addition to the muscle degeneration and photoreceptor axon pathfinding defects, mutations in *dystrophin* and *Dystroglycan* cause a visible phenotype manifested as alterations in the fly wing, particularly the posterior crossvein (Figure 2, [14]). Since this is an easily score-able, highly penetrant phenotype we undertook a dominant modifier screen approach and looked for flies that showed either a suppression of the crossvein phenotype or a noticeably altered crossvein. Importantly, crossvein development has been previously shown to require EGFR, TGF-β and Notch pathway activities and is therefore a sensitive place to observe potential interactions of the DGC with these signaling pathways [15,16,17]. In addition, hemocyte migration is shown to correlate with the crossvein development [14]. Therefore genes involved in correct migration processes might also be obtained by this approach. We screened P-element lethal as well as deficiency collections for interactors in addition to performing a classical ethylmethanesulfonate (EMS) screen for dominant modifiers.

Here we report that in using these screening strategies we have found modifiers that belong to six different functional groups. We have found genes involved in muscle development, neuronal/cell migration and motor function as well as cytoskeletal components and components of the TGF-β, EGFR and Notch pathways. A common theme among many of these interactors and Dg-Dys complex is their involvement in the cytoskeletal rearrangements controlled by extracellular cues.

Results

To identify genes that interact with *Dys* and *Dg*, we used the chromosomal lesion hypomorph *Dys^{E6}* as well as the RNAi knockdown mutants *Dys$^{N\ RNAi}$*, *Dys$^{C\ RNAi}$* and *Dg^{RNAi}*. These mutants exhibit age dependent muscle degeneration of indirect flight muscles (IFM, Figure 1B and 1C) as shown before for other *Dys* alleles [6].

In addition to the muscle phenotype, a visible wing vein phenotype was observed in these and previously analyzed alleles. In particular, the posterior crossvein (PCV) is defective in both *Dys* and *Dg* mutants, a disrupted anterior crossvein (ACV) and a partial duplication of L2, a longitudinal wing vein, can also be observed at a lower frequency (Figure 2). These phenotypes are present in the RNAi knockdown mutants, *Dys$^{N\ RNAi}$*, *Dys$^{C\ RNAi}$* and *Dg^{RNAi}* (Figure 2C, 2D and 2E, respectively) as well as the chromosomal lesion hypomorphs *Dys^{E6}* (Figure 2B), *DysDf*, *Dg^{O43}*, *Dg^{O55}* and *Dg^{O86}* [14]. *Dys* and *Dg* are expressed in third instar larval wing imaginal discs (Figure S1) and interact in the wing vein since the double mutant shows a new phenotype – a duplication of L3 longitudinal wing vein (Figure 2F and 2F').

We used the visual wing vein phenotypes in P-element lethal, EMS induced and deficiency screens to find *dystrophin* and *Dystroglycan* interactors in *Drosophila* that either increase or decrease the wing vein phenotype. Additional studies reveal that many of the interactors identified in the screens are required for proper function in other tissues that require Dys and Dg, such as muscle, brain and ovary.

Modifier Screens

To identify *Dys* and *Dg* interactors, we screened three different kinds of mutants; a collection of 800 FRT-P-element lethal lines, a deficiency collection of 216 lines and 43,000 EMS induced mutants. From these analyses, we identified 37 interacting genes that can be clustered into six different functional groups (Table 1).

P-element screen. We screened FRT-P-element lethal lines from the Kyoto Stock Collection (Japan). The homozygous lethality of the lines allows us to infer that the transposon is inserted in or near an essential gene. We set up crosses to search for modifiers of the RNAi mutants *Dys$^{C\ RNAi}$*, *Dys$^{N\ RNAi}$* and *Dg^{RNAi}* as well as *Dys^{E6}*, a hypomorph that removes DLP2, a specific long

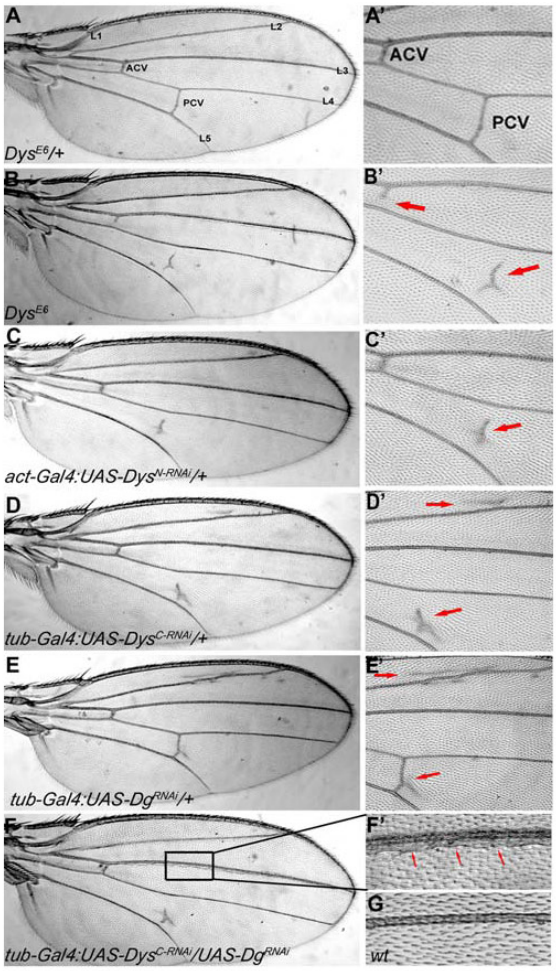

Figure 2. *Dys* and *Dg* are Required for Proper Wing Vein Formation and Interact in the Process. In a wild type like wing (A, genotype Dys^{E6}/+) there are 5 longitudinal veins (L1–L5). The anterior cross vein (ACV) forms between L3 and L4 and the posterior cross vein (PCV) forms between L4 and L5. (A') Higher magnification of the region of the wing that shows both cross veins, ACV and PCV. Dys^{E6}/Dys^{E6} homozygotes show defects in cross vein formation (B). The PCV is detached from L4 and L5 and at a lower frequency, the ACV fails to form a connection to L4. (B') Higher magnification of the image in B where arrows indicate the altered ACV and PCV. Arrows indicate altered cross veins. The RNAi mutant that knocks down long forms of *dystrophin* (*act-Gal4:UAS-*$Dys^{N\,RNAi}$/+) also shows a PCV mutant phenotype where the cross vein fails to attach to L4 and L5 (C). (C') Higher magnification of the image in (C) where the arrow indicates the altered PCV. (D) Shows the wing vein phenotype of the RNAi *dystrophin* mutant (*tub-Gal4:UAS-*$Dys^{C\,RNAi}$/+) that reduces the protein levels of all isoforms. Here the PCV is drastically affected and there is extra vein material above L2. (D') Higher magnification of the image in (D) where the arrows indicate the alterations. The upper arrow shows extra wing vein material above L2. The lower arrow indicates an altered PCV. The RNAi *Dystroglycan* mutant (*tub-Gal4:UAS-*Dg^{RNAi}/+) also shows a wing vein phenotype (E). In this case we see a branch off the PCV as well as extra material above L2. (E') Higher magnification of (E) where the upper arrow shows extra wing vein material above L2 and the lower arrow shows branching from the PCV. Finally, *Dys* and *Dg* interact in the *Drosophila* wing as the double mutant (*tub-Gal4:UAS-*$Dys^{C\,RNAi}$/*UAS-*Dg^{RNAi}) shows a novel phenotype (F, F'). The box indicates a thickened L3 vein. (F') Higher magnification of the box is shown in (F). Arrows indicate extra L3 longitudinal vein material. (G) Wild type L3 vein from the same region as shown in (F').

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isoform of *dystrophin* [7]. Modifiers were divided into phenotypic classes of enhancers, suppressors and suppressors with extra wing vein material (En, Su or Su+; Figure 3). From the P-element screen we obtained 33 modifiers of the *Dys* phenotypes (Table 1), 25 of which also showed interaction with Dg (Figure 4). Since the identity of these genes is known, we were able to organize these modifiers into 6 different functional categories (Table 1).

We were excited to find genes involved in both muscle and motor/cytoskeletal function as these are processes in which *Dys* and *Dg* are known to act (Table 1, functional group I; Figure 4). Of the muscle category genes, *muscleblind* (*mbl*) was the strongest suppressor in the wing vein. Weak suppressors of this group included *Calmodulin* (*Cam*) and the *nicotinic acetylcholine receptor α 30D* (*nAcRα-30D*) (Table 1, functional group I), though *nAcRα-30D* strongly interacted with *Dg* (Figure 4). Cytoskeletal and motor function genes (*Lissencephaly-1* (*Lis-1*), *Kinesin heavy chain* (*Khc*), *Dynamitin* (*Dmn*) and *Fhos* showed moderate to weak suppression with *dystrophin* mutants (Table 1, functional group I).

Another intriguing functional group that interacts with Dg-Dys complex includes genes known to be involved in neuronal migration (Table 1, functional group II). This group is composed of the genes which belong to Semaphorin-Plexin (*Sema-1a, Sema-2a*), Slit-Robo (*slit* (*sli*), *roundabout* (*robo*), *leak* (*lea*) – a robo2 homolog, *Syndecan* (*Sdc*)) and Netrin-Frazzlez (*frazzled* (*fra*)) pathways. In the wing vein most of these genes were scored as moderate to weak suppressors of the *Dys* RNAi mutants. Importantly, *Sema-2a* and *fra* modify Dys^{E6} (Table 1, functional group II; Figure S3). *Sema-1a* was found to moderately enhance the *Dys* wing vein phenotype. Using our Dg^{RNAi} mutant we found that *robo* and *Sema-2a* enhanced the *Dg* phenotype and *Sema-1a* and *lea* (robo2) suppressed the *Dg* phenotype (Table 1; Figure 4 and Figure S4). We have previously shown that *Dys* and *Dg* function in the *Drosophila* Photoreceptor axon pathfinding [6] in processes similar to Semaphorin-Plexin, Slit-Robo and Netrin-Frazzled pathways.

Functional groups IV and V (Table 1) contain interactors that belong to the TGF-β signaling pathway: *Daughters against dpp* (*Dad*), *decapentaplegic* (*dpp*), *thickveins* (*tkv*) and EGFR signaling pathway: *kekkon-1* (*kek1*) and *argos*. *Dad*, *dpp* and *kek1* were strong modifiers of the Dg^{RNAi} mutant (Table 1; Figure 4 and S4) and *Dad* and *tkv* of Dys^{E6} allele as well (Table 1, functional group IV; Figure S3). It has been known for some time that TGF-β, Notch and EGFR pathways are necessary for proper wing vein development ([18]; reviewed in [19,20]). In fact, many novel factors of these pathways have been identified through the analysis of wing vein mutants. The longitudinal wing veins that begin to form during late larval stages of development require EFGR and TGF-β signaling pathways for proper fating of cells in the region [15,21]. Crossveins appear in late pupal stages of development and require TGF-β signaling for formation and EGFR and Notch signaling for final patterning [17]. The posterior crossvein is particularly sensitive to different levels of TGF-β signaling and forms only after the proper formation of the longitudinal veins.

The last functional group (Table 1, functional group VI) contains a group of 12 genes with disparate functions. One enhancer, *glial cells missing* (*gcm*), was identified. Three strong suppressors of this group also interacted with Dys^{E6}, the *Drosophila* homolog of the homeodomain-interacting protein kinase (HIPK), *kismet* (*kis*) and *l(3)L4092*, which contains a zinc-finger motiff. *kis*, *deadlock* (*del*), *gcm*, *POSH* and *wunen* (*wun*) also strongly modified Dg^{RNAi} (Figure 4).

Table 1. Modifiers of Dg-Dys Complex

Functional groups	Gene name	Allele(s)	Function	*act-Gal4: UAS-Dys$^{N\text{-}RNAi}$/+*	*tub-Gal4: UAS-Dys$^{C\text{-}RNAi}$/+*	*Dys^{EG}/+* [1]	*Dg interactors*[2]
I. Muscle, motor and cyto-skeleton function	*Dys*	*Dys[EMS-ModE10]*	Dys-Dg complex	-	-	En (S)	+
	Cam	*Cam[k04213]*	Calmodulin, muscle contraction	Su (W)	-	-	
	mbl	*l(2)k04222b**	muscle development; splicing; RNA binding	Su (S)	Su+ (S)	-	
		*mbl[E27]***		Su (S)	Su+ (S)	-	+
	nAcRα-30D	*l(2)k14204**	acetylcholine receptor; muscle contraction	Su+ (W)	Su+ (W)	-	
		*nAcRα-30D[EY13897]***		Su+ (W)	Su+ (W)	Mod (W)	+
	Lis-1	*Lis-1[k13209]*	Dynein binding, WD repeats	-	Su+ (M)	-	-
	Khc	*Khc[k13219]*	Kinesin, microtubule motor	-	Su+ (M)	-	-
	Dmn	*Dmn[k16109]*	Subunit in Dynactin complex	-	Su+ (M)	-	-
	Fhos	*l(3)j5B6*	Actin organizing protein	Su (M)	Su+ (W)	-	
II. Neuro-nal migration or planar cell polarity genes	*sema-2a*	*sema-2a[k13416]*	ligand of PlexinB, axon guidance	Su (W)	Su+ (W)	Mod (M)	+
	sema-1a	*sema-1a[k13702]*	axon guidance	En (M)	En (M)	-	+
	fra	*l(2)k03003**	receptor of Netrin	Su+ (M)	Su+ (M)	Mod (W)	
		*fra[4]***		Su (W)		Mod (W)	
	sli	*l(2)k02205**	ligand (interects with Robo and Sdc)	Su (W)	Su+ (W)	Mod (W)	
		*sli[2]***		Su (W)	Su+ (W)	-	-
	robo	*robo[2]***	receptor	Su (W)	Su+ (W)	-	+
	robo2	*lea[2]***	receptor		Su+ (W)	-	+
	Sdc	*l(2)k10317**	Heparan sulphate proteoglycan (interacts with Sli and Robo)	Su+ (S)	Su+ (M)	-	
		*Sdc[10608]****		Su+ (M)	Su+ (W)	-	+
	stan	*stan[129]***	Receptor, Flamingo	Su+ (M)	Su+ (W)	-	+
	wg	*wg[spd-1]***	ligand	Su+ (S)	Su+ (S)	Mod (M)	+
	grh	*grh[s2140]*	transcription factor	Su+ (S)	Su+ (S)	Mod (W)	+
III. Notch signaling	*Dl*	*Dl[EMS-Mod130]*	ligand of Notch	Su+ (S)	Su+ (M)	Mod (S)	+
		Dl[EMS-Mod140]		Su+ (S)	Su+ (M)	Mod (S)	+
IV. TGF-β signaling	*Dad*	*Dad[j1E4]*	negative regulator	Su+ (S)	Su+ (S)	Mod (M)	+
	dpp	*dpp[KG08191]*	TGF-β homolog	Su+ (M)	Su+ (W)	-	+
	tkv	*tkv[k16713]*	type I receptor	-	-	Mod (M)	
	msk	*msk[EMS-Mod90]*	importin	En (S)	En (S)	En (S)	+
		*msk[5]***		Su (W)	Su+ (M)		+
V. EGFR signaling	*kek1*	*kek1[k07322]*	repressor of EGFR signaling	Su+ (S)	Su+ (M)	-	+
	argos	*l(3)j10E8**	repressor of EGFR signaling; ligand	Su+ (W)	-	-	
		*argos[Delta7]****		Su+ (S)	-	-	+
VI. Other	*Nrk*	*Nrk[k14301]*	receptor tyrosine kinase	Su (W)	Su+ (M)	-	-
	HIPK	*CG17090 [BG00855]*	serine/threonine kinase, death	Su+ (S)	Su+ (S)	Mod (S)	-
	kis	*kis [k13416]*	ATP helicase activity; chromatin binding	Su+ (S)	Su+ (S)	Mod (W)	+
	gcm	*gcm[KG01117]*	transcription factor activity	En (S)	En (M)	-	+
	CG4496	*CG4496[KG10365]*	zinc ion binding, nucleic acid binding	Su (S)	Su+ (S)		
	wun	*wun[k10201]*	lipid phosphate phosphatases	-	Su+ (W)	-	+
		*l(2)k11120a**		Su+ (W)	-	-	
	POSH	*POSH [15815]*	SH3 adaptor protein, JNK signaling	Su (W)	Su+ (M)	-	+
	vimar	*vimar[k16722]*	Ral GTPase binding	Su (W)	Su+ (M)	-	+
	del	*del[KG10262]*	oogenesis	-	Su+ (S)		+
	SP1070	*Poly-EGF [EMS-Mod29]*	Notch binding (predicted)	Su+ (M)	Su+ (M)	-	

Table 1. cont.

Functional groups	Gene name	Allele(s)	Function	act-Gal4: UAS-$Dys^{N\text{-}RNAi}$/+	tub-Gal4: UAS-$Dys^{C\text{-}RNAi}$/+	Dys^{E6}/+ [1]	Dg interactors[2]
	CG7845	*CG7845[EMS-Mod4]*	WD40 domain protein	Su+ (M)	Su+ (M)	-	
	l(3)L4092	*l(3)L4092*	Zn-finger protein	Su+ (S)	Su+ (S)	Mod (S)	

*, P-element insertion used in screen that may affect gene;
**, gene allele from Bloomington stock center; S, suppressor; Su+, suppressor of PCV with extra wing vein material; En, enhancer; Mod, modifier; (W), weak; (M), moderate; (S), strong; 1, phenotypic classes shown in Figure S3; 2, summary for the data in Figure 4; +, interact; -, does not interact.
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Deficiency screen. In addition to the P-element lethal collection, we also screened for the *Dys* interactors using a collection of deficiency lines, which covers about 30% of the 1st, 40% of the 2nd and 80% of the 3rd chromosome and found 10 regions on the 2nd and the 3rd chromosomes that interacted with $Dys^{N\ RNAi}$ in the wing vein (Figure 5A). Nine of these deficiencies suppressed the *Dys* PCV phenotype with formation of extra wing vein material and belonged to the Su+ class (Figure 3C and 5B) and one to the En class (Figure 3D and 5B). The *wg* and *stan* (*Flamingo, Fla*) genes, which belong to the planar cell polarity pathway (Table 1), were identified as $Dys^{N\ RNAi}$ interactors in [27D1-27F2] and [47A7-47C6] cytological regions respectively (Figure 5B). Both of these genes modify Dg^{RNAi} phenotype in wing vein as well (Figure 4). A third component of the planar cell polarity pathway, *grainy head* (*grh*), was identified from the P-element screen. Furthermore, *dystrophin* and *Dl* genes located in region [91F12-92A11], as well as *poly-EGF* and *CG4496* from [27D1-27F2] region were also independently identified in the P-element and EMS screens (Figure 5B and Table 1).

EMS mutagenesis screen. We analyzed ~37,000 chromosomes for modifiers of $Dys^{N\ RNAi}$ and $Dys^{N2\ RNAi}$ and over 6000 chromosomes for enhancers of Dys^{E6} and isolated 27 modifiers (Table S1). Eighteen of these were localized to the 2nd chromosome and nine were localized to the 3rd chromosome. The genes defective in eight of these mutants were identified and shown to correspond to five genes (Table 1). The modifier screens using EMS produced similar phenotypes to those observed in the P-element screen (Figure 4) and additionally produced a "posterior crossveinless" class (14/27 modifiers; Figure S2). These modifiers may be alleles of the *crossveinless* genes on the second and third chromosomes. There are eight previously identified *crossveinless* loci, *cv, cv-2, cv-3, cv-b, cv-c, cv-d, cvl-5* and *cvl-6*. Two, *cv* and *cvl-6*, are on the X chromosome. One, *cv-3* is on the second chromosome and the remaining five are on the third chromosome. Modulators of the TGF-β pathway are encoded by two of the eight *crossveinless* loci, *cv* and *cv-2*. Of the remaining six one other, *cv-c* has been molecularly characterized and encodes a *Drosophila* Rho GTPase Activating Protein. The others may also encode effectors of TGF-β signaling. Nevertheless, when *cv* and *cv-2* are lost the resultant aberrant TGF-β signaling results in the loss of both crossveins. Of our crossveinless like modifiers, one, Mod90 (Table 1 and Table S1), was mapped to cytological location 66B, two map units distal to the *hairy* gene, and so does not appear to be an allele of any of the *crossveinless* genes on the third chromosome. An attractive candidate for this gene is *moleskin* (*msk*) which encodes *Drosophila* Importin-7, a protein involved in nuclear translocation that has been shown to regulate the TGF-β pathway by controlling Mad localization. We further confirmed that *moleskin* (msk^5) interacts with *Dg* and *Dys* (Table 1).

The next largest class of modifiers (9/27 EMS induced modifiers; Table S1) belonged to the Suppressor+ (Su+) class with extra wing vein material (Figure 3) and showed a more global effect on the wing. Four of these modifier mutants, Mod59, Mod111, Mod130 and Mod140, had phenotypes in the absence of

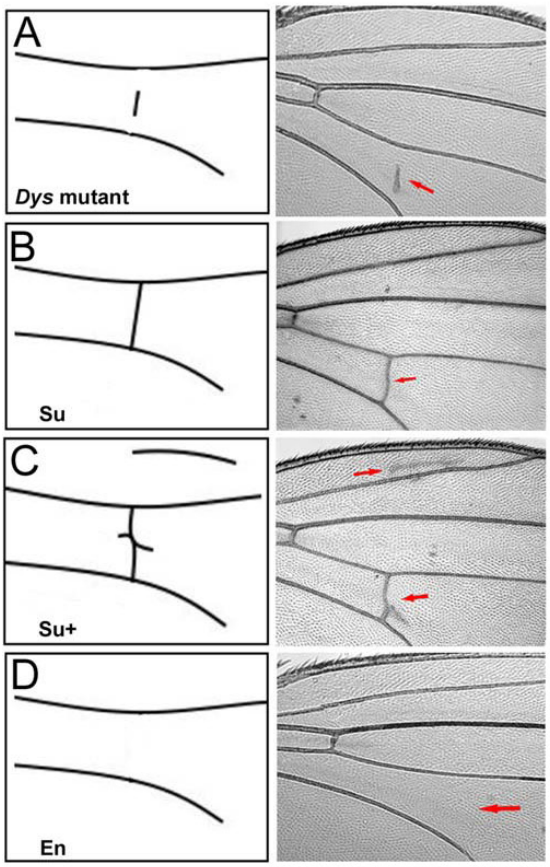


Figure 3. Wing Vein Phenotypes in *Dys* Modifier Classes. The posterior cross vein (PCV) was used in the screening process. The *Dys* mutant is depicted schematically in (A) with an actual fly wing (*act-Gal4:UAS-*$Dys^{N\ RNAi}$/+) shown to the right. Among the modifiers from the original mutant phenotype was the Su class or the completely suppressed class (B) where the PCV reverted to the wild type cross vein. Another class of interactors suppressed the detached PCV phenotype but also produced extra vein material, either as a branch or as an extra L2 vein (arrows, C). This group was classified as suppressor-plus (Su+). Finally, a group of modifiers showed a complete loss of the PCV (D, arrow) and this group was classified as enhanced (En).
doi:10.1371/journal.pone.0002418.g003

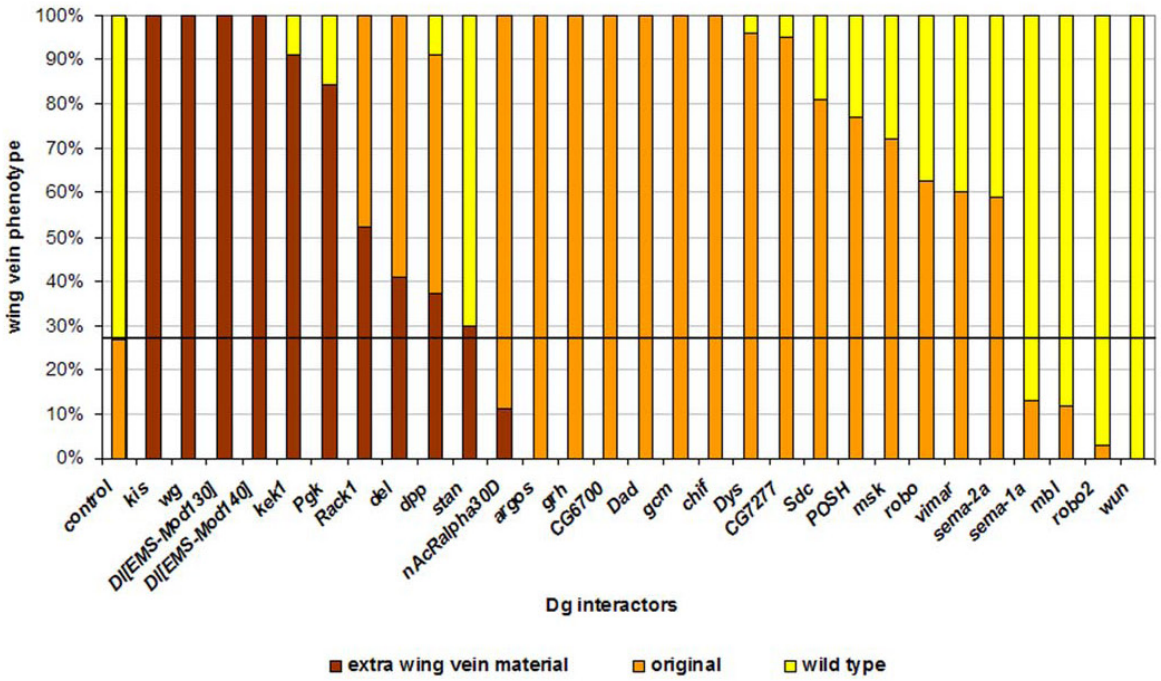
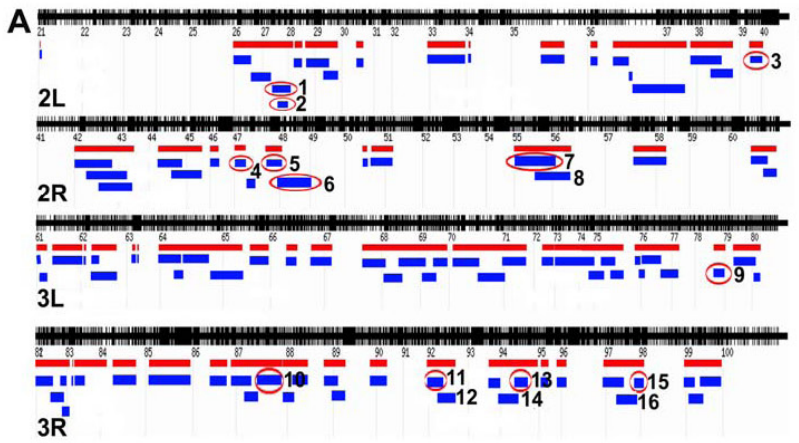


Figure 4. *Dg* Mutant Modifiers. The RNAi Dystroglycan mutant modifiers were scored either as an increase in penetrance of the Dg phenotype, or as an addition of extra vein material. These are represented in a bar graph. The ordinate indicates the percent penetrance of the wing vein phenotype. The abscissa indicates the genes that interact with *tub-Gal4:UAS-Dg^{RNAi}/+* as heterozygotes. The unmodified *Dg* mutant phenotype is at the far left (control) and shows that nearly 30% of *Dg^{RNAi}/+* flies show the Dg mutant phenotype (orange color) with the rest having wild type wing veins (yellow color). Modifiers that show an extra vein material are indicated in brown. Wing vein phenotypes are shown in Figure S4.
doi:10.1371/journal.pone.0002418.g004



B

Df	Modifier class	Dys interact-ting region	Dys Interact-ting genes
1	Su+	27D1-27F2	*wg*
2	Su+		*SP1070**
			*CG4496***
3	Su+	39B4-40A5	
4	Su+	47A7-47C6	*stan*
5	En	47D3-48A5	
6	En		
7	Su+	55A1-55E6	
8	-		
9	Su+	78D5-79A2	
10	Su+	87C7-87F6	
11	Su+	91F12-	*Dys**
12	-	92A11	*Dl**
13	Su+	93D4-93E10	
14	-		
15	Su+	97E2-98A2	
16	-		

Figure 5. Deficiency Screen. (A) Cytological map of 2nd and 3rd chromosomes. Red bars represent cytological regions that were screened for interaction with *Dys* in wing vein. Blue bars represent deficiencies tested in the screen. Deficiences that showed interactions are circled in red. Numbers next to the blue bars indicate deficiencies used to narrow down the *Dys* interacting region. (B) Numbers in column 1 describe the deficiencies used to narrow down region and respond to the following deficiencies: (1) *w*; Df(2L)spd^{i2}, wg$^{spd\,i2}$*, (2) *w^{1118}; Df(2L)ade3*, (3) *Df(2L)ED1473*, (4) *Df(2R)ED2098*, (5) *Df(2R)en-B, b^{1} pr^{1}*, (6) *Df(2R)en-A*, (7) *Df(2R)PC4*, (8) *y^{1}w*/Dp(1;Y)y^{+}; Df(2R)P34*, (9) *Df(3L)ED4978*, (10) *Df(3R)ED5612*, (11) *Df(3R)ED5942*, (12) *Df(3R)ED6025*, (13) *Df(3R)ED6069*, (14) *Df(3R)ED6076*, (15) *Df(3R)ED6265*, (16) *Df(3R)TI-P, e^{1} ca^{1}*. Class of wing vein phenotype modifiers is listed in second column. Cytology of 10 *Dys* interacting regions found in the screen is shown in column 3. Column 4 indicates Dys interacting genes found in deficiency, EMS (*) and P-element (**) screens.
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the *dystrophin* mutant. We mapped them to cytological location 92A and showed that they are tightly linked to the *Delta* gene (*Dl*, Table 1). Subsequent crosses to different *Dl* alleles yielded lethal phenotypes, suggesting that these mutants are alleles of *Dl*. Two of the modifiers Mod130 and Mod140 suppressed the posterior crossvein phenotype of $Dys^{N\text{-}RNAi}$, i.e. formed a complete crossvein from longitudinal vein L4 to longitudinal vein L5, in addition to generating extra wing vein material. We also found modifiers that belonged to the Enhancer as well as the Suppressor classes (Text S1).

Two other members of the class of mutants with extra wing vein material (Table S1 and Figure S2B), Mod4 and Mod29 were mapped to specific locations on the 2nd chromosome. Mod4 was mapped by following its lethality phenotype in crosses with deficiency lines. It was lethal in a cross with the *Df(2R)nap9/Dp(2;2)BG, In(2LR)Gla* line and was not lethal in a cross with the *Df(2R)ST1, Adh[n5] ltr[1] cn[*]/CyO* line. The available lethal mutants from cytological region 42A1-42B3 were crossed to Mod4 and using a P-element insertion line, *PBac{PB}CG7845c00845/CyO* we determined that the mutation was in gene *CG7845*. This gene codes for a WD40 containing protein whose function is not known (Table 1). The underlying common function of all WD-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions. Such a scaffolding protein may be utilized in the formation or stabilization of the DGC.

Following the wing vein phenotype seen in homozygous flies as well as its semi-lethality, Mod29 was mapped to cytological region 27D1-27D4. This region was independently identified to contain a *Dys* interactors through the Deficiency screen (Figure 5B). We further fine-mapped the region by showing that two smaller deficiency lines, *Df(2L)ade3/CyO,P{ftz/lacB}E3* and *Df(2L)Exel7029,P+PBac{XP5.WH5}Exel7029/CyO* phenocopy the semi-lethality/wing vein phenotypes seen with Mod29. Ultimately, Mod29 was determined to be a *poly-EGF* gene mutant. Other genes in the region were eliminated by complementation analysis. In addition to the wing vein phenotype, Mod29 also shows defects in muscles, in oogenesis and in the brain (photoreceptor axon termination; Figure S5). Additionally, these mutant animals have a very short lifespan. Since Poly-EGF is predicted to bind the Notch receptor further functional analysis will be very interesting.

As discussed above, the Dys^{E6} mutant does not show a wing vein phenotype as a heterozygote (Figure 2A). But as a homozygote it displays a disrupted posterior crossvein similar to $Dys^{N\text{-}RNAi}$ (Figure 2B and 2C). We reasoned that this heterozygote background was "sensitized" and therefore excellent for identifying enhancers. We screened 6000 chromosomes and found four modifiers for Dys^{E6}/+. Three of those modifiers showed some extra wing vein material in the posterior crossvein (Figure S2B). Two of the modifiers, on the third chromosome ModE21 and ModE26 showed a branched crossvein phenotype in the absence of Dys^{E6} though this dominant phenotype was not completely penetrant. The third of this group, ModE11 on the second chromosome, showed no wing vein phenotype in the absence of Dys^{E6}, however as a homozygotes ModE11 flies displayed a globally disrupted wing vein characterized by an abundance of extra vein material (data not shown). Modifier E10 was the only modifier that showed an enhanced phenotype (Figure S3A). $ModE10/Dys^{E6}$ flies phenocopied the Dys^{E6} homozygous phenotype which is a reduction in wing vein material (Figure 2B). Moreover, *ModE10/Df(3R)Exel6184* flies also gave a similar wing vein phenotype to Dys^{E6}*/Df(3R)Exel6184* flies strongly arguing that ModE10 is a *dystrophin* allele. Finding an additional allele of *Dys* confirms that the screening approach was successful and analysis of the EMS mutant will allow us to further characterize Dystrophin protein function. Furthermore, the newly identified *dystrophin* allele *ModE10* interacted strongly with the *Dg* RNAi mutant in the wing; instead of 27% penetrance, the phenotype was 96% penetrant in heterozygous *Dys[Mod-E10]* background (Figure 4). This further confirms that molecules acting closely with Dys and Dg can be found in this assay.

mbl Interacts with *Dys* in Muscles

These modifier screens led to the identification of genes that belong to different functional groups. Importantly, genes with known muscle function were identified in this screen, including *dystrophin* itself. In addition, the screen also identified muscle genes previously shown to interact with the Dg-Dys complex, *Cam*, *mbl* and *nAcRα-30D* [22,23,24]. *muscleblind* encodes a RNA binding protein that has shown to function in splicing [25]. As part of a secondary screen in muscle tissue, we tested whether *mbl*, a strong interactor with *Dys* in wing vein, also interacts with *Dys* in the indirect flight muscles.

Animals that have lost one copy of *mbl* appear to have normal muscle structure (Figure 6A), while *act-Gal4:UAS-*$Dys^{N\text{-}RNAi}$ mutants show moderate muscle degeneration (10 days old: 10%, n = 68; 18 days: 25%, n = 159; Figure 6B and 6D). However, the extreme muscle degeneration phenotype was not observed in 10 days old and at a low frequency in 18 day old $Dys^{N\text{-}RNAi}$ mutants (Figure 6B and 6D). In contrast, when *mbl* was reduced by one copy in a $Dys^{N\text{-}RNAi}$ mutant background, increase in muscles defects was observed. Furthermore, around 50% of the phenotypes observed were classified "extreme" both in 10 and 18 day timepoints (Figure 6C and 6D). These data suggest that *mbl* and *Dys* interact in the muscles and reduction of *muscleblind* level enhances the abnormal muscle phenotype in $Dys^{N\text{-}RNAi}$ mutants. These results show that the screen successfully identified genes that interact with *dystrophin* to establish normal muscle function.

Interactors Show Germline Phenotypes Similar to *Dys* and *Dg* Mutants

The *Dys* and *Dg* genes are required in the germ line for the establishment of oocyte polarity [6,8,9]. The oogenesis defects of *Dys* and *Dg* serve as an excellent test to identify whether the new modifiers might interact with Dg-Dys complex in other tissues as well as in wing development. The ovariole contains a progression of egg chambers at different stages of development (Figure 7A). An early oocyte polarity marker, Orb, is localized to the anterior side in early stages and then migrates to the posterior side of oocyte by stage 3 of oogenesis. Between stages 3 and 6, Orb is clearly localized to the posterior of the oocyte, making it an excellent marker to analyze the polarity of the oocyte (Figure 7A B). *Dg* loss-of-function germ line clones and homozygous point-mutants are arrested at early stages of oogenesis ([9]; Figure 7B) and show mislocalization of the Orb marker that is usually missing or diffused ([9]; Figure 7D). The oocyte polarity phenotype was also observed for viable *Dg* and *Dys* alleles (Figure 7B, 7D and 7E; 74%, n = 50 for Dg^{O86}/Dg^{O55}, 82%, n = 34 for Dg^{O43}/Dg^{O55} and 68%, n = 48 for Dg^{O86}/Dg^{O43}; the ovaries were dissected from 12 days old mutants).

Interestingly, many of the identified modifiers are genes previously shown to be required in germ line development. For example a scaffolding protein, Rack1 that contains multiple WD-domains and interacts with atypical protein kinase C (aPKC) has recently been shown to function during *Drosophila* oogenesis [26]. We now analyzed potential Rack1 function in germ line for posterior Orb localization. Importantly, this analysis revealed that Rack1 is required in the early oocyte polarity: lack of Rack1 in

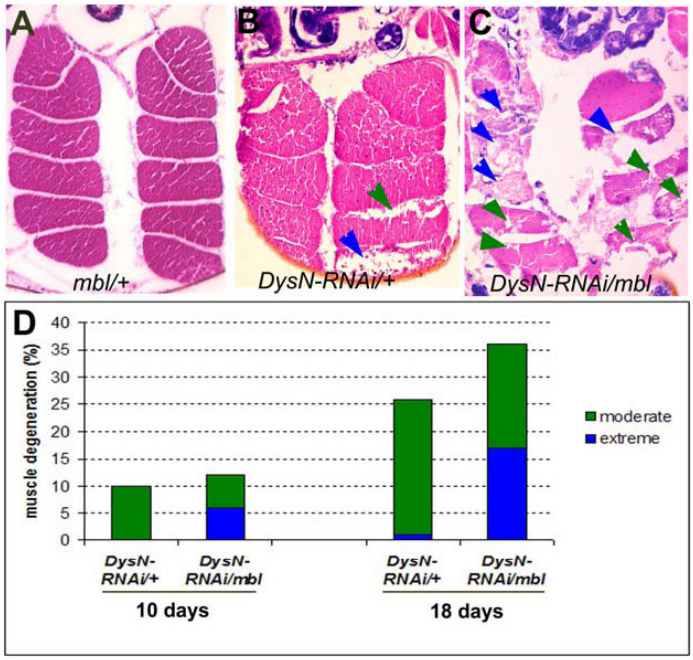

Figure 6. ***mbl*** **Interacts with** ***Dys*** **in Muscle.** (A–C) Transverse histological sections of indirect flight muscles of 18 days old flies. Reduction of *muscleblind* by one copy does not show obvious muscle defects (A). A stronger phenotype is observed in a *act-Gal4:UAS-Dys*$^{N\ RNAi}$/+ mutant where loss of muscle integrity is noticeable throughout the tissue (B) and a significantly higher level of muscle degeneration is observed if the level of *muscleblind* is reduced in a *Dys* mutant background (*act-Gal4:UAS-Dys*$^{N\ RNAi}$*/mbl*, C). A green arrows in B and C indicate moderate muscle degeneration and blue arrows extreme muscle degeneration phenotype. The bar graph (D) quantifies the percentage of muscles that yielded the muscle phenotypes in 10 and 18 days old flies. Green bars indicate moderate muscle degeneration and blue arrows extreme muscle degeneration phenotype.
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early oocytes resulted in Orb mislocalization in the majority of the eggchambers (76%, n = 13, Figure 8H). Consistent with these findings aPKC is required for early oocyte development in *Drosophila* [27]. Similarly, another *Dys* and *Dg* interacting protein, Deadlock has previously shown to be required for Orb localization [28]. It is, therefore, likely that other identified *Dys* and *Dg* interactors are required in early oocyte polarity, possibly in a process closely related to Dg-Dys function in oogenesis.

Utilizing the FRT sites in the P-lethal lines we made germ line clones to determine how many of the selected modifiers had a role in the establishment of early oocyte polarity. We analyzed germ line clones of 14 loss-of-function mutants and found 8 genes that showed a similar phenotype to that seen in *Dg* loss-of-function clones. One of these genes, *POSH*, is an SH3 adapter protein. Loss-of-function clones of *POSH* (Figure 8A, white arrow) were arrested prior to stage 3–4 of oogenesis and appeared to lack Orb entirely. Orb mislocalization was also found in *Sema-2a*, *kek1*, *kis*, *Lis-1*, *chif* and *Khc*. *Sema-2a* loss-of-function clones showed clear developmental arrest prior to stage 4–6 with diffused Orb staining (Figure 8B). *kek1*, a negative regulator of the EGFR pathway, also showed developmental arrest in loss-of-function clones (Figure 8C). Orb in *kek1* clones surround the oocyte in an irregular pattern. *kis* loss-of-function germline clones have shown developmental arrest and diffused Orb staining (Figure 8D). Similarly, the majority of *Lis-1*, *chif* and *Khc* germline clones showed abnormal Orb localization (Figure 8E–8G). Lis-1 and Khc have previously shown to be involved in oocyte microtubular motor activities and to interact in this process [29,30].

These data indicate that many of the genes that showed interactions with *Dys* and/or *Dg* in the wing vein assay, also showed early oocyte polarity phenotypes similar to *Dys* and *Dg* and potentially play a role in the establishment of early oocyte polarity.

Discussion

In mammalian systems the DGC is composed of Dystrophin, Dystroglycan, the Sarcoglycan complex (α, β, γ, δ, ε, and ζ), Sarcospan, the Syntrophins (α and β), the Dystrobrevins (α and β) and Caveolin-3 [2]. Presently, the regulation of Dg-Dys complex and its involvement in signaling are poorly understood. In this work, we have addressed these unknowns by using dominant modifier screens to find genes that may shed light on both of these processes. Our screens have revealed groups of modifiers that are components of canonical signaling pathways (TGF-β, EGFR, Wnt and Notch) as well as components involved in cell/neuronal migration, cytoskeletal rearrangements and most importantly muscle development.

New Components of the Dg-Dys Pathway?

The screens described in this paper revealed some expected interactors, *Dys*, *Cam* and *Khc*. Calmodulin, a calcium binding protein required for muscle and neuronal functions has previously been shown to interact with mammalian the Dg-Dys complex. However, whether reduction of *Cam* activities suppresses or enhances the muscular dystrophy phenotype is not totally clear. Targeted inhibition of *Cam* signaling exacerbates the dystrophic

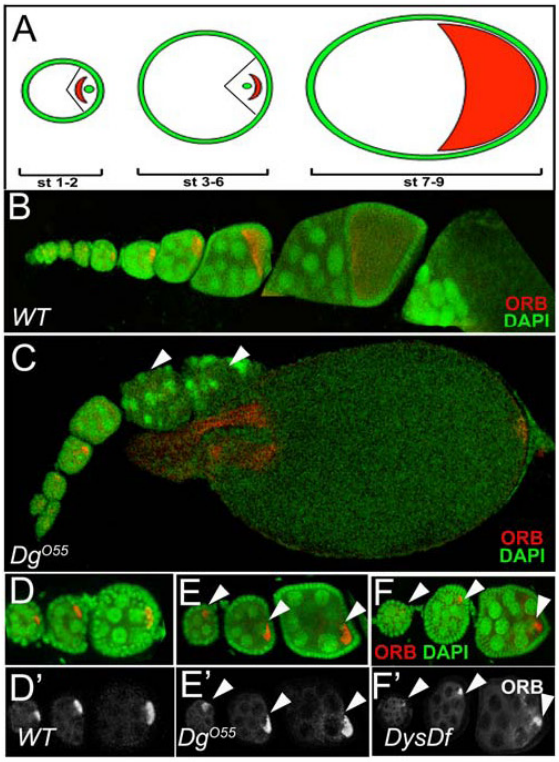

Figure 7. *Dys* and *Dg* Mutant Germline Phenotype. In the wild type ovariole, Orb localizes to the posterior of stage 3–6 oocytes (A, B). (C) In Dg^{O55} mutants, however, there is mislocalization of Orb and much degeneration is observed. Arrowheads indicate mutant egg chambers. Additional analyses of Dg^{O86}/Dg^{O55}, Dg^{O43}/Dg^{O55} and Dg^{O86}/Dg^{O43} show similar phenotypes (not shown). Stage 3–6 wild type egg chambers show posteriorly localized Orb staining (D, arrowheads, red staining). (D') shows Orb staining alone. Stage 3–6 Dg^{O55} mutant egg chambers show an abnormal lateral localization of Orb (E, arrowheads, red staining). (E') shows Orb staining alone. Stage 3–6 egg chambers from *DysDf* homozygotes where the Orb staining is defused. (E') more clearly shows the altered Orb staining.
doi:10.1371/journal.pone.0002418.g007

phenotype in mdx mouse muscle while genetic disruption of *Calcineurin* improves skeletal muscle pathology and cardiac disease in *ä-sarcoglycan* null mice [31,32]. Since reduction of *Cam* showed suppression of the phenotypes caused by reduction of the long forms of *dystrophin* in the *Drosophila* wing, it will be interesting to analyze whether reduction of *Cam* will suppress the *Drosophila Dys* muscle phenotype as well. *Khc* involvement in Dg-Dys complex is also expected since work in mammalian system has shown that Khc can bind Dystrobrevin, a component of Dg-Dys complex [33]. It will be interesting to test in the future whether *Drosophila* Dystrobrevin can similarly bind Khc and what the functional significance of this interaction is in muscles and neurons. We have already shown that in oocyte development *Khc* is required for the same early as *Dys* and *Dg* (Figure 7). It is, therefore, interesting to test the potential requirement of *dystrobrevin* in this process and to further dissect the *Khc* function in this complex during early polarity formation.

An unexpected new interactor was identified in our screens, the homeodomain interacting protein kinase, HIPK. In mammalian systems HIPK is involved in the cell death pathway by phosphorylating p53 [34]. Recently *Drosophila* HIPK has shown to be involved in a communal form of cell death, sudden, coordinated death among a community of cells without final engulfment step [35]. It remains to be seen whether this HIPK communal death pathway will utilize p53 phosphorylation. However, it is tempting to speculate that the cell death observed in muscular dystrophies use the newly described HIPK dependent communal death pathway. HIPK is shown to interact with a WD40-protein in mammalian system [35]. Since three WD40 proteins were identified in our screens, it will be interesting to test whether any of these interact with *Drosophila* HIPK.

Another interactor that might shed light in the pathways utilized by the Dg-Dys complex is an SH3-domain adapter-protein, POSH. Structure-function analysis of Dg protein has revealed that a potential SH3-domain binding site in Dg C-terminus is essential for Dg function [8]. However, the critical SH3-domain protein in this complex is still at large. The present screen revealed that POSH can interact with the Dg-Dys complex in the wing vein assay. It will now be interesting to determine whether POSH is the missing critical SH3-domain protein that interacts with Dg-Dys complex in *Drosophila*.

The Neuronal/Cell Migration Pathways Interact with the DGC

There are only a few examples of signaling pathways that have been shown to transmit information from outside the cell that results in cytoskeletal rearrangements inside the cell. Slit-Robo, Netrin-Frazzled and Semaphorin-Plexin pathways are examples of such activity. Dg-Dys complex appears also regulate the cytoskeleton based on extracellular information. Interestingly, the interaction screens described in this paper show that these aforementioned pathways are much more intricately connected than previously thought. The Robo and Netrin Receptor (DCC) pathways have previously been shown to interact [36], now we report that Dg-Dys complex interact with these pathways as well.

The interactions that we see in wing development involving the *Drosophila* DGC and the genes that affect neuronal guidance (*sli, robo, fra, sema-2a, sema-1a, Sdc*) might be explained by their possible role in hemocyte (insect blood cell) migration. Analysis done in *Drosophila* shows that known axon guidance genes (*sli, robo*) are also implicated in hemocyte migration during development of the central nervous system [37]. Similar findings have been reported in mammals, where blood vessel migration is linked to the same molecular processes as axon guidance [38]. Both *sli* and *robo* have been implicated in the vascularization system in vertebrates [39]. A recent study demonstrated that proper hemocyte localization is dependent upon *Dys* and *Dg* function in pupa wings. Mutations in these genes result in hemocyte migration defects during development of the posterior crossvein [14]. Hence, we speculate that the neuronal guidance genes that we have found may interact with the DGC in wing veins by having a role in the migration process.

Similar to *sli* and *robo* [40], the *Dys* and *Dg* mutants also affect photoreceptor axon pathfinding in *Drosophila* larvae [6]. It is therefore possible that this group of modifiers will interact with the DGC in axon pathfinding and other processes. Supportive of that notion is the fact that mammalian Syndecan-3 and Syndecan-4 are essential for skeletal muscle development and regeneration [41]. In addition *slit-Dg* interaction has previously been observed in cardiac cell alignment [42]. Sequence analysis of slit reveals that it possesses a laminin G-like domain at its C-terminus. Dystroglycan's extracellular domain has laminin G domain binding sites and has been shown to bind 2 of the five laminin G domains in laminin. It is therefore possible that slit, through its laminin G-like domain, binds to Dystroglycan and that Dystroglycan is a slit

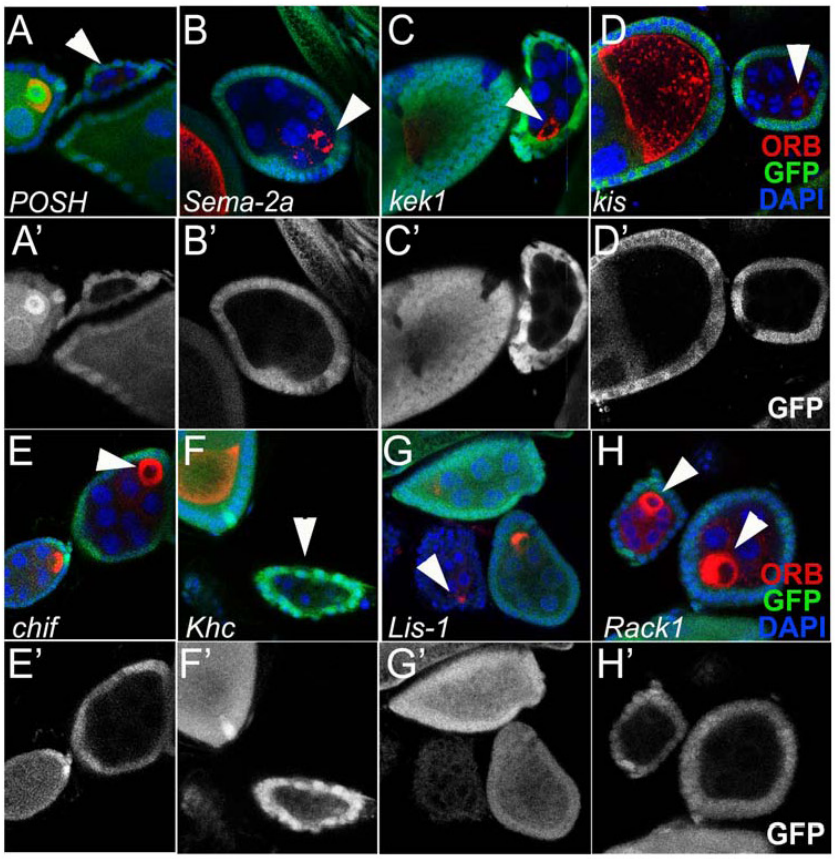

Figure 8. Interactors Show Similar Germ Line Phenotypes to *Dys* and *Dg*. *POSH* (*hsFlp, FRT42D*), loss of function clones show arrest at around stage 3–4 and appear to lack Orb staining completely (A). Loss of function clones of *Sema-2a* (*hsFlp, FRT42D Sema-2a[k13416]*) (B), *kek1* (*hsFlp, FRT40A kek1[k07322]*) (C), *kis* (*hsFlp, FRT40A kis[k13416]*) (D), *chif* (*hsFlp, FRT40A chif[BG02820]*) (E), *Khc* (*hsFlp, FRT42D Khc[k13219]*) (F), *Lis-1* (*hsFlp, FRT42D Lis-1[k13209]*) (G) and *Rack1* (*hsFlp, FRT40A Rack1[EY00128]*) (H) all show developmental arrest prior to stage 6. *Khc*, like *POSH* appears to lack any Orb staining (red) while the others have abnormal Orb staining ranging from punctate (*Sema-2a*) to completely surrounding the oocyte (*chif and Rack1*). GFP, green; Orb, red; DAPI, blue in (A–H). (A'–H') show GFP staining. Arrows indicate Orb staining in clones.
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receptor. It will be informative to reveal the mechanisms and nature of these interactions.

Oocyte Phenotypes

The establishment and formation of oocyte polarity during development is a complex multistep process (reviewed in [43]). In the anterior part of the germarium each stem cell undergoes asymmetric cell division to give rise to another stem cell and a cystoblast. The cystoblasts divide four times with incomplete cytokinesis to form a 16 cell cyst. The oocyte fate is determined when the cyst reaches the end of the germarium. At this point, BicD protein, Orb protein, the microtubule organizing center (MTOC) and the centrioles move from the anterior to the posterior of the oocyte (Figure 7A). These events mark the first sign of polarity in the oocyte. Subsequent Gurken signaling induces posterior follicle cells to signal back to the oocyte which repolarizes the microtubule cytoskeleton. This signal appears to require an intact extracellular matrix since *Laminin A* mutants do not undergo repolarization [44]. The outcome of the repolarization results in the disassembly of the MTOC at the posterior, nucleation of microtubules anteriorly and subsequent migration of the oocyte nucleus to an antero-lateral position.

Germ line clones that lack *Dg* show developmental arrest and mislocalization of the oocyte polarity marker Orb which is usually diffused or absent in the oocyte [9]. This phenotype might be due to *Dg* affecting the localization of the MTOC. But how exactly *Dg* is involved in this process is not clear. One possible explanation is that Dg is required for extracellular matrix (ECM) integrity since it is known to bind Laminin. Such a structural conduit may be necessary for proper signaling from the posterior follicle cells to the oocyte. This is supported by the fact that *Dg* loss-of-function mutants show defects in Actin accumulation. Another possibility is that *Dg* may be involved in mircrotubule organization. Since the regulation of actin- and microtubule-cytoskeleton are connected, these two models are not mutually exclusive.

Interestingly, in our screens we found several genes that showed phenotypes in oocyte development. One of these genes is *kek1*, a transmembrane protein of the *Drosophila* Kekkon family that has been reported to be a negative regulator of the EGFR receptor [45]. It has been previously shown that EGFR signaling regulates

the expression pattern of Dystroglycan to establish anterior-posterior polarity of oocyte [10]. Further study is required to determine if *kek1*, as an EGFR regulator controls *Dg* expression in the germ line.

Another interesting gene found in our screens is *POSH* (Plenty of SH3 domains), a *Drosophila* homologue of human SH3MD2 protein. Interestingly POSH is a multidomain scaffold protein that can interact with Rho related GTPase - Rac1 and promotes the activation of the JNK pathway [46] POSH has also shown to regulate POSH-MLK-MKK-JNK complex [47]. A defect in this complex can affect brain function. Furthermore, POSH and JNK-mediated cell death pathway is thought to play an important role in Parkinson's disease [48]. With many SH3 domains, POSH has the potential to bind *Dg* that has a predicted SH3-domain binding site and has been shown to be necessary for the establishment of oocyte polarity [8].

In addition, we have found interactions with *Khc*, *Lis-1* and *Dmn*, three genes known to be part of the Dynein-Dynactin complex which in addition to Kinesin microtubule motor activity have been shown to be necessary for establishment of intracellular polarity within the Drosophila oocyte [49,50]. In mid-oogenesis dynein, dynactin and kinesin are thought to act cooperatively in cargo transport [49,51,52]. Since these genes interact with *Dys* (Table 1) and show similar phenotypes in Orb localization (Figure 7), it will be interesting to dissect their potential functional interactions with *Dys* in early oocyte development. Furthermore, since mammalian Dystrobrevin physically interacts with Khc, it is plausible, that the Dynein-, Dynactin-, Kinesin-complex will utilize localization cues set-up by Dg-Dys Complex.

In addition to the interactions with microtubular motor-complexes, we also found interactions with a *Drosophila* Formin homologue, FHOS. Mammalian FHOS directly binds to F-actin and promotes actin fiber formation [53]. Recently *Drosophila* actin nucleators, Capu and Spire have shown to assemble a cytoplasmic actin mesh that maintains microtubular organization in the middle of oogenesis [54,55,56,57,58]. Therefore, it will be important to determine whether the actin nucleator, FHOS is also involved in actin nucleation that regulates microtubular activity in early oocyte development. Further study of these cytoskeletal genes will allow us to gain a more detailed understanding of how *Dg* and *Dys* function to ensure proper oocyte polarity during oogenesis.

Similar to microtubule and actin interplay in the regulation of oocyte polarity, the dynamic actin-microtubule interactions regulate growth cone steering at the growing axons [59]. It is therefore possible that similar mode of function for Dg-Dys interactions with these cytoskeletal modules is used in various cell types. Furthermore the axon pathfinding and oocyte polarity formation processes are similar in that they are controlled by extracellular information which is transmitted to the cell resulting in cytoskeletal rearrangement.

Planar Cell Polarity Genes

At the basal side of follicle epithelium, actin filaments exhibit a planar cell polarity that is perpendicular to the long axis, the AP axis, of the egg chamber. In *Dg* follicle cell clones the basal actin array is disrupted non-cell-autonomously [9]. Integrins and the receptor tyrosine phosphatase *Lar* are also involved in basal actin orientation [60]. It is unclear whether *Dg* and the other genes involved in basal actin polarity act together with the canonical planar cell polarity pathway or function independently of this pathway. Interestingly, we now report strong interactions between the DGC and *grainy head* (*grh*) a transcription factor which is required for several different processes during the differentiation including the function of the *frizzled* dependent tissue polarity pathway, epidermal hair morphogenesis and wing vein specification [61]. In the absence of *grh* function the Fz, Dsh and Vang proteins fail to accumulate apically and the levels of Stan (or Flamingo) protein are dramatically decreased. The interactions seen with *stan* (*Fla*) and *wg* in wing veins supports the hypothesis that Dg might act together with the *frizzled*-dependent tissue polarity pathway in coordinating the polarity of cells in epithelial sheets.

Conclusions

By screening for alterations of a dominant wing vein phenotype we have found modifiers of the DGC that are involved in cytoskeletal organization. Initial characterization of some of these genes revealed that they have phenotypes also in other tissues, in which the DGC is known to function. These tissue/cell types include the oocyte, the brain and the indirect flight muscles. This argues strongly that the identified interactors may be involved globally in DGC function. Further study is required to determine mechanistically how these modifiers work in the context of the Dg-Dys complex. However a common theme, already arising is that the identified interactors appear to regulate cytoskeletal rearrangement. Mechanistic understanding of how the new interactors might regulate Dg-Dys communication with cytoskeleton of muscle cells may serve as a basis for the development of novel therapeutic approaches that might improve the quality of life of individuals afflicted with muscular dystrophy.

Materials and Methods

Fly Strains and Genetics

The fly-strains used is in this study are: Dys^{E6}/*TM3*, *UAS-*$Dys^{N\text{-}RNAi}$/*CyO* (both kindly provided by L.Fradkin and described previously as $dys^{DLP2\ E6}$ and *RNAi-dysNH*$_2$ [62]), *UAS-*$Dys^{N2\text{-}RNAi}$/*TM3*, *UAS-*$Dys^{C\text{-}RNAi}$/*TM3*, *UAS-*Dg^{RNAi}/*TM3* (described previously as *UASdsDysN-term*, *UASdsDysC-term* and *UASdsDg*, respectively [6]), *DysDf*, Dg^{O43}/*CyO*, Dg^{O55}/*CyO*, Dg^{O86}/*CyO* (kindly provided by R. Ray [14]), *KX43/TM6C*, *Dys8-2/TM3* [6], *act-Gal4/CyO*, *tub-Gal4/TM3*, *Ubi-GFP FRT42D,*Dg^{323}/*CyO*, *hsFLP; FRT40A Ubi-GFP/CyO*, *hsFLP; FRT42D Ubi-GFP/CyO* and *hsFLP; FRT82B/TM3*. Alleles used in this study *w[1118]*, *msk*[5], *nAcRα-30D[EY13897]*, *fra*[4], *mbl[E27]*, *sli*[2], *robo*[2], *lea*[2], *Sdc[10608]*, *stan[129]*, *wg[spd-1]*, *argos[Delta7]* and *Df(3R)Exel6184*, the deficiency for *dystrophin* were obtained from Bloomington Stock Center.

The following *Dys* and *Dg* mutants were used to screen for modifiers of the wing vein phenotype: Dys^{E6}, an hypomorphic allele of *dystrophin*, *act-Gal4:UAS-*$Dys^{N\text{-}RNAi}$/*CyO*, *tub-Gal4:UAS-*$Dys^{N2\text{-}RNAi}$/*TM3*, *tub-Gal4:UAS-*$Dys^{C\text{-}RNAi}$/*TM3* which are three *Dys* RNAi mutants recombined onto chromosomes with actin and tubulin Gal4 drivers, respectively; and *tub-Gal4:UAS-*Dg^{RNAi}/*TM3*, a *Dg* RNAi mutant recombined onto a chromosome with the tubulin Gal4 driver. $Dys^{N\text{-}RNAi}$ is a transgene on the second chromosome that encodes an inverted repeat that forms a double stranded RNA hairpin complementary to the corresponding N-terminus of the protein. It reduces the protein levels of all the known long isoforms. $Dys^{N2\text{-}RNAi}$ is a transgene on the third chromosome that encodes a different inverted repeat than $Dys^{N\text{-}RNAi}$ but still reduces the protein levels of the known long isoforms. $Dys^{C\text{-}RNAi}$ is a transgene on the third chromosome that encodes sequence complementary to the corresponding C-terminal region of *dystrophin* and reduces the protein levels of all isoforms. All *Dys* RNAi mutants exhibited 100% penetrance of the wing vein phenotype. The *Dg* RNAi mutant exhibited ~30% penetrance of the wing vein phenotype.

P-element screen. The FRT P-element insertion lethal lines used in this study were obtained from the Kyoto Stock Center (Japan). From this collection, 800 lines were screened. Two-to-four day-old males carrying P-lethal insertions over balancers (or virgin females if the P-element was on the X chromosome) were mated to *Dys^{E6}/TM3; act-Gal4:UAS-$Dys^{N\text{-}RNAi}$/CyO; tub-Gal4:UAS-$Dys^{C\text{-}RNAi}$/TM3* and *tub-Gal4:UAS-Dg^{RNAi}/TM3* virgin females (or males). Non-balancer F_1 progeny were screened for dominant modification of the wing vein phenotype. Modifiers were divided into phenotypic classes based on the alterations of their wing veins as compared to wing veins of *act-Gal4:UAS-$Dys^{N\text{-}RNAi}$/(+ or CyO), tub-Gal4:UAS-$Dys^{C\text{-}RNAi}$/(+ or TM3)* and *tub-Gal4:UAS-Dg^{RNAi}/(+ or TM3)* sibling flies. Suppressors were identified when wing veins of F_1 flies phenocopied the wing veins seen in wild type flies. Since *Dys^{E6}*/+ had wild-type wing veins, non-balancer F_1 progeny from the *Dys^{E6}/TM3* and P-lethal insertion cross were analyzed for the presence or absence of a mutant posterior crossvein which is altered in *Dys^{E6}* homozygotes. All the genes found in this screen were crossed to *w[1118]* to verify the absence of a dominant wing vein phenotype. None modifiers showed a dominant posterior wing vein phenotype. Since P-element insertion lethal lines were on chromosomes with FRT sites, we made mosaics and determined if selected modifiers possessed an oocyte polarity phenotype (see below).

EMS screen. For the EMS mutagenesis 2 4 day old *w^{1118}* males were place in yeasted bottles overnight. Twenty males were placed in each vial and starved for 6 8 hours. The flies were then provided access to a solution of 22 25 mM EMS (ethyl methansulfonate; Sigma) in a 5% sucrose solution for 18 hrs. The flies were placed in new vials and allowed to recover for 1 3 hrs. Each vial of flies was transferred to yeasted bottles with 40 virgin females of the desired genotype per bottle. The females were allowed to lay eggs for two days then transferred to new bottles daily for two more days. Mutagenized *w^{1118}* males were crossed to *act-Gal4:UAS-$Dys^{N\text{-}RNAi}$/CyO* or *tub-Gal4:UAS-$Dys^{N2\text{-}RNAi}$/TM3* females. Males of these crosses were scored for a modification of the wing vein phenotype, either a novel alteration or suppression. These were picked and backcrossed to the parental *Dystrophin* mutant females to determine whether the modification was on the second or third chromosomes. Once this was done these modifiers were balanced either with *CyO* or *TM6B* and maintained as a stock. The subset of modifiers that produced phenotypes in the absence of the *Dys* mutants were crossed to meiotic mapping stocks and subsequently mapped using those phenotypes. The remainders were crossed to meiotic mapping stocks with a *dystrophin* mutant in the background and mapped using the modification phenotypes. In addition to the two RNAi *dystrophin* mutants mentioned above, enhancers of the *Dys^{E6}* mutant were also screened for.

Obtained modifiers were mapped meiotically using the lines from Bloomington Stock Center *al[1] dp[ov1] b[1] pr[1] c[1] px[1] sp[1]* and *ru[1] h[1] th[1] st[1] cu[1] sr[1] e[s] ca[1]* .

Deficiency screen. A collection of 216 deficiency lines were screened for modifiers of *act-Gal4:UAS-$Dys^{N\text{-}RNAi}$/CyO* mutant. These lines were a mixed collection of flies from the Bloomington Stock Center, the Exelixis (Harvard) and Drosdel (Cambridge) collections. Additionally, these lines were used for mapping modifiers (Mod29 and Mod4) from the EMS screen. Deficiencies which show interaction in screen: *w^*; Df(2L)spd^2, $wg^{spd\text{-}j2}$; w^{1118}; Df(2L)ade3; Df(2L)ED1473; Df(2R)ED2098; Df(2R)en-B, b^1 pr^1; Df(2R)en-A; Df(2R)PC4; y^1w^*/Dp(1;Y)y^+; Df(2R)P34; Df(3L)ED4978; Df(3R)ED5612; Df(3R)ED5942; Df(3R)ED6025; Df(3R)ED6069; Df(3R)ED6076; Df(3R)ED6265; Df(3R)Tl-P, e^1 ca^1*.

Loss of Function Mosaic Analysis

To obtain germline clones of the modifiers found in the FRT P-element lethal screen, they were crossed to corresponding chromosomal FRT stocks. Third instar larvae and early pupae from this cross were heat-shocked at 37°C for 2 hours. After eclosion, they were placed in vials with fresh yeast paste for 4 5 days before dissection.

Dissections and Immunohistochemistry

Drosophila ovaries and wing imaginal discs were dissected rapidly in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Adult's brains were dissected in PBS and fixed in PFA for 30 minutes. All antibody stainings were performed as described previously [6], except with the Dystrophin antibody with which a protocol provided by L. Fradkin was used [62]. Ovaries, brains and wing imaginal discs were mounted in 70% glycerol in PBS for analysis using a Leica TSC SPE confocal microscope. The following antibodies were used: rabbit anti-Dg (1:3000; [9]), anti-Dys CO_2H (1:3000; [62]), mouse anti-Orb (1:20; Developmental Studies Hybridoma Bank), mouse anti-24B10 (1:20; Developmental Studies Hybridoma Bank), Alexa 488, 568, or 633 goat anti-mouse, Alexa 488, 568 goat anti rabbit. To mount adult wings, flies were pre-incubated in 80% ethanol and 20% glycerol solution, then dissected and mounted in 70% glycerol before analysis using a Leica light microscope.

Muscle Analysis

Histological sections of muscle were prepared from wax-embedded material as described previously [6], stained with hematoxyline and eosin (H&E staining) and analyzed using light microscopy.

Supporting Information

Text S1

Found at: doi:10.1371/journal.pone.0002418.s001 (0.03 MB DOC)

Table S1

Found at: doi:10.1371/journal.pone.0002418.s002 (0.14 MB DOC)

Figure S1 Dys and Dg Expression in Wild Type and Mutant Wing Discs. In wild-type larvae, Dys is expressed in all cells of the wing disc (A) and is strongly reduced in the wing disc of the DysC-RNAi mutant (B; tubGal4:UAS-DysC-RNAi/+). Dystroglycan localization in wild type imaginal discs is enriched at the basal surface of the epithelial cells. Dg expression is more intense in folds formed from the contact of basal surfaces and is less visible in the folds made from apical surfaces of wing disc cells (C). The DgRNAi mutant also shows a reduction of Dystroglycan protein in the wing disc (D; tub-Gal4:UAS-DgRNAi/+). (A′ D′) Enlarged images of framed areas on (A D). (A" B") Enlarged images of framed areas on (A B) show Dystrophin single channel staining. (C" D") Enlarged images of framed areas on (C D) show Dystroglycan single channel staining.

Found at: doi:10.1371/journal.pone.0002418.s003 (6.06 MB TIF)

Figure S2 Subclasses of Dys(RNAi) Modifiers. (A) Example of enhancers that lack the posterior cross vein (PCV) and belong to phenotypic class En. Arrow indicates where the PCV should be. (B) Wing representing Su+ class phenotype that shows the PCV attached to L4 and L5 and extra wing vein material can be seen below L5.

Found at: doi:10.1371/journal.pone.0002418.s004 (0.94 MB TIF)

Figure S3 DysE6/+ Modifiers. DysE6/+ flies have normal posterior cross veins. (A) Represents an enhancer (ModE10) of this phenotype which phenocopies the wing veins from DysE6/DysE6 flies. (B) Shows an extra vein modification of the DysE6/+ posterior cross vein. Arrows indicate altered cross veins.
Found at: doi:10.1371/journal.pone.0002418.s005 (1.94 MB TIF)

Figure S4 Dg(RNAi) Modifiers. (A) Wild type fly wing with normal posterior cross vein (PCV) as indicated by the arrow. (B) DgRNAi mutant PCV (arrow) with a branch. (C D) represent modifiers that produce extra vein material (indicated by arrows. In one case (C) the branch is elongated with extra material also seen above L2 (upper arrow). (D) Shows extra material below L5 (lower arrow).
Found at: doi:10.1371/journal.pone.0002418.s006 (1.77 MB TIF)

Figure S5 Phenotypes Observed in poly-EGF Mutants. (A) Fly wing from Mod29/Df flies. Arrows indicate extra vein material. (B) Transverse histological section of the indirect flight muscle in Mod29 homozygotes. (B') Higher magnification of the region indicated by the box which indicates the separation of the muscle into individual fibers. (C) Ovariole from a Mod29 homozygote. Anterior to the left. The germarium is at the anterior most tip of the ovariole with developing egg chambers progressing to the right (posteriorly). The egg chambers undergo developmental arrest in later stages. Actin-Green, Adducin-Red, DAPI-purple. (D) 24B10 antibody staining of the adult wild type brain. Arrows indicate photoreceptor axon termination sites where the R8 photoreceptor axon (left arrow) terminates before the R7 axon (right arrow). (E) Mod29/Df adult brain. Termination of the R8 and R7 axons are indicated by the red arrows. White arrows indicate non termination of two axons that protrude deeper into the brain. (F) Dg323/Dg323 clone in the adult brain. Red arrows indicate where the R8 and R7 axons should terminate. Upper white arrows indicate a general disruption of axon termination in the R8/R7 termination region. Lower right arrow indicates a non terminating axon that proceeds deeper into the brain.
Found at: doi:10.1371/journal.pone.0002418.s007 (3.58 MB TIF)

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Author Contributions

Conceived and designed the experiments: HR MK MP AY HS. Performed the experiments: HR MK MP AY HS KF DM YC. Analyzed the data: HR MK MP AY HS KF DM. Contributed reagents/materials/analysis tools: HR HS YC. Wrote the paper: HR MK MP AY HS.

References

1. Haenggi T, Fritschy JM (2006) Role of dystrophin and utrophin for assembly and function of the dystrophin glycoprotein complex in non-muscle tissue. Cell Mol Life Sci 63: 1614 1631.
2. Lapidos KA, Kakkar R, McNally EM (2004) The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. Circ Res 94: 1023 1031.
3. Davies KE, Nowak KJ (2006) Molecular mechanisms of muscular dystrophies: old and new players. Nat Rev Mol Cell Biol 7: 762 773.
4. Kanagawa M, Toda T (2006) The genetic and molecular basis of muscular dystrophy: roles of cell-matrix linkage in the pathogenesis. J Hum Genet 51: 915 926.
5. Allikian MJ, Bhabha G, Dospoy P, Heydemann A, Ryder P, et al. (2007) Reduced life span with heart and muscle dysfunction in Drosophila sarcoglycan mutants. Hum Mol Genet 16: 2933 2943.
6. Shcherbata HR, Yatsenko AS, Patterson L, Sood VD, Nudel U, et al. (2007) Dissecting muscle and neuronal disorders in a Drosophila model of muscular dystrophy. Embo J 26: 481 493.
7. van der Plas MC, Pilgram GS, de Jong AW, Bansraj MR, Fradkin LG, et al. (2007) Drosophila Dystrophin is required for integrity of the musculature. Mech Dev 124: 617 630.
8. Yatsenko AS, Gray EE, Shcherbata HR, Patterson LB, Sood VD, et al. (2007) A putative Src homology 3 domain binding motif but not the C-terminal dystrophin WW domain binding motif is required for dystroglycan function in cellular polarity in Drosophila. J Biol Chem 282: 15159 15169.
9. Deng WM, Schneider M, Frock R, Castillejo-Lopez C, Gaman EA, et al. (2003) Dystroglycan is required for polarizing the epithelial cells and the oocyte in Drosophila. Development 130: 173 184.
10. Poulton JS, Deng WM (2006) Dystroglycan down-regulation links EGF receptor signaling and anterior-posterior polarity formation in the Drosophila oocyte. Proc Natl Acad Sci U S A 103: 12775 12780.
11. Taghli-Lamallem O, Akasaka T, Hogg G, Nudel U, Yaffe D, et al. (2008) Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. Aging Cell 7: 237 249.
12. Carbonetto S, personal communication.
13. Batchelor CL, Winder SJ (2006) Sparks, signals and shock absorbers: how dystrophin loss causes muscular dystrophy. Trends Cell Biol 16: 198 205.
14. Christoforou CP, Greer CE, Challoner BR, Charizanos D, Ray RP (2008) The detached locus encodes Drosophila Dystrophin, which acts with other components of the Dystrophin Associated Protein Complex to influence intercellular signalling in developing wing veins. Dev Biol 313: 519 532.
15. Guichard A, Biehs B, Sturtevant MA, Wickline L, Chacko J, et al. (1999) rhomboid and Star interact synergistically to promote EGFR/MAPK signaling during Drosophila wing vein development. Development 126: 2663 2676.
16. Johannes B, Preiss A (2002) Wing vein formation in Drosophila melanogaster: hairless is involved in the cross-talk between Notch and EGF signaling pathways. Mech Dev 115: 3 14.
17. Ralston A, Blair SS (2005) Long-range Dpp signaling is regulated to restrict BMP signaling to a crossvein competent zone. Dev Biol 280: 187 200.
18. Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. Science 284: 770 776.
19. Bier E (2000) Drawing lines in the Drosophila wing: initiation of wing vein development. Curr Opin Genet Dev 10: 393 398.
20. De Celis JF (2003) Pattern formation in the Drosophila wing: The development of the veins. Bioessays 25: 443 451.
21. de Celis JF (1997) Expression and function of decapentaplegic and thick veins during the differentiation of the veins in the Drosophila wing. Development 124: 1007 1018.
22. Kalamida D, Poulas K, Avramopoulou V, Fostieri E, Lagoumintzis G, et al. (2007) Muscle and neuronal nicotinic acetylcholine receptors. Structure, function and pathogenicity. Febs J 274: 3799 3845.
23. Madhavan R, Massom LR, Jarrett HW (1992) Calmodulin specifically binds three proteins of the dystrophin-glycoprotein complex. Biochem Biophys Res Commun 185: 753 759.
24. Newbell BJ, Anderson JT, Jarrett HW (1997) Ca2+-calmodulin binding to mouse alpha1 syntrophin: syntrophin is also a Ca2+-binding protein. Biochemistry 36: 1295 1305.
25. Pascual M, Vicente M, Monferrer L, Artero R (2006) The Muscleblind family of proteins: an emerging class of regulators of developmentally programmed alternative splicing. Differentiation 74: 65 80.
26. Kadrmas JL, Smith MA, Pronovost SM, Beckerle MC (2007) Characterization of RACK1 function in Drosophila development. Dev Dyn 236: 2207 2215.
27. Cox DN, Seyfried SA, Jan LY, Jan YN (2001) Bazooka and atypical protein kinase C are required to regulate oocyte differentiation in the Drosophila ovary. Proc Natl Acad Sci U S A 98: 14475 14480.
28. Wehr K, Swan A, Schupbach T (2006) Deadlock, a novel protein of Drosophila, is required for germline maintenance, fusome morphogenesis and axial patterning in oogenesis and associates with centrosomes in the early embryo. Dev Biol 294: 406 417.
29. Swan A, Nguyen T, Suter B (1999) Drosophila Lissencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. Nat Cell Biol 1: 444 449.
30. Taya S, Shinoda T, Tsuboi D, Asaki J, Nagai K, et al. (2007) DISC1 regulates the transport of the NUDEL/LIS1/14-3-3epsilon complex through kinesin-1. J Neurosci 27: 15 26.
31. Chakkalakal JV, Michel SA, Chin ER, Michel RN, Jasmin BJ (2006) Targeted inhibition of Ca2+/calmodulin signaling exacerbates the dystrophic phenotype in mdx mouse muscle. Hum Mol Genet 15: 1423 1435.
32. Parsons SA, Millay DP, Sargent MA, Naya FJ, McNally EM, et al. (2007) Genetic disruption of calcineurin improves skeletal muscle pathology and cardiac disease in a mouse model of limb-girdle muscular dystrophy. J Biol Chem 282: 10068 10078.

33. Macioce P, Gambara G, Bernassola M, Gaddini L, Torreri P, et al. (2003) Beta-dystrobrevin interacts directly with kinesin heavy chain in brain. J Cell Sci 116: 4847 4856.
34. Rinaldo C, Prodosmo A, Siepi F, Soddu S (2007) HIPK2: a multitalented partner for transcription factors in DNA damage response and development. Biochem Cell Biol 85: 411 418.
35. Link N, Chen P, Lu WJ, Pogue K, Chuong A, et al. (2007) A collective form of cell death requires homeodomain interacting protein kinase. J Cell Biol 178: 567 574.
36. Furrer MP, Kim S, Wolf B, Chiba A (2003) Robo and Frazzled/DCC mediate dendritic guidance at the CNS midline. Nat Neurosci 6: 223 230.
37. Magalhaes TR, Palmer J, Tomancak P, Pollard KS (2007) Transcriptional control in embryonic Drosophila midline guidance assessed through a whole genome approach. BMC Neurosci 8: 59.
38. Carmeliet P, Tessier-Lavigne M (2005) Common mechanisms of nerve and blood vessel wiring. Nature 436: 193 200.
39. Park KW, Morrison CM, Sorensen LK, Jones CA, Rao Y, et al. (2003) Robo4 is a vascular-specific receptor that inhibits endothelial migration. Dev Biol 261: 251 267.
40. Tayler TD, Robichaux MB, Garrity PA (2004) Compartmentalization of visual centers in the Drosophila brain requires Slit and Robo proteins. Development 131: 5935 5945.
41. Cornelison DD, Wilcox-Adelman SA, Goetinck PF, Rauvala H, Rapraeger AC, et al. (2004) Essential and separable roles for Syndecan-3 and Syndecan-4 in skeletal muscle development and regeneration. Genes Dev 18: 2231 2236.
42. Qian L, Liu J, Bodmer R (2005) Slit and Robo control cardiac cell polarity and morphogenesis. Curr Biol 15: 2271 2278.
43. Steinhauer J, Kalderon D (2006) Microtubule polarity and axis formation in the Drosophila oocyte. Dev Dyn 235: 1455 1468.
44. Deng WM, Ruohola-Baker H (2000) Laminin A is required for follicle cell-oocyte signaling that leads to establishment of the anterior-posterior axis in Drosophila. Curr Biol 10: 683 686.
45. Alvarado D, Rice AH, Duffy JB (2004) Bipartite inhibition of Drosophila epidermal growth factor receptor by the extracellular and transmembrane domains of Kekkon1. Genetics 167: 187 202.
46. Kukekov NV, Xu Z, Greene LA (2006) Direct interaction of the molecular scaffolds POSH and JIP is required for apoptotic activation of JNKs. J Biol Chem 281: 15517 15524.
47. Figueroa C, Tarras S, Taylor J, Vojtek AB (2003) Akt2 negatively regulates assembly of the POSH-MLK-JNK signaling complex. J Biol Chem 278: 47922 47927.
48. Zhou W, Zhu M, Wilson MA, Petsko GA, Fink AL (2006) The oxidation state of DJ-1 regulates its chaperone activity toward alpha-synuclein. J Mol Biol 356: 1036 1048.
49. Januschke J, Gervais L, Dass S, Kaltschmidt JA, Lopez-Schier H, et al. (2002) Polar transport in the Drosophila oocyte requires Dynein and Kinesin I cooperation. Curr Biol 12: 1971 1981.
50. Lei Y, Warrior R (2000) The Drosophila Lissencephaly1 (DLis1) gene is required for nuclear migration. Dev Biol 226: 57 72.
51. Brendza RP, Serbus LR, Saxton WM, Duffy JB (2002) Posterior localization of dynein and dorsal-ventral axis formation depend on kinesin in Drosophila oocytes. Curr Biol 12: 1541 1545.
52. Duncan JE, Warrior R (2002) The cytoplasmic dynein and kinesin motors have interdependent roles in patterning the Drosophila oocyte. Curr Biol 12: 1982 1991.
53. Takeya R, Sumimoto H (2003) Fhos, a mammalian formin, directly binds to F-actin via a region N-terminal to the FH1 domain and forms a homotypic complex via the FH2 domain to promote actin fiber formation. J Cell Sci 116: 4567 4575.
54. Bosch M, Le KH, Bugyi B, Correia JJ, Renault L, et al. (2007) Analysis of the function of Spire in actin assembly and its synergy with formin and profilin. Mol Cell 28: 555 568.
55. Dahlgaard K, Raposo AA, Niccoli T, St Johnston D (2007) Capu and Spire assemble a cytoplasmic actin mesh that maintains microtubule organization in the Drosophila oocyte. Dev Cell 13: 539 553.
56. Quinlan ME, Hilgert S, Bedrossian A, Mullins RD, Kerkhoff E (2007) Regulatory interactions between two actin nucleators, Spire and Cappuccino. J Cell Biol 179: 117 128.
57. Rosales-Nieves AE, Johndrow JE, Keller LC, Magie CR, Pinto-Santini DM, et al. (2006) Coordination of microtubule and microfilament dynamics by Drosophila Rho1, Spire and Cappuccino. Nat Cell Biol 8: 367 376.
58. Wang Y, Riechmann V (2007) The role of the actomyosin cytoskeleton in coordination of tissue growth during Drosophila oogenesis. Curr Biol 17: 1349 1355.
59. Kalil K, Dent EW (2005) Touch and go: guidance cues signal to the growth cone cytoskeleton. Curr Opin Neurobiol 15: 521 526.
60. Bateman J, Reddy RS, Saito H, Van Vactor D (2001) The receptor tyrosine phosphatase Dlar and integrins organize actin filaments in the Drosophila follicular epithelium. Curr Biol 11: 1317 1327.
61. Lee E, Salic A, Kruger R, Heinrich R, Kirschner MW (2003) The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. PLoS Biol 1: E10.
62. van der Plas MC, Pilgram GS, Plomp JJ, de Jong A, Fradkin LG, et al. (2006) Dystrophin is required for appropriate retrograde control of neurotransmitter release at the Drosophila neuromuscular junction. J Neurosci 26: 333 344.

Supplementary Information

Figure S1

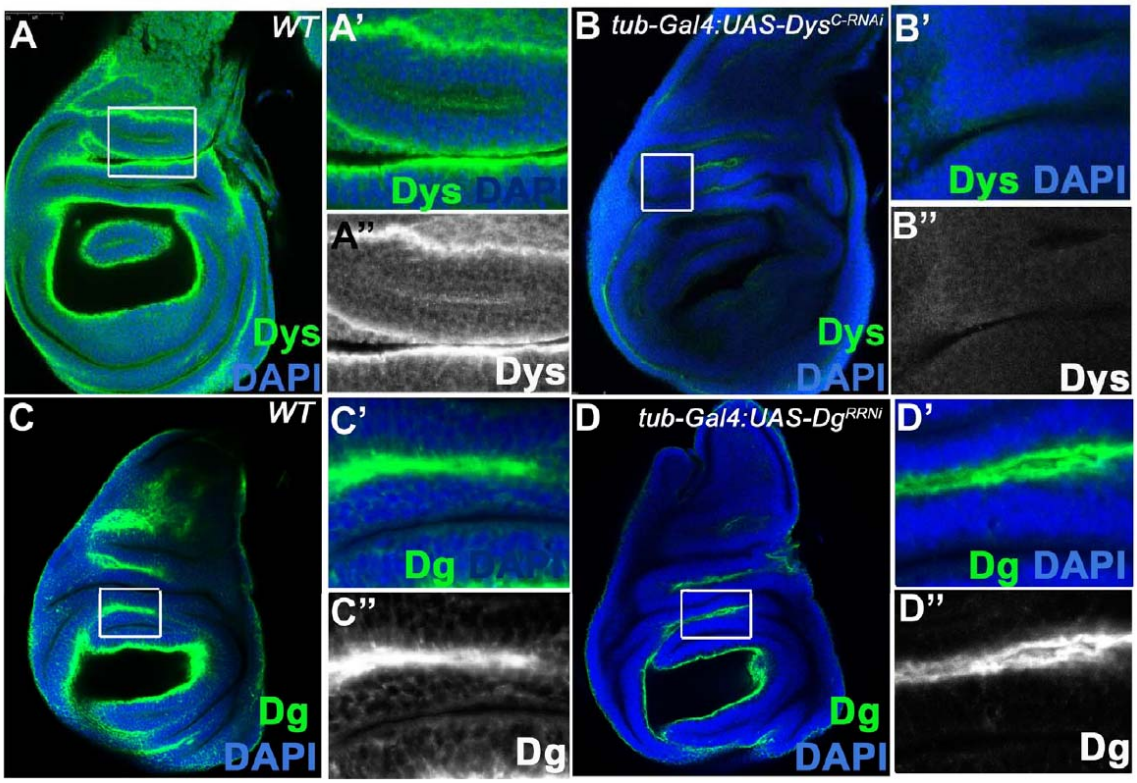


Fig. S1. *Dys* and *Dg* Expression in Wild Type and Mutant Wing Discs. In wild-type larvae, *Dys* is expressed in all cells of the wing disc (A) and is strongly reduced in the wing disc of the *Dys$^{C\text{-}RNAi}$* mutant (B; *tubGal4:UAS-Dys$^{C\text{-}RNAi}$*/+). Dystroglycan localization in wild type imaginal discs is enriched at the basal surface of the epithelial cells. *Dg* expression is more intense in folds formed from the contact of basal surfaces and is less visible in the folds made from apical surfaces of wing disc cells (C). The *Dg^{RNAi}* mutant also shows a reduction of Dystroglycan protein in the wing disc (D; *tub-Gal4:UAS-Dg^{RNAi}*/+). (A'-D') Enlarged images of framed areas on (AD). (A"-B") Enlarged images of framed areas on (A-B) show Dystrophin single channel staining. (C"-D") Enlarged images of framed areas on (C-D) show Dystroglycan single channel staining.

Figure S2

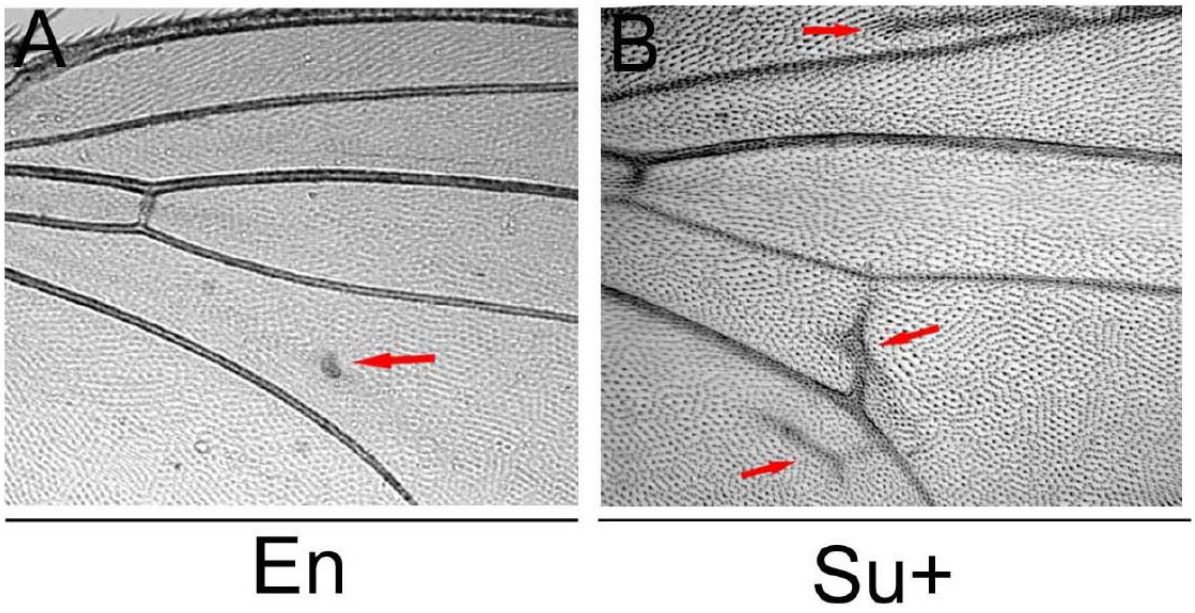


Fig. S2. Subclasses of *Dys(RNAi)* Modifiers. (A) Example of enhancers that lack the posterior cross vein (PCV) and belong to phenotypic class En.Arrow indicates where the PCV should be. (B) Wing representing Su+ class phenotype that shows the PCV attached to L4 and L5 and extra wing vein material can be seen below L5.

Figure S3

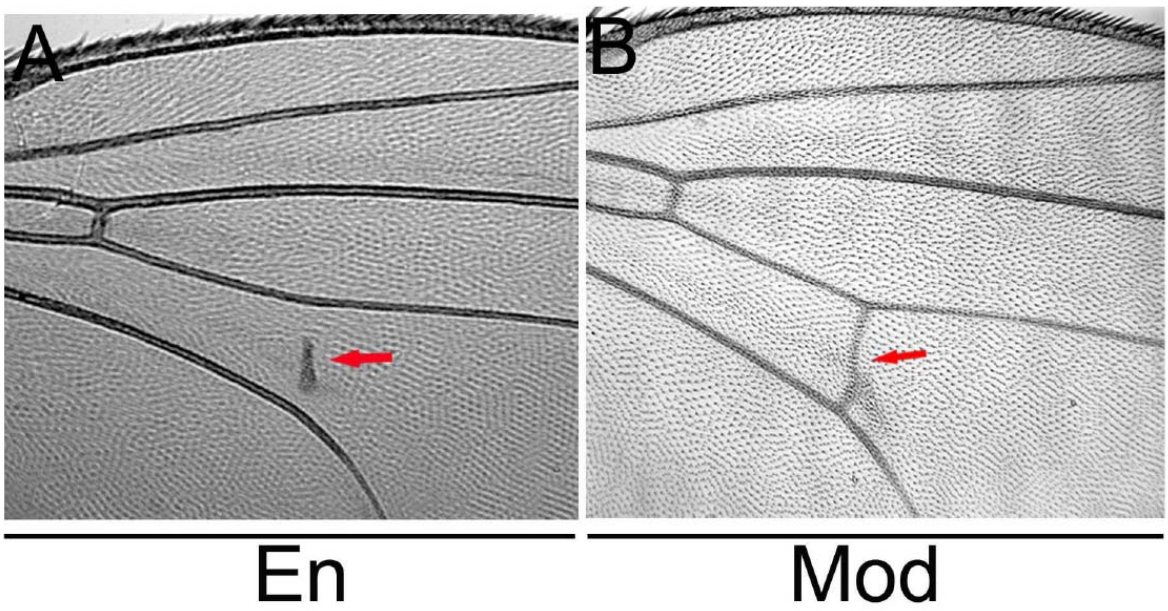


Fig. S3. *Dys*^{E6}/+ Modifiers. *Dys*^{E6}/+ flies have normal posterior cross veins. (A) Represents an enhancer (ModE10) of this phenotype which phenocopies the wing veins from *Dys*^{E6}/*Dys*^{E6} flies. (B) Shows an extra vein modification of the *Dys*^{E6}/+ posterior cross vein. Arrows indicate altered cross veins.

Figure S4

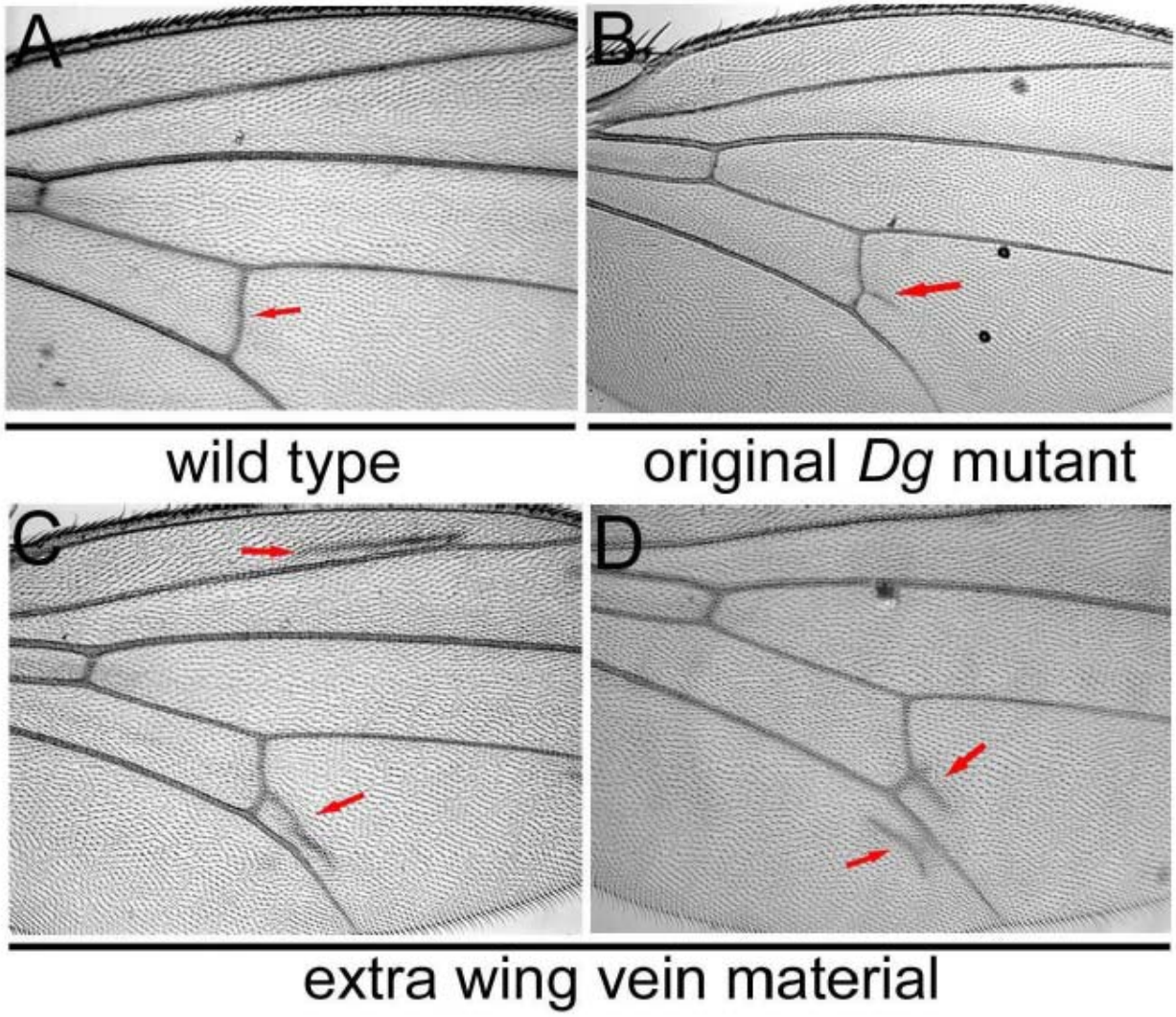


Fig. S4. *Dg(RNAi)* Modifiers. (A) Wild type fly wing with normal posterior cross vein (PCV) as indicated by the arrow. (B) Dg^{RNAi} mutant PCV (arrow) with a branch. (C-D) represent modifiers that produce extra vein material (indicated by arrows. In one case (C) the branch is elongated with extra material also seen above L2(upper arrow). (D) Shows extra material below L5 (lower arrow).

Figure S5

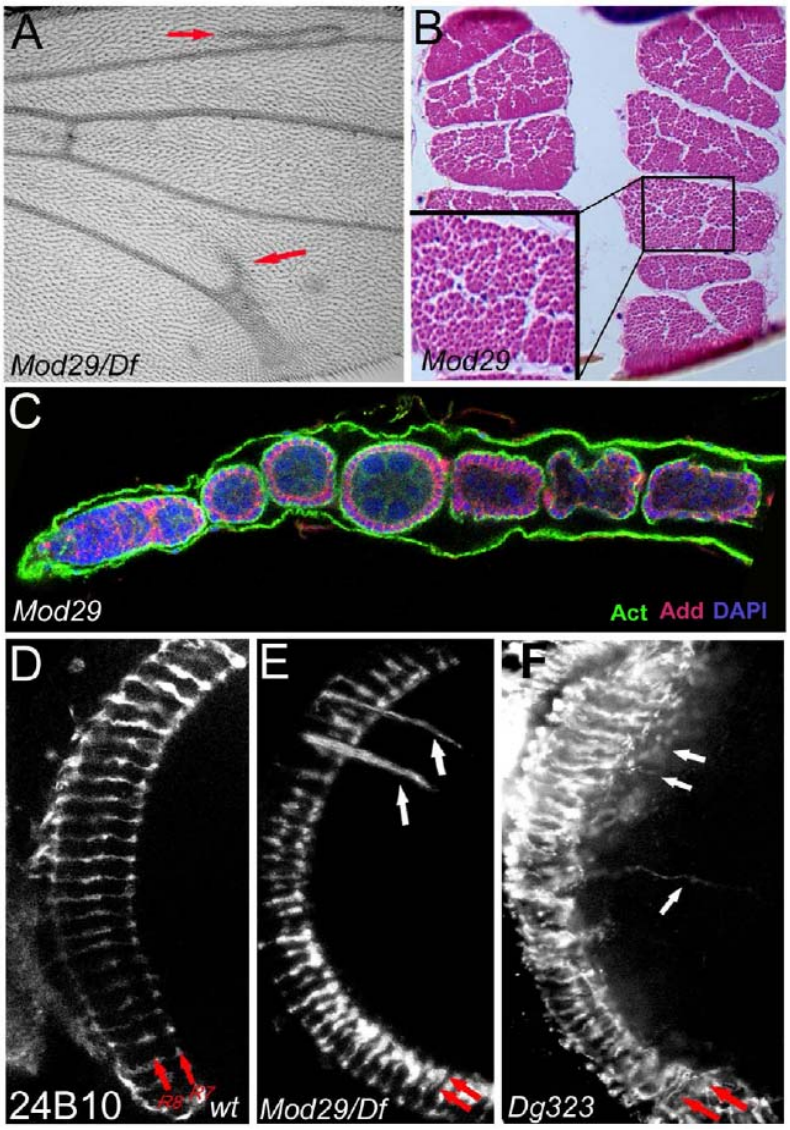


Fig. S5. Phenotypes Observed in *poly-EGF* Mutants. (A) Fly wing from Mod29/Df flies. Arrows indicate extra vein material. (B) Transverse histologicalsection of the indirect flight muscle in Mod29 homozygotes. (B') Higher magnification of the regionindicated by the box which indicates the separation of the muscle into individual fibers. (C) Ovariole from a Mod29 homozygote. Anterior to the left. The germarium is at the anterior most tip of the ovariole with developing egg chambers progressing to the right (posteriorly). The egg chambers undergo developmental arrest in later stages. Actin-Green, Adducin-Red, DAPI-purple. (D) 24B10 antibody staining of the adult wild type brain. Arrows indicate photoreceptor axon termination sites where the R8 photoreceptor axon (left arrow) terminates before the R7 axon (right arrow). (E) Mod29/Df adult brain. Termination of the R8 and R7 axons are indicated by the red arrows. Whitearrows indicate non termination of two axons that protrude deeper into the brain. (F) *Dg323/ Dg323* clone in the adult brain. Red arrows indicate where the R8 and R7 axons should terminate. Upper white arrows indicate a general disruption of axon termination in the R8/R7 termination region. Lower rightarrow indicates a non terminating axon that proceeds deeper into the brain.

3.4. New modulators of the Dystrophin-Dystroglycan dependent muscle degeneration are identified in a *Drosophila* muscular dystrophy model

In my previous work (Kucherenko et al., 2008) the dominant modifiers of the *Drosophila* wing vein phenotype caused by down regulation of Dystrophin and Dystroglycan were found. Interestingly, that among identified modifiers we isolated components, whose role in modulating the DGC function can be quite novel. Since defects in the DGC functioning cause different forms of muscular dystrophy in humans, I wanted to investigate if found modifiers show a genetic interaction with *Dys* and *Dg* in muscles in order to get closer to understanding the mechanisms of muscle degeneration. It has been shown (Shcherbata et al., 2007) that both *Drosophila Dys* and *Dg* mutants manifest age-dependent muscle degeneration. Before starting the secondary screen, I analyzed if Dys and Dg, the two major components of the DGC, genetically interact in the muscle degeneration process and therefore confirmed the capability to select modifiers of the Dys/Dg-dependent muscle phenotype using the chosen strategy. Since *Dys* and *Dg* showed a genetic interaction in the muscle degeneration process, the rest of the pre-selected (Kucherenko et al., 2008) components were tested for the ability to suppress or enhance the muscle phenotype in *Dys* and *Dg* RNAi mutants and to interact in heterozygote with *Dys* and *Dg* loss-of-function alleles. As a result, mutations that interact with Dys and Dg in maintaining the muscle integrity were identified. Further studies of found modifiers showed their requirement in either muscle or nervous tissue, where the specific interaction with the DGC components may occur. The novel components that contribute to DGC-dependent muscle maintenance are being analyzed.

New modulators of the Dystrophin-Dystroglycan dependent muscle degeneration are identified in a *Drosophila* muscular dystrophy model

Mariya M. Kucherenko, April Marrone, Halyna R. Shcherbata

Author contribution to the practical work:

Mariya M. Kucherenko	All the experiments
April Marrone	Part of the analysis

Status: in preparation

Methods

Fly Strains and Genetics

The fly strains used in this study were: $Dys^{N\text{-}RNAi}$*:act-Gal4*, Dg^{RNAi}*:tub-Gal4* (used previously by (Kucherenko et al., 2008)), Dg^{323} (Deng et al., 2003), *DysDf*, Dg^{O86} and Dg^{O55} (Christoforou et al., 2008), enhancer trap lines – *act-Gal4*, *tub-Gal4*, *24B-Gal4*, *MHC-Gal4* and *D42-Gal4* (Bloomington Drosophila Stock Center), mutants and RNAi lines used for the screen were obtained from Drosophila stock centers (Vienna *Drosophila* RNAi Center, *Drosophila* Genetic Resourse Center (Kyoto) and Bloomington *Drosophila* Stock Center). Mutant alleles $CG7845^{EMS\text{-}Mod4}$ and $SP1070^{EMS\text{-}Mod29}$ were found at the primary screen (Kucherenko et al., 2008), $Fkbp13^{23m}$ (a gift from Dr. O´Conner), $SP1070^{Uif\text{-}E(br)155}$ and $SP1070^{Uif\text{-}2B7}$ (gifts from Dr. Ward). For control crosses *Oregon R* and w^{1118} flies were used.

Genetic screen. In order to identify dominant suppressors/enhancers of the muscle degeneration phenotype virgin females with genotype $Dys^{N\text{-}RNAi}$*:act-Gal4* and Dg^{RNAi}*:tub-Gal4* were crossed to males carrying the mutation of interest. The progeny with both, transgenic construct and the screened mutation were collected for muscle analysis. To identify heterozygous interaction in muscles loss-of-function *DysDf*, Dg^{O86} and Dg^{O55} mutant females were crossed to males carrying the mutation of interest. The F1 progeny heterozygous for *Dys* or *Dg* and the screened allele were collected for muscle analysis. Mutations, which showed an effect on the *Dys* and/or *Dg* phenotype in muscles were crossed to w^{1118} flies to exclude the possibility that screened mutants have their own dominant phenotype in muscles. All crosses were kept at 25°C. Flies with the correct genotype selected for muscles analysis were aged for three weeks at 25°C.

Muscle analysis

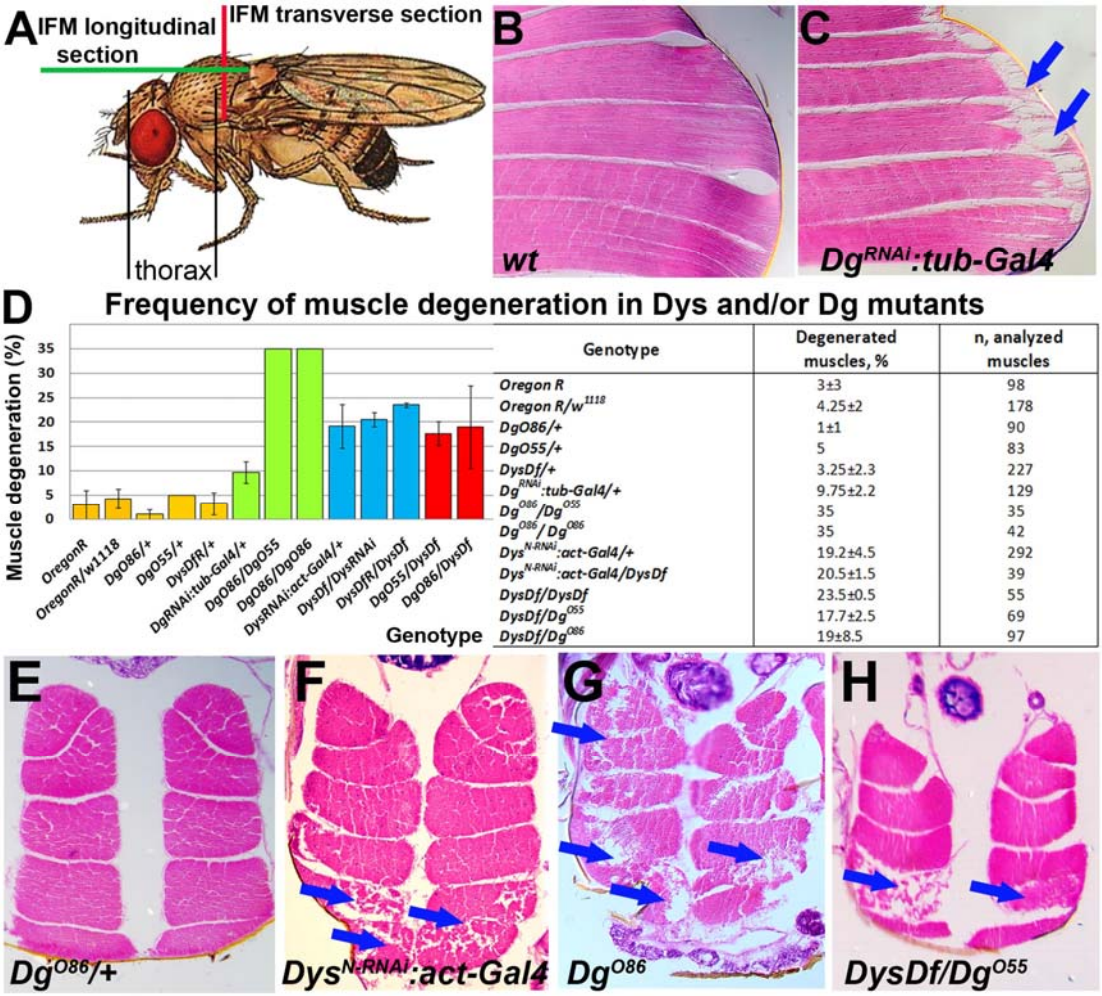
In order to prepare *Drosophila* muscle tissue for analysis, bodies of 3 week old flies were placed into collars in the required orientation and fixed in Carnoy fixative solution (6:3:1 = Ethanol : Chloroform : Acetic acid) at 4˚C overnight. Then tissue dehydration and paraffinisation was performed as described previously (Shcherbata et al., 2007). Histological sections (8 mkm) of *Drosophila* indirect flight muscles (IFM) were prepared from wax-embedded material using Hyrax M25 (Zeiss) microtome and stained with hematoxyline and eosin (H&E staining). All chemicals for these procedures were obtained from Sigma Aldrich. Muscle analysis was done using light microscopy. The frequency of muscle degeneration was quantified as a ratio of degenerated muscles to the total number of analyzed muscles. The analyzyd IFM sections were located at the position 200-250 mkm from the posterior of the fly thorax.

Results

Dystrophin and Dystroglycan interact in muscle degeneration process

Drosophila melanogaster has been proven to be a remarkably good model for age-dependent progression of muscle degeneration (Shcherbata et al., 2007). It has been shown that *Drosophila Dystrophin* and *Dystroglycan* mutants exhibit the muscle degeneration and lack of climbing ability. These similarities to human muscular dystrophy together with numerous advantages of *Drosophila* as a genetic model make the fruit fly an attractive system for genetic screenings in order to identify components which regulate DGC function and have an effect on muscle degeneration. Before starting the screen, we analyzed if Dystrophin and Dystroglycan, two major components of the DGC, genetically interact in the muscle degeneration process in order to confirm the capability to select modifiers of the Dys/Dg-dependent muscle phenotype. To test if *Dys* and *Dg* interact in muscles we made a transverse section of *Drosophila* indirect flight muscles (IFM) of the *Dystrophin* and *Dystroglycan* RNAi mutants ($Dys^{N\text{-}RNAi}$*:act-Gal4* and Dg^{RNAi}*:tub-Gal4*), homozygous viable loss-of-function alleles (*DysDf*, Dg^{O86} and Dg^{O55}) and transheterozygous animals (*DysDf*/Dg^{O86} and *DysDf*/Dg^{O55}). As a control *Oregon R* and w^{1118} flies were used, as well as the flies from crosses of mutant alleles with w^{1118} (Figure 1). During analysis we noticed that in mutant flies the degeneration process starts from muscle termini and then spreads along the muscle fibers, which can be observed in the longitudinal sections through the fly thorax (Figure 1A-C). In our studies we have used transverse sections of *Drosophila* IFM, therefore in order to obtain comparable data we restricted our analysis to the sections made through the fly thorax in the region 200-250mkm from the posterior end of the thorax (Figure 1A).

The *Dys* and *Dg* RNAi alleles exhibited a moderate muscle degeneration phenotype: 19.2±4.5% (n=292) for $Dys^{N\text{-}RNAi}$*:act-Gal4* and 9.7±2.2% (n=227) for Dg^{RNAi}*:tub-Gal4* (Figure 1D,F) in comparison to control flies that mostly had normal tissue structure (1-5%, Figure 1D-E). More significant muscle abnormalities were observed in *Dys* and *Dg* loss-of-function mutants: 35% for homozygous Dg^{O86} (n=42) and heterozygous *Dystroglycan* allelic combination Dg^{O86}/Dg^{O55} (n=35) and 23.5±0.5% (n=55) for homozygous *DysDf* (Figure 1D,G). Reduction by one copy of both *Dys* and *Dg* also caused muscle degeneration: 19±8% (n=97) for Dg^{O86}*/DysDf* and 17.5±2.5% (n=69) for Dg^{O55}*/DysDf* (Figure 1D,H). Obtained results indicate that both, Dystrophin and Dystroglycan are important for proper muscle maintenance and both DGC components genetically interact in the muscle degeneration process. This also proves that the strategy chosen for the screening has a potential to reveal interactors involved in the same pathway as the DGC to provide normal muscle function.



Genotype	Degenerated muscles, %	n, analyzed muscles
Oregon R	3±3	98
Oregon R/w^{1118}	4.25±2	178
DgO86/+	1±1	90
DgO55/+	5	83
DysDf/+	3.25±2.3	227
Dg^{RNAi}:tub-Gal4/+	9.75±2.2	129
Dg^{O86}/Dg^{O55}	35	35
Dg^{O86}/ Dg^{O86}	35	42
Dys^{N-RNAi}:act-Gal4/+	19.2±4.5	292
Dys^{N-RNAi}:act-Gal4/DysDf	20.5±1.5	39
DysDf/DysDf	23.5±0.5	55
DysDf/Dg^{O55}	17.7±2.5	69
DysDf/Dg^{O86}	19±8.5	97

Figure 1. The *Drosophila Dystrophin* and *Dystroglycan* genetically interact in muscles

(A) Location of longitudinal and transverse sections of indirect flight muscles (IFM) in the *Drosophila* thorax. (B) Longitudinal section of IFM of the wild type fly with properly organized muscle tissue. (C) Longitudinal muscle section of *Dystroglycan* RNAi mutant exhibits muscle degeneration (indicated by arrows), which starts from the muscle termini. (D) Bar graph represents the frequency of muscle degeneration in control flies (yellow bars), *Dystroglycan* mutants (green bars), *Dystrophin* mutants (blue bars) and heterozygous flies with reduced *Dys* and *Dg* by one copy (red bars). The table shows quantity of analyzed muscles and percentage of degenerated muscles in *Dys* and/or *Dg* mutants and control flies. (E) The IFM transverse section of a control fly (*Dg ^{O86}*/+) shows normal muscle structure. (F) The IFM transverse section of *Dystrophin* RNAi mutant exhibits moderate degeneration of muscle tissue. (G) Muscles of *Dystroglycan* loss-of-function mutant *Dg^{O86}* with abnormalities in muscle architecture. (I) The transverse muscle section of the fly with heterozygous combination *DysDf/Dg^{O55}*; defects in muscle structure indicate Dys-Dg genetic interaction in muscle degeneration process. Arrows show the degenerated muscle tissue.

Genetic modifiers screen for components that interact with *Dys* and *Dg* in muscles

In order to identify components that modify the muscle degeneration phenotype, which occurs as a result of Dys and/or Dg reduction, we have screened gene-candidates preselected previously (Kucherenko et al., 2008). At the primary large-scale screen we have analyzed modification in *Drosophila Dys* and *Dg* mutants wing veins with a purpose of finding components which interact with the DGC or regulate its function. As a result, the DGC modifiers were selected and classified to the functional groups: genes involved in muscle function, cellular and/or axonal migration, components of Notch, TGF-β, EGFR signaling pathways and others. In the present work, we addressed the question if found modifiers of the DGC in the wing veins can modulate the muscle degeneration phenotype in a *Drosophila* muscular dystrophy model. In our secondary screen *Dys* and *Dg* RNAi mutants ($Dys^{N\text{-}RNAi}$*:act-Gal4* and Dg^{RNAi}*:tub-Gal4*) that manifested moderate muscle degeneration (Figure 1D-E,G) were crossed to the mutants, which carried the mutation of interest in order to select both, dominant enhancers and suppressors of muscle degeneration. In addition, *Dys* and *Dg* loss-of-function alleles (*DysDf,* Dg^{323} and Dg^{O86}) were used to identify heterozygous interactions. Flies lacking one copy of the gene-candidate in the *Dys* and/or *Dg* mutant background were aged for three weeks and the frequency of muscle degeneration was quantified (Table 1). Mutations, which showed an effect on the *Dys* and/or *Dg* phenotype in muscles were crossed to w^{1118} flies with a purpose to exclude the possibility that screened mutants have their own dominant phenotype in muscles. This control analysis eliminated *Lis-1, SP1070* and *wun* from the list of gene-interactors. Moreover, we have used different alleles of the same gene to confirm or disprove found interactions. In total, we screened 22 gene-candidates and selected 14 modifiers of *Dys* and/or *Dg*-dependent muscle degeneration (Table 1).

Modification of Dys and Dg RNAi mutant phenotype

The $Dys^{N\text{-}RNAi}$*:act-Gal4/*w^{1118} mutant shows ≈ 20% degeneration, therefore all the modifiers, which in cross with *Dys* RNAi mutant had an increased frequency of muscle degeneration were classified as enhancers and the ones that caused reduction of the muscle degeneration were called suppressors (Table 1, Figure 2A). A similar classification was used for modifiers of *Dg* RNAi mutants; in this case Dg^{RNAi}*:tub-Gal4/*w^{1118} exhibits ≈ 10% of muscle degeneration (Table 1, Figure 2D). In our previous work (Kucherenko et al., 2008) it has been demonstrated that reduction of *Mbl* strongly enhances *Dys* phenotype in muscles. Now it has been found that *Mbl,* as well as *Fhos*, *Cam*, *Rack1*, *CG34400* and *capt* increase the muscle degeneration in both *Dys* and *Dg* RNAi mutants (Figure 2A,D). Flies with reduction by one copy of before-mentioned genes in a *Dys* and/or *Dg* RNAi mutant background appear to have higher frequency of muscle a degeneration (Figure 2A,D-F).

Table 1. Frequency of muscle degeneration phenotypes caused by reduction by one copy of screened genes in *Dys* and *Dg* mutant background

Gene name	Allele	*Dys$^{N\text{-}RNAi}$:act-Gal4*		*DysDf* x		*Dg^{RNAi}:tub-Gal4*		*Dg^{323}* x		*Dg^{O86}* x		*w^{1118}* x	
		degenerated muscles, %	n, analyzed muscles	degenerated muscles, %	n, analyzed muscles	degenerated muscles, %	n, analyzed muscles	degenerated muscles, %	n, analyzed muscles	degenerated muscles, %	n, analyzed muscles	degenerated muscles, %	n, analyzed muscles
w	***[1118]***	**19.2±4.5**	**n=292**	**3.3±3.3**	**n=227**	**9.7±2.2**	**n=129**	**5**	**n=112**	**1±1**	**n=90**	**4.2±2**	**n=98**
Cam	***[n339]***	74	n=35	21	n=55	20	n=104	0	n=20	9	n=45	4	n=27
capt	***[E593]***	45	n=137	31	n=35	7	n=67	0	n=22	0	n=60	14.5	n=69
	[E636]	64±14	n=107	43	n=50	22	n=96	13	n=106	12	n=58	0	n=7
CG34400	***[c03838]***	37	n=16	15.2	n=33	74	n=23	43	n=12	32	n=28	10	n=11
CG7845	***[EMS-Mod4]****	16	n=12	25	n=36	28	n=35	31	n=51	37	n=54		
chif	***[BG02820]****	15	n=32	52	n=80	7.5±7.5	n=100	36	n=22	0	n=25	6.4	n=47
	[BG02820]	17	n=60	32	n=25	17±7	n=165	1	n=69	20	n=29	9.5	n=84
	[EY05746]	3±3	n=82	26	n=42	40±6	n=134	12	n=70	9	n=114	11	n=82
	[A507], CyO	§	§	21	n=42	20±2	n=87	§	§	§	§	0	n=31
del	***[3]***	14	n=47	NA	-	0	n=39	14	n=50	NA	-	NA	-
	[KG10262]	17	n=113	0	n=50	6	n=17	4	n=17	3	n=38	NA	-
Dmn	***[k16109]****	20	n=20	3	n=33	6.5	n=123	3	n=33	NA	-	NA	-
Fhos	***[k15815]****	22	n=12	18	n=59	0	n=60	40	n=44	NA	-	NA	-
	[EY09842]	31±5	n=122	18	n=57	28	n=14	5	n=116	9±2	n=155	0	n=36
	[A055]	12	n=47	32	n=25	24±5	n=154	21	n=47	15	n=71	2	n=44
Fkbp13	***[23]m*****	5	n=44	0	n=45	6	n=85	31	n=59	17	n=24	3.4	n=88
	[P962]	0	n=13	2.9	n=34	0	n=41	4	n=74	25	n=28	3.4	n=29
gcm	***[KG01117]****	26	n=113	8	n=50	26	n=38	0	n=32	0	n=72	NA	-
	[KG01117]	7	n=71	32	n=25	13	n=46	10	n=38	0	n=44	NA	-
	[rA87]	13	n=61	0	n=85	8	n=26	12	n=41	NA	-	NA	-
Grh	***[IM]***	8	n=52	0	n=21	5	n=56	3	n=58	3	n=72	NA	-
Lis-1	***[k13209]****	31	n=32	24	n=53	5±5	n=138	23	n=29	NA	-	13	n=117
	[k13209]	22	n=36	17±6	n=58	24	n=57	0	n=12	9	n=109	NA	-
	[k11702]	0	n=52	32	n=22	15	n=62	0	n=24	12	n=58	9	n=103
	[G10.14]	19	n=68	37	n=16	10	n=81	NA	-	0	n=24	0	n=72
mbl	***[E27]***	40	n=70	23	n=42	23±2	n=149	28	n=60	12	n=25	1	n=98
nAcRα-30D	***[EY13897]****	2	n=51	50	n=48	7	n=52	13	n=53	7	n=71	NA	-
	[EY13897]	16	n=95	10	n=20	11	n=38	0	n=40	1	n=105	NA	-
	[KG05852]	14	n=91	9	n=53	6	n=72	2	n=67	8	n=187	NA	-
Nrk	***[k14301]****	0	n=21	37	n=48	17±5	n=230	47	n=34	19	n=58	7±1	n=185
POSH	***[k15815]****	10	n=37	1	n=90	18	n=33	0	n=75	NA	-	NA	-
Rack1	***[EY00128]****	12±8	n=112	18	n=83	11±7	n=133	33	n=15	6	n=107	4	n=105
	[1.8]	5	n=44	11	n=27	12±1	n=122	9	n=45	11	n=68	0	n=127
	[EE]	78±3	n=39	14	n=36	19±2	n=216	7	n=27	20	n=132	7	n=82
robo	***[2]***	4	n=45	NA	-	1	n=107	0	n=20	1	n=110	4	n=84
SP1070	***[EMS-Mod29]****	20	n=68	8	n=49	18	n=32	0	n=124	NA	-	4	n=124
	UifE(br)155**	31±4	n=88	5	n=74	44	n=9	4	n=20	16	n=91	18	n=105
	Uif2B7**	38±6	n=117	14±4	n=142	14	n=94	4	n=91	13	n=81	21	n=90
SP2353	***[MB00605]***	11	n=27	NA	-	NA	-	15	n=40	NA	-	1.6	n=62
vimar	***[k16722]****	18	n=11	8	n=24	30	n=27	68	n=37	4	n=55	0	n=39
	[k16722]	8	n=60	0	n=22	37	n=38	42	n=36	NA	-	22	n=50
	[09]	13	n=70	0	n=84	33	n=49	16	n=110	9	n=32	1	n=142
	[EY09646]	6	n=65	26	n=54	18±5	n=136	16	n=19	23	n=30	31	n=29
wun	***[EMS23]***	23	n=30	11	n=34	14	n=65	10	n=62	13	n=62	5	n=83

all mutant alleles obtained from BDSC, except

(*) – described previously by *Kucherenko et al, 2008*, (**) – obtained from DGRC, (***) – gifts from Drs. O´Conner and Ward, respectively;

(NA) – not analyzed;

(§) – cannot be analyzed

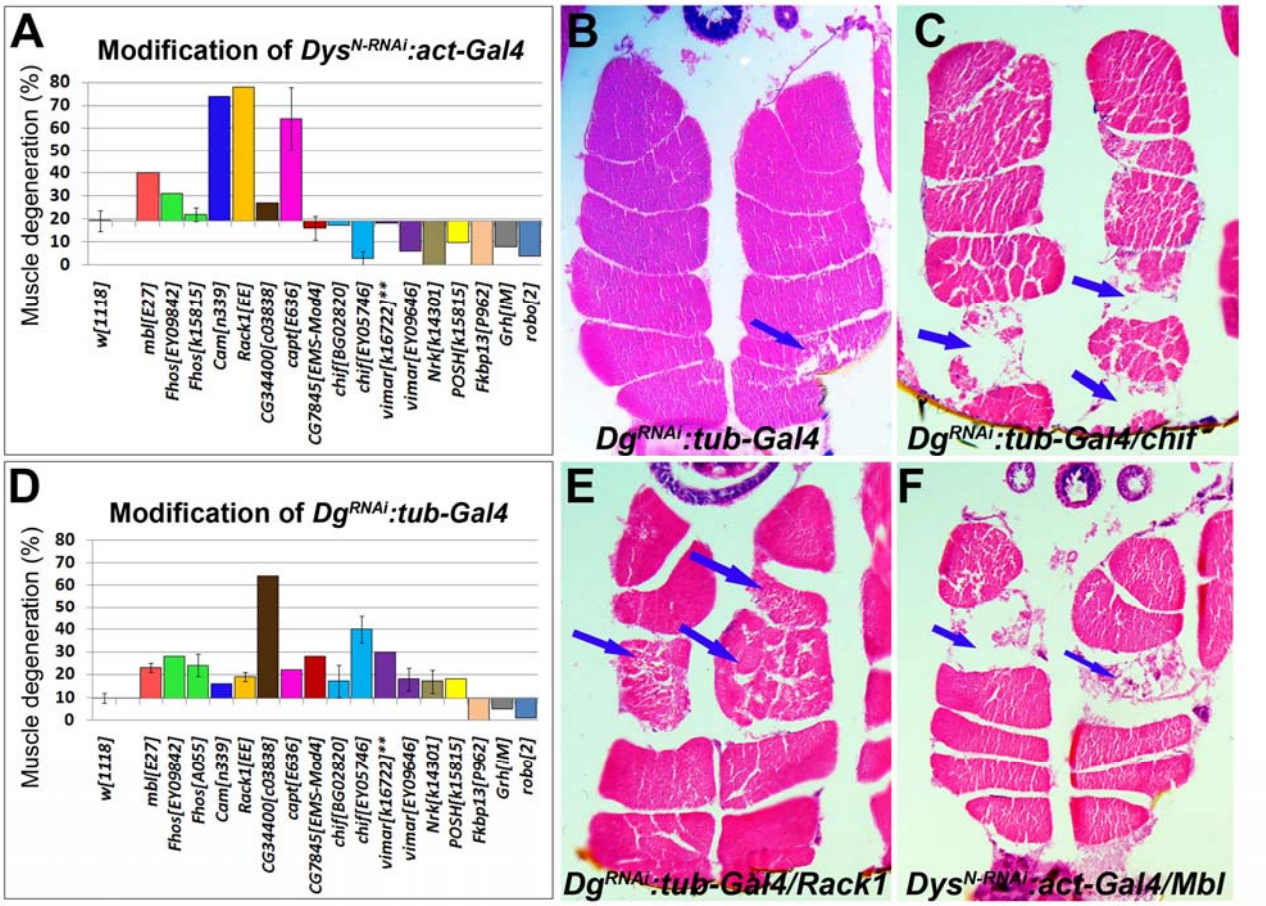


Figure 2. Modification of Dys and Dg RNAi mutant muscle phenotype

(A) Bar graph represents frequencies of muscle degeneration in mutants with lacking one copy of screened genes in *Dys* RNAi background. The $Dys^{N\text{-}RNAi}$*:act-Gal4/*w^{1118} shows ≈ 20% degeneration. All bars above or below control indicate enhancement or suppression of *Dys* muscle degeneration phenotype, respectively. (B) Transverse section of the *Dg* RNAi mutant IFM with moderate muscle tissue degeneration. (C) Transverse section of the *Dg* RNAi mutant IFM, where muscle degeneration is enhanced by reduction by one copy of *chif* gene. (D) Bar graph represents frequencies of muscle degeneration in mutants with reduced one copy of screened genes in *Dg* RNAi background. The Dg^{RNAi}*:act-Gal4/*w^{1118} shows ≈ 10% degeneration. All bars above or below control indicate enhancement or suppression of *Dg* muscle phenotype. (E) The transverse section of the *Dg* RNAi mutant IFM with a phenotype modulated by reduction one copy of the *Rack1* gene; arrows indicate the abnormal muscle architechture is indicated with arrows. (F) Transverse section of the *Dys* RNAi mutant IFM, where muscle degeneration is enhanced by reduction by one copy of the *Mbl* gene. Arrows indicate the muscle degeneration.

Interestingly, the modifier *CG7845* increased the phenotype in *Dg* RNAi mutant background, but did not modulate the *Dys* RNAi mutant phenotype in muscles (Figure 2A,D). Additionally, we have observed that mutations in *chif*, *vimar*, *Nrk* and *POSH* genes manifested an opposed effect on *Dys* and *Dg* mutants: reduction of these genes suppressed the muscle degeneration in *Dys* mutants, but enhanced it in *Dg* mutant flies (Figure 2A,C,D). Moreover, we have identified three genes *Fkbp13*, *Grh* and *robo,* reduction of which in *Dys* and/or *Dg* RNAi mutant background suppressed muscle degeneration (Figure 1A,D).

Heterozygous interaction with Dys and Dg loss-of-function alleles

A powerful approach to identify genes that act in the same pathway is to test for trans-heterozygous interaction. If the genes function in parallel pathways, and neither pathway is impaired enough to show a phenotype, then we would predict that trans-heterozygotes, in which one allele of each gene is mutant, would also have no phenotype. However, if the genes function in the same pathway, then mutations in two steps of one pathway may enhance each other and lead to a phenotype. Based on this, in addition to *Dys* and *Dg* RNAi mutants we have used loss-of-function alleles *DysDf,* Dg^{323} and Dg^{O86} in our secondary screen in order to identify transheterozygous interactions (Table 1). In heterozygous *Dys* and *Dg* mutants (*DysD/+f,* Dg^{323}/+ and Dg^{O86}/+) no obvious changes in muscle structure were observed (Figure 1D-E,F; Table 1; Figure 3A-C), while reduction by one copy of *Mbl*, *Fhos*, *Cam*, *Rack1*, *CG34400*, *capt*, *CG7845*, *chif*, *vimar* and *Nrk* in the *Dys* and *Dg* heterozygous background resulted in an appearance of 10-45% of muscle degeneration (Table 1, Figure 3). Interestingly, *Fkbp13* showed the interaction with *Dg* only, while *POSH* and *Grh*, the modifiers of *Dys* and *Dg* RNAi mutant phenotype, exhibited no transheterozygous interaction with either *Dys* or *Dg* loss-of-function alleles (Figure 3A-B). These findings suggest that components identified as transheterozygous interactors are involved together with Dys and Dg in the same pathway to cause muscle degeneration. However, the components, which were identified as modifiers of muscle degeneration caused by reduction of Dys and Dg in RNAi mutants, but did not show transheterozygous interactions are more likely implicated in parallel to the DGC pathways to provide stability for muscle structure.

The role of newly-found DGC modulators in muscle and neural tissue

To start investigation of the mechanisms of found interactions we have addressed the question whether identified modulators of the *Dys* and/or *Dg* phenotype have their own function directly in muscles or in neurons, which provide muscle innervations.

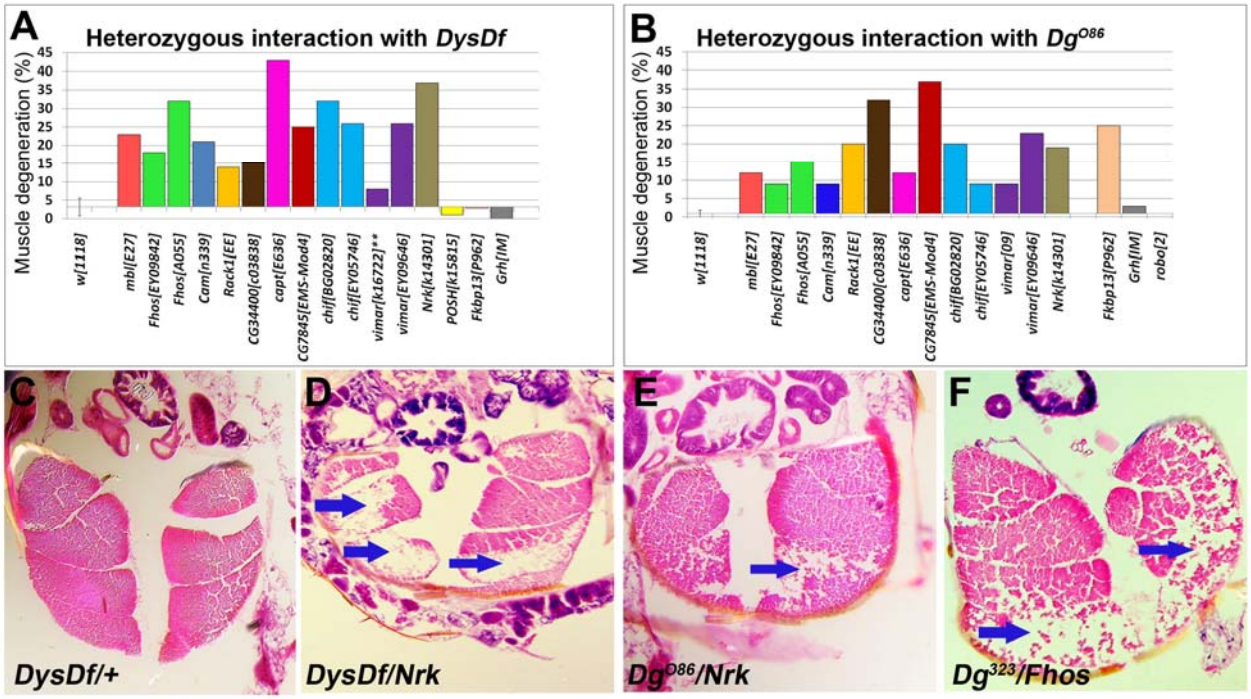


Figure 3. Heterozygous interaction with Dys and Dg loss-of-function mutants

(A) Bar graphs show frequency of degenerated muscles in the flies with heterozygous combination of screened gene and *DysDf*. (B) Bar graphs show frequency of degenerated muscles in the flies with heterozygous combination of screened gene and Dg^{O86}. (C) The transverse section of control fly (*DysDf/+*) shows normal muscle structure. (D) Muscle section of the fly with heterozygous gene combination (*DysDf/Nrk*) demonstrates muscle tissue degeneration. (E) Muscle section of the fly with heterozygous gene combination (Dg^{O86}*/Nrk*) exhibits abnormal muscle architecture. (F) Muscle section of the fly with heterozygous gene combination (Dg^{323}*/Fhos*) with degenerated muscles. Arrows indicate areas with muscle degeneration.

For this purpose we have used RNA interference lines to target novel components in a tissue-specific manner. To reveal if the modifier has its own muscle degeneration phenotype, we have used the *tub-Gal4* enhancer trap line, which down-regulates the protein of our interest ubiquitously in all tissues. For the specific knock-down in muscles the *MHC-Gal4* line was used and to decrease protein level in nerves we have applied motor neuron-specific driver *D42-Gal4*. In the present work, we have tested four interactors *Fkbp13*, *Mbl*, *Nrk* and *vimar* and found that the reduction of the protein levels achieved by using *tub-Gal4* driver resulted in development of the muscle degeneration phenotype for all of the transgenic constructs. The frequency of muscle degeneration was 26% for *Fkbp13/tub-Gal4*, 56% for *Mbl/tub-Gal4*, 60% for *Nrk/tub-Gal4* and 28% for *vimar/tub-Gal4.* Interestingly, the reduction of Fkbp13 and Nrk specifically in muscles did not affect much muscle structure. 6% of muscle degeneration was observed in *Fkbp13/MHC-Gal4* mutants and 3% in *Nrk/MHC-Gal4,* while 1-5% of the phenotype was observed in control flies. However, the reduction of Mbl in muscles caused moderate muscle degeneration (11%) and down-regulation of *vimar* using *MHC-Gal4* developed a strong tissue degeneration, indicating that these two components are required for normal muscles functioning. When the down-regulation of found interactors was introduced in the motor neurons, the moderate muscle phenotype was observed for *Fkbp13/D42-Gal4*, *Mbl/D42-Gal4* and *vimar/D42-Gal4* mutants (12, 9 and 14%, respectively), however strong muscle degeneration was noticed in *Nrk/D42-Gal4* mutant flies. These findings suggest that all of the tested components are important for muscle tissue maintenance. Mbl is required in both neurons and muscles for their normal function, Fkbp13 and Nrk functions are restricted to the motor neurons, whereas vimar is required in both types of tissue, however its down-regulation in muscles leads to an extremely strong phenotype.

Continuing of these analyses should help to clarify the mechanisms of the DGC interaction with the newly-found components.

4. Discussion

Muscular dystrophies are a group of inherited neuromuscular disorders that share the same basic phenotype – progressive loss of muscle integrity. Most types of MD are multi-system diseases with manifestations in the heart, nervous system, eyes and other organs. Duchenne, Becker and limb-girdle muscular dystrophies are human myopathies caused by disruption in genes encoding for dystrophin or other components of the Dystrophin-Glycoprotein Complex. The use of animal models has led to clarification of the role of specific gene products in maintaining muscle integrity and function; however, the regulation of this complex is largely unknown and there is no specific treatment for any of the forms of MD. The fly genome contains many highly conserved orthologues to human disease genes (Bier, 2005; Reiter et al., 2001), including components of the DGC (Deng and Ruohola-Baker, 2000; Deng et al., 2003; Greener and Roberts, 2000). A *Drosophila melanogaster* model with muscular, neuronal and cardiovascular defects caused by mutations in the DGC components was developed recently (Allikian et al., 2007; Bogdanik et al., 2008; Shcherbata et al., 2007; Taghli-Lamallem et al., 2008; van der Plas et al., 2007) and it is useful for studying the DGC role in the neuromuscular disorders.

4.1. Importance of WW-domain binding sites in the Dystroglycan C-terminus for the DGC function

Now I have used the *Drosophila* model of MD to dissect in more detail the interaction between the two major components of the complex – Dystrophin and Dystroglycan. The C-terminal part of *Drosophila* Dystroglycan is conserved between human and fly (31% identity and 46% similarity). Especially well conserved are the protein-protein interaction sites in the cytoplasmic domain of Dg, including the binding site for Dystrophin WW domain (Deng et al., 2003). The type I WW domains interact with the ligand PPxY, and the proline rich cytoplasmic tail of Dystroglycan contains two such potential motifs (PPPY and PPEY – in human or PPPY and PPSY in *Drosophila*) (reviewed in (Ilsley et al., 2002)). Previous studies have indicated that Dystrophin primarily binds to the first but not the second PPxY motif of Dg (Huang et al., 2000; Jung et al., 1995; Rentschler et al., 1999). In this work I have demonstrated *in vitro* that Dystrophin can bind the second WW domain binding site at the C-terminal end of Dystroglycan as well. In addition, I confirmed the importance of each WW binding site for Dystroglycan function by studying *in vivo* their role in oocyte polarity establishment (Yatsenko et al., 2009). The functional redundancy of the WW binding sites poses interesting questions: have both binding sites survived through evolution to protect organisms from the mutations in an essential complex or does each binding site have a

specific function in different tissues and/or developmental stages. Mutations in the DGC cause muscular dystrophies; however only mutations in dystrophin, but not dystroglycan per se, are associated with known types of muscular dystrophies in vertebrates. In mice, mutations in dystroglycan are embryonic lethal, which suggests that Dg is an essential gene. Therefore it was assumed that lack of MD patients with any mutations in *dystroglycan* could be explained by its lethality. However, the results presented suggest a potentially new explanation; perhaps WWbsI and WWbsII are redundant. It is possibly that humans with a mutation in WWbsI exist, but they do not show any MD phenotypes because WWbsII can substitute in place of the mutant WW domain binding site. In addition, it has been shown in vertebrate that dystroglycan can bind caveolin-3 involving the same PPxY motif suggesting that dystrophin and caveolin-3 compete for the same binding site (reviewed in (Ilsley et al., 2002)). Identification of the functional necessity for the second WWbs provides a more complex possibility for this interaction, as well as suggests an additional means for the DGC regulation by other WW domain containing molecules. The comparative sequence analysis of *Drosophila* and human WW binding motifs revealed a very high conservation. However, each WWbs resides in a specific protein micro-environment, which also may suggest that each site has specific binding partners. Therefore it will be important in the future to test if WW binding sites have specific and independent biological functions in different tissues.

The identification of the tyrosine residue within the type I WW domain binding motif led to the hypothesis that the WW/PPxY interaction could be regulated by tyrosine phosphorylation (James et al., 2000), in a manner analogous to the regulation of SH3 domain interactions. As discussed previously, *in vitro* work suggests that when the tyrosine of the PPPY motif (WWbsI) is phosphorylated the binding between dystrophin and dystroglycan is abolished (Sotgia et al., 2001), signifying that the DGC may be regulated in a tyrosine phosphorylation-dependent manner. In my *in vitro* study I showed, using a quantitative assay that the tyrosine phosphorylation of either of the two WW domain binding sites, PPPY motif or PPSY motif, does reduce the binding affinity (Yatsenko et al., 2007), suggesting fine regulation. From the point of view of the WW domain biology, perhaps a most exciting finding was that the ability of the caveolin-3 WW domain to interact with β-dystroglycan was insensitive to the phosphorylation of the tyrosine 892 in the β-dystroglycan PPPY motif (Sotgia et al., 2001). This leads to the possible situation, whereby the phosphorylation of dystroglycan would act as an effective switch to alter the affinity of dystroglycan from dystrophin (or utrophin) to caveolin. In addition, *in vivo* studies in *Drosophila* indicate that a putative SH3 domain binding site is required for proper function of the protein (Yatsenko et al., 2007). These data suggest a more specific mechanism of regulation. One possibility is that an SH3 domain containing tyrosine kinase may dock to the SH3 domain binding site in Dg. This may result in a kinase activation and phosphorylation of the WW domain binding

site in Dg, thereby reducing dystrophin binding to this site. The evidence thus far regarding the regulation of the Dg-Dys interaction depicts a model that strikingly resembles what we know about integrin-talin interactions (Kinashi, 2005). Integrins are heterodimeric, transmembrane proteins that like dystroglycan link the extracellular matrix to the intracellular cytoskeleton. The NPxY motif on the integrin subunit interacts with talin, an actin-binding protein, via the F3 subdomain within the FERM domain of talin, a PTB-like domain (Campbell and Ginsberg, 2004). Talin plays a role analogous to dystrophin by binding the NPxY motif on integrin cytoplasmic tails and linking integrins to the actin cytoskeleton. Binding of talin to the NPxY motif is required for energy dependent activation of integrins (Calderwood, 2004). In addition to performing analogous structural roles, a similar regulatory mechanism may exist. It is known that integrin-talin interaction is mediated in a phosphorylation-dependent manner. When the tyrosine of the NPxY motif is phosphorylated, binding of the integrin to talin is abolished. Focal adhesion kinase and integrin-linked kinase bind to integrins *in vitro* and may regulate integrin-talin interaction, although this remains to be demonstrated *in vivo*. Furthermore, several other proteins, including platelet myosin, SHC, and Grb2, have been shown to bind integrins in their phosphorylated state *in vitro* (Liu et al., 2000). This study provides evidence that a similar mechanism may act to regulate Dg-Dys interaction. Other signaling molecules may then interact with Dg in a phosphorylation-dependent manner. Previous work has revealed SH3 domain-mediated interaction between Dg and Grb2 (Yang et al., 1995). However, I have not been able to observe direct binding between Dg and Drk, a *Drosophila* homologue of Grb2, suggesting a different candidate for SH3 domain interaction in *Drosophila.* Taken together, my findings suggest the additional possibility of potential regulation within the DGC and more options for interaction of the DGC with other cell components.

4.2. Novel elements that interact with the *Drosophila* DGC

Presently, the regulation of the DGC and its involvement in signaling are poorly understood. In this work I have addressed these unknowns by using dominant modifier screens to find genes that may shed light on both of these processes. The large-scale screens for modifiers of Dys/Dg-related wing vein phenotype have revealed groups of modifiers that are components of canonical signaling pathways (TGF-β, EGFR, Wnt and Notch) as well as components involved in cell/neuronal migration, cytoskeletal rearrangements and, most importantly, muscle development. The secondary screen performed in muscle tissue allowed me to identify the components, which interact with Dys and Dg to provide the normal muscle function. I have found human homologues of identified components by comparing protein sequences (Table 2).

Table 2

Human homologues to identified DGC modifiers

Drosophila protein	Human homologue	Identity, %	Positive, %	Gaps, (%)	Function	Involvement in disorders
Cam	**CAM2**	97	98	0	Interact with DGC components (Ilsley et al., 2002), (regulate calcium dependent pathways (Ye et al., 1997)	Enhances muscular dystrophy in mice (Chakkalakal et al., 2006)
capt	**CAP1**	48	64	4	Actin polymerization, signaling (Moriyama and Yahara, 2002)	Unknown
CG34400	**DFNB31**	36	56	18	Actin cytoskeleton organization (Mburu et al., 2003)	Deafness (Mburu et al., 2003)
CG7845	**WDR74**	31	50	4	Unknown	Unknown
chif	**DBF4**	25	42	23	Regulatory subunit of Cdc 7 kinase (Charych et al., 2008)	Cancer (Charych et al., 2008)
Fhos	**FHOD1**	ND	ND	ND	Actin polymerization, MTs organization, signaling (Gasteier et al., 2005), affect muscle function (Montana and Littleton, 2006)	Unknown
Fkbp13	**FKBP14**	44	64	4	Unknown	Unknown
Grh	**GRHL1**	50	66	4	Transcription regulation, affect PCP (Lee and Adler, 2004)	Defects in neural closure and skin development (Gustavsson et al., 2008; Wilanowski et al., 2008)
Mbl	**MBNL**	43	54	12	Splicing factor (Vicente et al., 2007)	Myotonic dystrophy (Vicente et al., 2007)
Nrk	**MuSK**	60	75	5	Receptor, function in NMJ (Meriggioli and Sanders, 2009)	Congenital myasthenic syndrome (Huze et al., 2009)
POSH	**SH3RF3**	38	54	15	Signaling, JNK pathway (Zhang et al., 2005)	Global cerebral ischemia (Zhang et al., 2005)
Rack1	**GNB2L1**	77	87	0	Signaling (Bourd-Boittin et al., 2008; Egidy et al., 2008)	Cancer (Bourd-Boittin et al., 2008; Egidy et al., 2008)
robo	**ROBO**	38	55	4	Cytoskeleton rearrangement, axon guidance receptor (Tayler et al., 2004)	Unknown
Sp2353	**EGFLAM**	32	49	5	Unknown	Unknown
vimar	**RAP1**	30	50	10	GTP-GDP dissociation stimulator (Bailey et al., 2009)	Cancer (Bailey et al., 2009)

4.2.1. Components involved in Ca^{2+} dependent pathways

From the screen I have identified two components, *Calmodulin* and *Fkbp13*, which might modulate *Dys* and *Dg*-dependent muscle degeneration due to their involvement in Ca^{2+} dependent pathways.

Calmodulin was one of the expected interactors, since its binding to Syntrophin (a component of the DGC) was previously shown (reviewed in (Ilsley et al., 2001)). Calmodulin is a highly conserved protein. The amino acid sequence of *Drosophila* Calmodulin is almost identical to CAM2 in human (97% identity). CAM belongs to the EF-hand family of Ca^{2+}-binding proteins and it is found in all eukaryotic cells at varying concentration. CAM modulates the activities of several key enzymes and cellular processes including regulation of cell growth, division, differentiation and cell death (Ye et al., 1997). Many of Ca^{2+} dependent pathways are channeled through the same Ca^{2+} sensor – calmodulin. CAM mediates differential sensitivity of CaMKII (CAM-dependent kinase) to the local Ca^{2+} in cardiac myocytes (Saucerman and Bers, 2008). Moreover, it has been shown *in vitro* that CaMKII is involved in phosphorylation of dystrophin and syntrophin. The association of dystrophin and syntrophin sequences is inhibited by CaMKII phosphorylation (Madhavan and Jarrett, 1999). The CaMKII mediated phosphorylation of dystrophin Dp71 isoform modulates its nuclear localization in PC12 cells (Calderilla-Barbosa et al., 2006). Also, CAM is able to interact with the ZZ domain of dystrophin and utrophin in a calcium-dependent manner and thereby regulate the WW domain mediated interaction between dystrophin/utrophin and dystroglycan. The ZZ domain together with the EF-hand has been shown to stabilize Dys-Dg binding via WW domain (reviewed in (Ilsley et al., 2001)). Targeted inhibition of CAM signaling exacerbates the dystrophic phenotype in *mdx* mouse muscle (Chakkalakal et al., 2006). Now I have demonstrated that reduction of *Cam* in *Drosophila* enhances age-dependent muscle degeneration in *Dys* and *Dg* mutants.

It is known that the loss of dystrophin from human muscles results in disruption of the DGC, which in turn leads to pathological Ca^{2+} dependent signals that damage muscle cells. Recently, in the *mdx* mouse model of muscular dystrophy, the structural and functional defects in a sarcoplasmic reticulum Ca^{2+} channel, ryanodine receptor (RyR1), were identified (Bellinger et al., 2008). RyR1 functions in complex with stabilizing subunit – calstabin-1 (FKBP12). The depletion of FKBP12 results in «leaky» channels that contributes to alterations in Ca^{2+} homeostasis in dystrophic muscles (Bellinger et al., 2008). Additionally, the mutation that causes left ventricular non-compaction cardiomyopathy was recently associated with the *FKBP12* gene (Moric-Janiszewska and Markiewicz-Loskot, 2008). In contrast, another FK506 binding protein (FKBP51) has been shown to be over-expressed in skeletal muscles of the *mdx* mouse (Fisher et al., 2005). Now I have

identified a *Drosophila* Fkbp13, a homologue to human FK506 binding protein 14 (FKBP14) as an interactor with Dys and Dg in muscles. Taken together, these findings suggest the important role of FK506 binding proteins in muscle function, possibly due to Ca^{2+} homeostasis regulation. Further studies of the found interaction are interesting in view of the fact that Fkbp13 is likely to be involved in positive regulation of degeneration, since mutation in *Drosophila Fkbp13* caused suppression of the muscle phenotype in *Dys* and *Dg* RNAi mutants.

4.2.2. Factors with a function in neuromuscular junctions (NMJs)

The studies in *Drosophila* have revealed the DGC function in the neuromuscular junctions (Bogdanik et al., 2008; van der Plas et al., 2006), emphasizing the important role of Dystrophin and Dystroglycan together with Laminin and Coracle in maintaining *Drosophila* NMJs. Dystrophin and Dystroglycan are localized postsynaptically. The reduction of these components from the postsynapse affects synaptic neurotransmitter release (Bogdanik et al., 2008; van der Plas et al., 2006). Moreover, the mutations in DGC components affect neurotransmitter receptors clustering. In humans the heterogeneous disorders with defects in NMJ transmission, that leads to muscle weakness, are classified as congenital myasthenic syndromes (CMS). So far, 12 disease-causing genes coding for proteins that have a key role at the human NMJ have been identified: *CHAT, CHRNA1, CHRNB1, CHRND, CHRNE, COLQ, RAPSN, SCN4A, MuSK, DOK7, LAMB2* and *AGRN* (Huze et al., 2009). However, for half of CMS patients, genes causing the disease have not yet been found. Now I have shown that *Drosophila* Nrk (neurospecific receptor kinase), a protein highly homologous (60% identity) to human MuSK (muscle receptor kinase), interacts with *Dys* and *Dg* in the muscles. MuSK is an essential receptor tyrosine kinase for establishment and maintenance of the neuromuscular junctions (Meriggioli and Sanders, 2009). During *Drosophila* embryogenesis, the *Nrk* gene is expressed specifically in the developing nervous system (Oishi et al., 1997). Furthermore, I have shown that the specific knock-down of Nrk in the *Drosophila* motor neurons causes strong muscle degeneration. This suggests that the *Drosophila* homologue of human MuSK plays a similar role in NMJ function. It has also been shown that MuSK can bind agrin and activation of MuSK by agrin leads to clustering of acetylcholine receptors on the postsynaptic side of NMJs (Stiegler et al., 2009). Furthermore, it is known that agrin and laminin B can directly interact with dystroglycan and both of these ligands have been shown to be involved in congenital myastenic syndrome development (Huze et al., 2009). It is becoming clear that dystroglycan and MuSK (Nrk) are two receptor proteins important for transferring signals necessary for normal NMJ function. However, whether these pathways share the same ligand components to provide neuron-muscle communication has to be studied further.

In addition, I have identified the agrin-like protein SP2353 as an interactor with *Dystroglycan* in muscles. Since this molecule contains a Laminin G domain, it is a potential new extracellular binding partner for Dystroglycan. I have found that humans have a homologue to *Drosophila* SP2353, whose function is still unknown. It will be interesting to study the SP2353 role as an extracellular matrix protein in the NMJ maintenance using a *Drosophila* model.

4.2.3. Cytoskeleton rearrangement components

Another interesting group of genes, identified from the screen, plays an important role in the assembly of the actin cytoskeleton and in the transferring signals that cause cytoskeleton reconstruction. This group contains *CG34400*, *Fhos*, *capt*, *robo* and *Mbl* genes.

The *Drosophila CG34400* gene encodes a protein homologues to human DFNB31 (Deafness, autosomal recessive 31) or mouse whirlin. Defects in whirlin, a PDZ domain molecule, causes deafness in the whirler mouse and congenital hearing impairment in DFNB31 deficient people. It has been shown that DFNB31 acts as an organizer of submembranous molecular complexes that control the coordinated actin polymerization and membrane growth of the stereocilia (Mburu et al., 2003). The gain-of-function screen performed in *Drosophila* reveals that *CG34400* strongly affects muscle pattern formation during embryogenesis (Staudt et al., 2005) and our studies indicate that reduction of CG34400 specifically in *Drosophila* muscles results in their degeneration.

An additional gene identified from the screen, which modulates the cytoskeleton, is *Fhos.* This gene encodes the protein homologues to human FHOD1 (a formin homology containing protein). Formins are conserved in eukaryotes from yeasts to mammals. They control cell polarity during processes such as motility, cytokinesis, and differentiation by organizing the actin cytoskeleton and microtubules (MTs) (Gasteier et al., 2005). Recently a direct interaction between dystrophin and MTs (Prins et al., 2009) was identified. Since *Fhos* came from the screen as an interactor of *Dys*, this proposes that the DGC and Fhos may act as a team in the MTs organizing procedure. Furthermore, it has been shown that mammalian formin FHOD1 interacts with the ERK MAP kinase pathway (Boehm et al., 2005). The involvement of dystroglycan in the same signaling pathway is described above, where the proposed role of dystroglycan is to act as a protein scaffold that sequesters ERK protein in a separate cellular location to regulate their further activation (Spence et al., 2004). This suggests that FHOD1 is involved in the pathway, in which dystroglycan seems to be an up-stream component and raises the question, is dystroglycan required for further FHOD1 function. Moreover, the microarray screen identified an up-regulated Fhos transcript in *Drosophila Myosin heavy chain (Mhc)* mutants and further *in situ* analysis revealed strong expression of Fhos in somatic muscles and putative midline mesodermal cells (Montana and

Littleton, 2006). I have identified now a *Drosophila* formin as a strong enhancer of muscle phenotype caused by a reduction of either *Drosophila* Dystrophin or Dystroglycan. Further studies of the mechanisms of DGC-Fhos interaction are important in different aspects: function in signaling, establishment of cellular polarity and muscle maintenance.

Another gene required for actin polymerization that was identified in our screen is *capulet* (*capt*). Capt (cyclase-associated protein) is a *Drosophila* homologue of human CAP1, which has been shown to play a key role in speeding up the turnover of actin filaments by effectively recycling cofilin and actin (Moriyama and Yahara, 2002). Additionally, CAP proteins have a domain structure, which makes them attractive components to be involved in signal transduction. This may link the cell signaling with actin polymerization (Hubberstey and Mottillo, 2002). Studies in *Drosophila* showed that *capt* genetically interacts with Abl and Slit in providing a midline axon pathfinding (Wills et al., 2002). In addition, it has been reported that the genetic interaction of *capt* with microtubule motor kinesin plays a role in maintaining neuronal dendrite homeostasis (Medina et al., 2008) and that *capt* is involved in organization of actin cytoskeleton during eye development (Benlali et al., 2000) and in oocyte polarity establishment (Baum et al., 2000). Now I have found that reduction of *capt* by one copy enhances muscle degeneration in *Dys* and *Dg* mutants. Since Dys has also been shown to bind actin it may suggest that both proteins, Dys and capt, interact in the process of regulation of the actin cytoskeleton organization.

The Slit-Robo pathway has been shown to transmit information from the ECM to the inside of the cell, causing actin cytoskeleton rearrangement. Such a process is required during axonal growth and their proper migration and termination. The DGC, as well as the Slit-Robo pathway, is also required for neuron pathfinding, which has been shown for the photoreceptor axons in *Drosophila* (Shcherbata et al., 2007; Tayler et al., 2004). From the screen I found both, *slit* and *robo* as interactors with *Dg* and *Dys* in wing veins (Kucherenko et al., 2008) and also showed that *robo* genetically interacts with DGC components in muscles. In addition, *Drosophila* slit-Dg interaction has previously been observed in cardiac cell alignment (Qian et al., 2005) and Dg requirement for slit localization during cardiac tube formation was reported recently (Medioni et al., 2008). Sequence analysis of slit reveals that it possesses a Laminin G domain at its C-terminus, which theoretically can interact with the Laminin G binding sites at the extracellular domain of Dg, making Dg the second possible receptor for slit.

Additionally, my studies demonstrated a strong interaction in muscles between *Dys/Dg* and *Mbl* (*muscleblind*) (Kucherenko et al., 2008). Splicing factors, *Drosophila* Mbl proteins control terminal muscle and neuronal differentiation and are implicated in alternative splicing of α-actinin (Pascual et al., 2006). α-actinin is a cytoskeletal actin-binding protein and a member of a spectrin superfamily, which comprises spectrin, dystrophin and their homologues and isoforms. In muscle

cells α-actinin is localized at the Z-disk, where it forms a lattice-like structure and stabilizes the muscle contractile apparatus (reviewed in (Sjoblom et al., 2008)). Human muscleblind-like homologues (MBNL1-3) are implicated in the pathogenesis of the inherited muscular developmental and a degenerative disease, myotonic dystrophy (Vicente et al., 2007). It would be interesting in the future to dissect DGC-Mbl relations in more detail and to address the question if the DGC-Mbl strong interaction is due to Mbl effect on α-actinin and cytoskeletal actin destabilization, or if it directly interacts with the DGC components, for example, participates in Dystrophin isoforms splicing.

4.2.4. Components implicated in signaling

The Dystrophin-Glycoprotein Complex provides a strong mechanical link between the intracellular cytoskeleton and the extracellular matrix. Recent structural studies have provided new insights into the molecular interactions between Dystroglycan and other key components of the DGC (reviewed in (Winder, 2001)), suggesting regulation of the complex. Increasing understanding of the DGC function at the molecular level shows its involvement not only in MD disease, but in development, adhesion and signaling; however, the exact DGC function in these processes is still unclear.

One of the components found in the screen, which may act together with DGC in signaling, is POSH (plenty of SH3), a *Drosophila* homologue of human SH3RF3 protein. POSH is a multidomain scaffold protein comprised of four Src homology 3 domains, a Rac binding domain and a RING domain. It has been shown that POSH and the JNK pathway are important in mediating death in a Parkinson disease model (Wilhelm et al., 2007). POSH interacts with activated Rac and functions as a proapoptotic protein by activating JNK signaling. Knockdown of POSH attenuates JNK and c-jun activation in the hippocampus following global cerebral ischemia and protects from ischemic neuronal cell death (Zhang et al., 2005). It has been reviewed above the involvement of the DGC in JNK signaling (Zhou et al., 2007). With many SH3 domains, POSH has the potential to bind dystroglycan, but whether these two components interact directly needs to be investigated. Moreover, it has been recently shown that POSH assembles the inhibitory complex that leads the actin-myosin network to regulate neuronal process outgrowth (Taylor et al., 2008). In addition, POSH was also identified in a genetic modifier screen in *Drosophila* for mutants with morphological defects in dorsal appendages (Schnorr et al., 2001) and my studies reveal that if *POSH* mutation is introduced in the *Drosophila* germline, abnormalities in oocyte polarity are observed (Kucherenko et al., 2008). All these results suggest a role for POSH in regulating cell

polarity and cell shape change, but it is unknown in which pathway POSH is involved to mediate these processes.

Another component identified from the screen is a transcription regulator *Grainy head (Grh)*. The mammalian homologue of *Drosophila* Grh, GRHL1 (Grainy head like 1), is involved in regulation of neural tube closure (Gustavsson et al., 2008) and skin development (Wilanowski et al., 2008). *Grh* in *Drosophila* is required for several different processes during differentiation including planar cell polarity (PCP) establishment, epidermal hair morphogenesis and wing vein specification (Lee et al., 2003). At the basal side of follicle epithelium actin filaments exhibit a planar cell polarity. In *Dg* follicle cell clones the basal actin is disrupted non-cell-autonomously (Deng et al., 2003). However, it is unclear whether Dg acts together with the canonical PCP pathway or functions independently of this pathway. In my large-scale screen, for DGC interacting components in wing vein, a group of genes with PCP function was identified including *stan* (*Flamingo*) and *wingless* (*wg*) (Kucherenko et al., 2008). In the absence of Grh function the Frizzled protein fails to accumulate apically and the levels of Stan is dramatically decreased. These interactions support the hypothesis that Dg might act together with the *frizzled*-dependent tissue polarity pathway in coordinating the polarity of cells in epithelial sheets.

Additionally, I have identified several proteins – the recepror of activated protein kinase C (Rack1), visceral mesoderm armadillo repeats (vimar) and chiffon (chif) – whose mammalian homologues are involved in different types of cancer. Rack1 protein, as well as CG7845, a protein with unknown function that has been found in the screen, both contain WD40-repeats and have their homologues in human, GNB2L1 and WDR74, respectively. The underlying common function of all WD-repeat proteins is coordinating multi-protein complex assemblies, where the repeating units serve as a rigid scaffold for protein interactions (Garcia-Higuera et al., 1998). The GNB2L1 protein is involved in numerous cancers: it has been shown to be up-regulated in superficial spreading melanoma cells (Egidy et al., 2008) and in the patients with hepatocellular carcinoma (Bourd-Boittin et al., 2008).

Furthermore, I have identified *vimar* and *chif* as interactors with the DGC in muscles. *Drosophila* vimar (visceral mesoderm armadillo repeats) is homologous to human RAP1, which functions as a transcription regulator and is also implicated in cancer formation (Bailey et al., 2009). Chiffon function is not very well clarified in *Drosophila*; however the human homologue of chif, DBF4 is a regulatory subunit of Cdc7 kinase, whose phosphorylative function is involved in cancer formation (Charych et al., 2008). Likewise, the loss of Dg often is associated with the progression of various cancers (Barresi and Campbell, 2006; Sgambato et al., 2004), specifically, Dg is down-regulated in breast and prostate cancers (Henry et al., 2001; Muschler et al., 2002). However, the exact mechanism and pathway, through which Dg is involved in cancer formation, remains elusive.

It seems to be of great importance to reveal the mechanisms of the DGC signaling in more detail and to study the role of new interacting genes found in this work in the regulation and function of the DGC.

5. References

Allikian, M.J., Bhabha, G., Dospoy, P., Heydemann, A., Ryder, P., Earley, J.U., Wolf, M.J., Rockman, H.A. and McNally, E.M. (2007) Reduced life span with heart and muscle dysfunction in Drosophila sarcoglycan mutants. *Hum Mol Genet*, **16**, 2933-2943.

Bailey, C.L., Kelly, P. and Casey, P.J. (2009) Activation of Rap1 promotes prostate cancer metastasis. *Cancer Res*, **69**, 4962-4968.

Barresi, R. and Campbell, K.P. (2006) Dystroglycan: from biosynthesis to pathogenesis of human disease. *J Cell Sci*, **119**, 199-207.

Baum, B., Li, W. and Perrimon, N. (2000) A cyclase-associated protein regulates actin and cell polarity during Drosophila oogenesis and in yeast. *Curr Biol*, **10**, 964-973.

Bellinger, A.M., Mongillo, M. and Marks, A.R. (2008) Stressed out: the skeletal muscle ryanodine receptor as a target of stress. *J Clin Invest*, **118**, 445-453.

Benlali, A., Draskovic, I., Hazelett, D.J. and Treisman, J.E. (2000) act up controls actin polymerization to alter cell shape and restrict Hedgehog signaling in the Drosophila eye disc. *Cell*, **101**, 271-281.

Bier, E. (2005) Drosophila, the golden bug, emerges as a tool for human genetics. *Nat Rev Genet*, **6**, 9-23.

Bier, E. and Bodmer, R. (2004) Drosophila, an emerging model for cardiac disease. *Gene*, **342**, 1-11.

Blake, D.J., Weir, A., Newey, S.E. and Davies, K.E. (2002) Function and genetics of dystrophin and dystrophin-related proteins in muscle. *Physiol Rev*, **82**, 291-329.

Boehm, M.B., Milius, T.J., Zhou, Y., Westendorf, J.J. and Koka, S. (2005) The mammalian formin FHOD1 interacts with the ERK MAP kinase pathway. *Biochem Biophys Res Commun*, **335**, 1090-1094.

Bogdanik, L., Framery, B., Frolich, A., Franco, B., Mornet, D., Bockaert, J., Sigrist, S.J., Grau, Y. and Parmentier, M.L. (2008) Muscle dystroglycan organizes the postsynapse and regulates presynaptic neurotransmitter release at the Drosophila neuromuscular junction. *PLoS One*, **3**, e2084.

Bonini, N.M. and Fortini, M.E. (2003) Human neurodegenerative disease modeling using Drosophila. *Annu Rev Neurosci*, **26**, 627-656.

Bourd-Boittin, K., Le Pabic, H., Bonnier, D., L'Helgoualc'h, A. and Theret, N. (2008) RACK1, a new ADAM12 interacting protein. Contribution to liver fibrogenesis. *J Biol Chem*, **283**, 26000-26009.

Bredt, D.S. (1999) Knocking signalling out of the dystrophin complex. *Nat Cell Biol*, **1**, E89-91.

Bushby, K.M. (1999) Making sense of the limb-girdle muscular dystrophies. *Brain*, **122 (Pt 8)**, 1403-1420.

Calderilla-Barbosa, L., Ortega, A. and Cisneros, B. (2006) Phosphorylation of dystrophin Dp71d by Ca2+/calmodulin-dependent protein kinase II modulates the Dp71d nuclear localization in PC12 cells. *J Neurochem*, **98**, 713-722.

Calderwood, D.A. (2004) Talin controls integrin activation. *Biochem Soc Trans*, **32**, 434-437.

Campbell, I.D. and Ginsberg, M.H. (2004) The talin-tail interaction places integrin activation on FERM ground. *Trends Biochem Sci*, **29**, 429-435.

Campbell, K.P. (1995) Three muscular dystrophies: loss of cytoskeleton-extracellular matrix linkage. *Cell*, **80**, 675-679.

Chakkalakal, J.V., Michel, S.A., Chin, E.R., Michel, R.N. and Jasmin, B.J. (2006) Targeted inhibition of Ca2+ /calmodulin signaling exacerbates the dystrophic phenotype in mdx mouse muscle. *Hum Mol Genet*, **15**, 1423-1435.

Charych, D.H., Coyne, M., Yabannavar, A., Narberes, J., Chow, S., Wallroth, M., Shafer, C. and Walter, A.O. (2008) Inhibition of Cdc7/Dbf4 kinase activity affects specific phosphorylation sites on MCM2 in cancer cells. *J Cell Biochem*, **104**, 1075-1086.

Christoforou, C.P., Greer, C.E., Challoner, B.R., Charizanos, D. and Ray, R.P. (2008) The detached locus encodes Drosophila Dystrophin, which acts with other components of the Dystrophin Associated Protein Complex to influence intercellular signalling in developing wing veins. *Dev Biol*, **313**, 519-532.

Collins, C.A. and Morgan, J.E. (2003) Duchenne's muscular dystrophy: animal models used to investigate pathogenesis and develop therapeutic strategies. *Int J Exp Pathol*, **84**, 165-172.

Cote, P.D., Moukhles, H., Lindenbaum, M. and Carbonetto, S. (1999) Chimaeric mice deficient in dystroglycans develop muscular dystrophy and have disrupted myoneural synapses. *Nat Genet*, **23**, 338-342.

Crosbie, R.H., Lebakken, C.S., Holt, K.H., Venzke, D.P., Straub, V., Lee, J.C., Grady, R.M., Chamberlain, J.S., Sanes, J.R. and Campbell, K.P. (1999) Membrane targeting and stabilization of sarcospan is mediated by the sarcoglycan subcomplex. *J Cell Biol*, **145**, 153-165.

Davies, K.E., Pearson, P.L., Harper, P.S., Murray, J.M., O'Brien, T., Sarfarazi, M. and Williamson, R. (1983) Linkage analysis of two cloned DNA sequences flanking the Duchenne muscular dystrophy locus on the short arm of the human X chromosome. *Nucleic Acids Res*, **11**, 2303-2312.

Deng, W.M. and Ruohola-Baker, H. (2000) Laminin A is required for follicle cell-oocyte signaling that leads to establishment of the anterior-posterior axis in Drosophila. *Curr Biol*, **10**, 683-686.

Deng, W.M., Schneider, M., Frock, R., Castillejo-Lopez, C., Gaman, E.A., Baumgartner, S. and Ruohola-Baker, H. (2003) Dystroglycan is required for polarizing the epithelial cells and the oocyte in Drosophila. *Development*, **130**, 173-184.

Durbeej, M. and Campbell, K.P. (2002) Muscular dystrophies involving the dystrophin-glycoprotein complex: an overview of current mouse models. *Curr Opin Genet Dev*, **12**, 349-361.

Egidy, G., Jule, S., Bosse, P., Bernex, F., Geffrotin, C., Vincent-Naulleau, S., Horak, V., Sastre-Garau, X. and Panthier, J.J. (2008) Transcription analysis in the MeLiM swine model identifies RACK1 as a potential marker of malignancy for human melanocytic proliferation. *Mol Cancer*, **7**, 34.

Fisher, I., Abraham, D., Bouri, K., Hoffman, E.P., Muntoni, F. and Morgan, J. (2005) Prednisolone-induced changes in dystrophic skeletal muscle. *Faseb J*, **19**, 834-836.

Garcia-Higuera, I., Gaitatzes, C., Smith, T.F. and Neer, E.J. (1998) Folding a WD repeat propeller. Role of highly conserved aspartic acid residues in the G protein beta subunit and Sec13. *J Biol Chem*, **273**, 9041-9049.

Gasteier, J.E., Schroeder, S., Muranyi, W., Madrid, R., Benichou, S. and Fackler, O.T. (2005) FHOD1 coordinates actin filament and microtubule alignment to mediate cell elongation. *Exp Cell Res*, **306**, 192-202.

Gieseler, K., Grisoni, K. and Segalat, L. (2000) Genetic suppression of phenotypes arising from mutations in dystrophin-related genes in Caenorhabditis elegans. *Curr Biol*, **10**, 1092-1097.

Gonzalez-Ramirez, R., Morales-Lazaro, S.L., Tapia-Ramirez, V., Mornet, D. and Cisneros, B. (2008) Nuclear and nuclear envelope localization of dystrophin Dp71 and dystrophin-associated proteins (DAPs) in the C2C12 muscle cells: DAPs nuclear localization is modulated during myogenesis. *J Cell Biochem*, **105**, 735-745.

Greener, M.J. and Roberts, R.G. (2000) Conservation of components of the dystrophin complex in Drosophila. *FEBS Lett*, **482**, 13-18.

Groh, S., Zong, H., Goddeeris, M.M., Lebakken, C.S., Venzke, D., Pessin, J.E. and Campbell, K.P. (2009) Sarcoglycan complex: implications for metabolic defects in muscular dystrophies. *J Biol Chem*, **284**, 19178-19182.

Gustavsson, P., Copp, A.J. and Greene, N.D. (2008) Grainyhead genes and mammalian neural tube closure. *Birth Defects Res A Clin Mol Teratol*, **82**, 728-735.

Henry, M.D., Cohen, M.B. and Campbell, K.P. (2001) Reduced expression of dystroglycan in breast and prostate cancer. *Hum Pathol*, **32**, 791-795.

Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M. and Eck, M.J. (2000) Structure of a WW domain containing fragment of dystrophin in complex with beta-dystroglycan. *Nat Struct Biol*, **7**, 634-638.

Hubberstey, A.V. and Mottillo, E.P. (2002) Cyclase-associated proteins: CAPacity for linking signal transduction and actin polymerization. *Faseb J*, **16**, 487-499.

Huze, C., Bauche, S., Richard, P., Chevessier, F., Goillot, E., Gaudon, K., Ben Ammar, A., Chaboud, A., Grosjean, I., Lecuyer, H.A., Bernard, V., Rouche, A., Alexandri, N., Kuntzer, T., Fardeau, M., Fournier, E., Brancaccio, A., Ruegg, M.A., Koenig, J., Eymard, B., Schaeffer, L. and Hantai, D. (2009) Identification of an agrin mutation that causes congenital myasthenia and affects synapse function. *Am J Hum Genet*, **85**, 155-167.

Ilsley, J.L., Sudol, M. and Winder, S.J. (2001) The interaction of dystrophin with beta-dystroglycan is regulated by tyrosine phosphorylation. *Cell Signal*, **13**, 625-632.

Ilsley, J.L., Sudol, M. and Winder, S.J. (2002) The WW domain: linking cell signalling to the membrane cytoskeleton. *Cell Signal*, **14**, 183-189.

James, M., Nuttall, A., Ilsley, J.L., Ottersbach, K., Tinsley, J.M., Sudol, M. and Winder, S.J. (2000) Adhesion-dependent tyrosine phosphorylation of (beta)-dystroglycan regulates its interaction with utrophin. *J Cell Sci*, **113 (Pt 10)**, 1717-1726.

Jung, D., Yang, B., Meyer, J., Chamberlain, J.S. and Campbell, K.P. (1995) Identification and characterization of the dystrophin anchoring site on beta-dystroglycan. *J Biol Chem*, **270**, 27305-27310.

Kato, Y., Ito, M., Kawai, K., Nagata, K. and Tanokura, M. (2002) Determinants of ligand specificity in groups I and IV WW domains as studied by surface plasmon resonance and model building. *J Biol Chem*, **277**, 10173-10177.

Kinashi, T. (2005) Intracellular signalling controlling integrin activation in lymphocytes. *Nat Rev Immunol*, **5**, 546-559.

Kucherenko, M.M., Pantoja, M., Yatsenko, A.S., Shcherbata, H.R., Fischer, K.A., Maksymiv, D.V., Chernyk, Y.I. and Ruohola-Baker, H. (2008) Genetic modifier screens reveal new components that interact with the Drosophila dystroglycan-dystrophin complex. *PLoS One*, **3**, e2418.

Langenbach, K.J. and Rando, T.A. (2002) Inhibition of dystroglycan binding to laminin disrupts the PI3K/AKT pathway and survival signaling in muscle cells. *Muscle Nerve*, **26**, 644-653.

Lapidos, K.A., Kakkar, R. and McNally, E.M. (2004) The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. *Circ Res*, **94**, 1023-1031.

Lee, E., Salic, A., Kruger, R., Heinrich, R. and Kirschner, M.W. (2003) The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway. *PLoS Biol*, **1**, E10.

Lee, H. and Adler, P.N. (2004) The grainy head transcription factor is essential for the function of the frizzled pathway in the Drosophila wing. *Mech Dev*, **121**, 37-49.

Liu, S.K., Smith, C.A., Arnold, R., Kiefer, F. and McGlade, C.J. (2000) The adaptor protein Gads (Grb2-related adaptor downstream of Shc) is implicated in coupling hemopoietic progenitor kinase-1 to the activated TCR. *J Immunol*, **165**, 1417-1426.

Madhavan, R. and Jarrett, H.W. (1999) Phosphorylation of dystrophin and alpha-syntrophin by Ca(2+)-calmodulin dependent protein kinase II. *Biochim Biophys Acta*, **1434**, 260-274.

Mburu, P., Mustapha, M., Varela, A., Weil, D., El-Amraoui, A., Holme, R.H., Rump, A., Hardisty, R.E., Blanchard, S., Coimbra, R.S., Perfettini, I., Parkinson, N., Mallon, A.M., Glenister, P., Rogers, M.J., Paige, A.J., Moir, L., Clay, J., Rosenthal, A., Liu, X.Z., Blanco, G., Steel, K.P., Petit, C. and Brown, S.D. (2003) Defects in whirlin, a PDZ domain molecule involved in stereocilia elongation, cause deafness in the whirler mouse and families with DFNB31. *Nat Genet*, **34**, 421-428.

Medina, P.M., Worthen, R.J., Forsberg, L.J. and Brenman, J.E. (2008) The actin-binding protein capulet genetically interacts with the microtubule motor kinesin to maintain neuronal dendrite homeostasis. *PLoS One*, **3**, e3054.

Medioni, C., Astier, M., Zmojdzian, M., Jagla, K. and Semeriva, M. (2008) Genetic control of cell morphogenesis during Drosophila melanogaster cardiac tube formation. *J Cell Biol*, **182**, 249-261.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E. and Rudnicki, M.A. (1996) MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev*, **10**, 1173-1183.

Meriggioli, M.N. and Sanders, D.B. (2009) Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity. *Lancet Neurol*, **8**, 475-490.

Mirouse, V., Christoforou, C.P., Fritsch, C., St Johnston, D. and Ray, R.P. (2009) Dystroglycan and perlecan provide a basal cue required for epithelial polarity during energetic stress. *Dev Cell*, **16**, 83-92.

Montana, E.S. and Littleton, J.T. (2006) Expression profiling of a hypercontraction-induced myopathy in Drosophila suggests a compensatory cytoskeletal remodeling response. *J Biol Chem*, **281**, 8100-8109.

Moore, C.J., Goh, H.T. and Hewitt, J.E. (2008) Genes required for functional glycosylation of dystroglycan are conserved in zebrafish. *Genomics*, **92**, 159-167.

Moric-Janiszewska, E. and Markiewicz-Loskot, G. (2008) Genetic heterogeneity of left-ventricular noncompaction cardiomyopathy. *Clin Cardiol*, **31**, 201-204.

Moriyama, K. and Yahara, I. (2002) Human CAP1 is a key factor in the recycling of cofilin and actin for rapid actin turnover. *J Cell Sci*, **115**, 1591-1601.

Muntoni, F. (2004) Journey into muscular dystrophies caused by abnormal glycosylation. *Acta Myol*, **23**, 79-84.

Muschler, J., Levy, D., Boudreau, R., Henry, M., Campbell, K. and Bissell, M.J. (2002) A role for dystroglycan in epithelial polarization: loss of function in breast tumor cells. *Cancer Res*, **62**, 7102-7109.

Neuman, S., Kovalio, M., Yaffe, D. and Nudel, U. (2005) The Drosophila homologue of the dystrophin gene - introns containing promoters are the major contributors to the large size of the gene. *FEBS Lett*, **579**, 5365-5371.

Oishi, I., Sugiyama, S., Liu, Z.J., Yamamura, H., Nishida, Y. and Minami, Y. (1997) A novel Drosophila receptor tyrosine kinase expressed specifically in the nervous system. Unique structural features and implication in developmental signaling. *J Biol Chem*, **272**, 11916-11923.

Pascual, M., Vicente, M., Monferrer, L. and Artero, R. (2006) The Muscleblind family of proteins: an emerging class of regulators of developmentally programmed alternative splicing. *Differentiation*, **74**, 65-80.

Prins, K.W., Humston, J.L., Mehta, A., Tate, V., Ralston, E. and Ervasti, J.M. (2009) Dystrophin is a microtubule-associated protein. *J Cell Biol*, **186**, 363-369.

Qian, L., Liu, J. and Bodmer, R. (2005) Slit and Robo control cardiac cell polarity and morphogenesis. *Curr Biol*, **15**, 2271-2278.

Reiter, L.T., Potocki, L., Chien, S., Gribskov, M. and Bier, E. (2001) A systematic analysis of human disease-associated gene sequences in Drosophila melanogaster. *Genome Res*, **11**, 1114-1125.

Rentschler, S., Linn, H., Deininger, K., Bedford, M.T., Espanel, X. and Sudol, M. (1999) The WW domain of dystrophin requires EF-hands region to interact with beta-dystroglycan. *Biol Chem*, **380**, 431-442.

Saucerman, J.J. and Bers, D.M. (2008) Calmodulin mediates differential sensitivity of CaMKII and calcineurin to local Ca2+ in cardiac myocytes. *Biophys J*, **95**, 4597-4612.

Schnorr, J.D., Holdcraft, R., Chevalier, B. and Berg, C.A. (2001) Ras1 interacts with multiple new signaling and cytoskeletal loci in Drosophila eggshell patterning and morphogenesis. *Genetics*, **159**, 609-622.

Sgambato, A., Camerini, A., Faraglia, B., Pavoni, E., Montanari, M., Spada, D., Losasso, C., Brancaccio, A. and Cittadini, A. (2004) Increased expression of dystroglycan inhibits the growth and tumorigenicity of human mammary epithelial cells. *Cancer Biol Ther*, **3**, 967-975.

Shcherbata, H.R., Yatsenko, A.S., Patterson, L., Sood, V.D., Nudel, U., Yaffe, D., Baker, D. and Ruohola-Baker, H. (2007) Dissecting muscle and neuronal disorders in a Drosophila model of muscular dystrophy. *Embo J*, **26**, 481-493.

Sjoblom, B., Salmazo, A. and Djinovic-Carugo, K. (2008) Alpha-actinin structure and regulation. *Cell Mol Life Sci*, **65**, 2688-2701.

Sotgia, F., Lee, H., Bedford, M.T., Petrucci, T., Sudol, M. and Lisanti, M.P. (2001) Tyrosine phosphorylation of beta-dystroglycan at its WW domain binding motif, PPxY, recruits SH2 domain containing proteins. *Biochemistry*, **40**, 14585-14592.

Spence, H.J., Dhillon, A.S., James, M. and Winder, S.J. (2004) Dystroglycan, a scaffold for the ERK-MAP kinase cascade. *EMBO Rep*, **5**, 484-489.

St Johnston, D. (2002) The art and design of genetic screens: Drosophila melanogaster. *Nat Rev Genet*, **3**, 176-188.

Staudt, N., Molitor, A., Somogyi, K., Mata, J., Curado, S., Eulenberg, K., Meise, M., Siegmund, T., Hader, T., Hilfiker, A., Bronner, G., Ephrussi, A., Rorth, P., Cohen, S.M., Fellert, S., Chung, H.R., Piepenburg, O., Schafer, U., Jackle, H. and Vorbruggen, G. (2005) Gain-of-function screen for genes that affect Drosophila muscle pattern formation. *PLoS Genet*, **1**, e55.

Stiegler, A.L., Burden, S.J. and Hubbard, S.R. (2009) Crystal Structure of the Frizzled-Like Cysteine-Rich Domain of the Receptor Tyrosine Kinase MuSK. *J Mol Biol*.

Straub, V. and Campbell, K.P. (1997) Muscular dystrophies and the dystrophin-glycoprotein complex. *Curr Opin Neurol*, **10**, 168-175.

Straub, V., Ettinger, A.J., Durbeej, M., Venzke, D.P., Cutshall, S., Sanes, J.R. and Campbell, K.P. (1999) epsilon-sarcoglycan replaces alpha-sarcoglycan in smooth muscle to form a unique dystrophin-glycoprotein complex. *J Biol Chem*, **274**, 27989-27996.

Taghli-Lamallem, O., Akasaka, T., Hogg, G., Nudel, U., Yaffe, D., Chamberlain, J.S., Ocorr, K. and Bodmer, R. (2008) Dystrophin deficiency in Drosophila reduces lifespan and causes a dilated cardiomyopathy phenotype. *Aging Cell*, **7**, 237-249.

Tayler, T.D., Robichaux, M.B. and Garrity, P.A. (2004) Compartmentalization of visual centers in the Drosophila brain requires Slit and Robo proteins. *Development*, **131**, 5935-5945.

Taylor, J., Chung, K.H., Figueroa, C., Zurawski, J., Dickson, H.M., Brace, E.J., Avery, A.W., Turner, D.L. and Vojtek, A.B. (2008) The scaffold protein POSH regulates axon outgrowth. *Mol Biol Cell*, **19**, 5181-5192.

van der Plas, M.C., Pilgram, G.S., de Jong, A.W., Bansraj, M.R., Fradkin, L.G. and Noordermeer, J.N. (2007) Drosophila Dystrophin is required for integrity of the musculature. *Mech Dev*, **124**, 617-630.

van der Plas, M.C., Pilgram, G.S., Plomp, J.J., de Jong, A., Fradkin, L.G. and Noordermeer, J.N. (2006) Dystrophin is required for appropriate retrograde control of neurotransmitter release at the Drosophila neuromuscular junction. *J Neurosci*, **26**, 333-344.

Vicente, M., Monferrer, L., Poulos, M.G., Houseley, J., Monckton, D.G., O'Dell K, M., Swanson, M.S. and Artero, R.D. (2007) Muscleblind isoforms are functionally distinct and regulate alpha-actinin splicing. *Differentiation*, **75**, 427-440.

Wagner, K.R., Lechtzin, N. and Judge, D.P. (2007) Current treatment of adult Duchenne muscular dystrophy. *Biochim Biophys Acta*, **1772**, 229-237.

Wairkar, Y.P., Fradkin, L.G., Noordermeer, J.N. and DiAntonio, A. (2008) Synaptic defects in a Drosophila model of congenital muscular dystrophy. *J Neurosci*, **28**, 3781-3789.

Wilanowski, T., Caddy, J., Ting, S.B., Hislop, N.R., Cerruti, L., Auden, A., Zhao, L.L., Asquith, S., Ellis, S., Sinclair, R., Cunningham, J.M. and Jane, S.M. (2008) Perturbed desmosomal cadherin expression in grainy head-like 1-null mice. *Embo J*, **27**, 886-897.

Wilhelm, M., Xu, Z., Kukekov, N.V., Gire, S. and Greene, L.A. (2007) Proapoptotic Nix activates the JNK pathway by interacting with POSH and mediates death in a Parkinson disease model. *J Biol Chem*, **282**, 1288-1295.

Wills, Z., Emerson, M., Rusch, J., Bikoff, J., Baum, B., Perrimon, N. and Van Vactor, D. (2002) A Drosophila homolog of cyclase-associated proteins collaborates with the Abl tyrosine kinase to control midline axon pathfinding. *Neuron*, **36**, 611-622.

Winder, S.J. (2001) The complexities of dystroglycan. *Trends Biochem Sci*, **26**, 118-124.

Xiong, Y., Zhou, Y. and Jarrett, H.W. (2009) Dystrophin glycoprotein complex-associated Gbetagamma subunits activate phosphatidylinositol-3-kinase/Akt signaling in skeletal muscle in a laminin-dependent manner. *J Cell Physiol*, **219**, 402-414.

Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G. and Campbell, K.P. (1995) SH3 domain-mediated interaction of dystroglycan and Grb2. *J Biol Chem*, **270**, 11711-11714.

Yatsenko, A.S., Gray, E.E., Shcherbata, H.R., Patterson, L.B., Sood, V.D., Kucherenko, M.M., Baker, D. and Ruohola-Baker, H. (2007) A putative Src homology 3 domain binding motif but not the C-terminal dystrophin WW domain binding motif is required for dystroglycan function in cellular polarity in Drosophila. *J Biol Chem*, **282**, 15159-15169.

Yatsenko, A.S., Kucherenko, M.M., Pantoja, M., Fischer, K.A., Madeoy, J., Deng, W.M., Schneider, M., Baumgartner, S., Akey, J., Shcherbata, H.R. and Ruohola-Baker, H. (2009)

The conserved WW-domain binding sites in Dystroglycan C-terminus are essential but partially redundant for Dystroglycan function. *BMC Dev Biol*, **9**, 18.

Ye, Q., Wei, Y., Fischer, R., Borner, C. and Berchtold, M.W. (1997) Expression of calmodulin and calmodulin binding proteins in rat fibroblasts stably transfected with protein kinase C and oncogenes. *Biochim Biophys Acta*, **1359**, 89-96.

Zhang, Q.G., Wang, R.M., Yin, X.H., Pan, J., Xu, T.L. and Zhang, G.Y. (2005) Knock-down of POSH expression is neuroprotective through down-regulating activation of the MLK3-MKK4-JNK pathway following cerebral ischaemia in the rat hippocampal CA1 subfield. *J Neurochem*, **95**, 784-795.

Zhou, Y., Jiang, D., Thomason, D.B. and Jarrett, H.W. (2007) Laminin-induced activation of Rac1 and JNKp46 is initiated by Src family kinases and mimics the effects of skeletal muscle contraction. *Biochemistry*, **46**, 14907-14916.

6. Appendix

6.1. Abbreviations

BMD Becker muscular dystrophy

BDSC Bloomington *Drosophila* stock center

CAP Cyclase associated protein

C.elegans *Caenorhabditis elegans*

CMD Congenital muscular dystrophy

CMS Congenital myastenic syndrome

D.melanogaster *Drosophila melanogaster*

Dg Dystroglycan

DGC Dystrophin-Glycoprotein Complex

DGRC Drosophila genomics resource center

DMD Duchenne muscular dystrophy

DmWWbs *Drosophila melanogaster* WW binding site

DRP Dystrophin related protein

Dys Dystrophin

ECM Extracellular matrix

IFM Indirect flight muscles

JNK c-jun N-terminal kinase

Kd Constant of dissociation

LGMD Limb-girdle muscular dystrophy

MD Muscular dystrophy

MEB Muscle-eye-brain disease

MTs microtubules

NMJ Neuromuscular junction

NOS Nitric oxide synthase

Nrk Neuronal receptor kinase

PCP Planar cell polarity

PCR Polymerase chain reaction

POMT Protein-O-mannosyl transferase

POSH Plenty of SH3

Rack1 Receptor of activated protein kinase C1

SH2 Src homologue 2

SH3 Src homologue 3

vimar visceral mesoderm armadillo repeats

WWbs WW binding site

WWS Walker-Warburg syndrome

7. Curriculum Vitae

MARIYA M. KUCHERENKO
Max Planck Institute for Biophysical Chemistry
Research Group, Gene expression and signaling,
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Education and Employment:

2009 **PhD. Degree in Biology**

Dissertation thesis: «Determination of Genetic Interactions Required for Dystrophin-Dystroglycan Complex Function and Regulation in a *Drosophila* Model of Muscular Dystrophy» (George August University, Goettingen, Germany)

2008 – 2009 **Graduate student** at George-August University (Goettingen, Germany) and in Max Planck Institute for Biophysical Chemistry, Gene expression and signaling research group (Goettingen, Germany) supervised by Dr. Halyna R.Shcherbata

2005 - 2008 **Visiting scientist** in the Ruohola-Baker Lab, Biochemistry Department, University of Washington, Seattle, WA, USA

2004 - 2009 **Graduate studen**t at Ivan Franko National University of Lviv, Genetics and Biotechnology Department (Ukraine) supervised by Dr. Yaroslava I. Chernyk

2004 **M.Sc. Degree**

Master thesis: "The influence of neuroprotectors on degenerative processes in *Drosophila melanogaster* brain" (Ivan Franko National University of Lviv, Ukraine)

2003 - 2004 **Master student** at Ivan Franko National University of Lviv, Genetics and Biotechnology Department (Ukraine) supervised by Dr. Yaroslava I. Chernyk

1999 - 2003 **Undergraduate student** at Ivan Franko National University of Lviv, Genetics and Biotechnology Department (Ukraine)

1988 - 1999 **Secondary School** №92 (Lviv, Ukraine)

Additional skills

Languages: Ukrainian (native language), English and Russian (fluent written and spoken), Polish (fluent spoken), German (basic).

Honors

2008 – GSA travel award for graduate students

2005 – WUBMRC (West-Ukrainian Biomedical Research Center) award for graduate students

Skills and techniques useful in *Drosophila* research
Strong background in Drosophila genetics
Good skills in dissecting of *Drosophila* tissues (larvae and adults),
Histology and immunohistochemistry
Microscopy analysis (light and confocal)
Practical basis of molecular biology (

Participation in Conferences

- 49th Annual *Drosophila* Research Conference, San-Diego, California, USA (2008)
- 48th Annual *Drosophila* Research Conference, Philadelphia, Pennsylvania, USA (2007)
- "Genetic analysis: model organisms to human biology", San Diego, California, USA (2005)
- II Interrnational Research Conference for students, graduate students and young scientists "Biodiversity, Ecology, Evolution, Adaptation", Odesa, Ukraine (2005)
- The First International Conference for Students and PhD Students, Lviv, Ukraine (2005)

Publications

- AS Yatsenko*, **MM kucherenko**,* M Pantoja, KA Fischer, J Madeoy, W-M Deng, M Schneider, S Baumgartner, J Akey, HR Shcherbata and H Ruohola-Baker // The conserved WW-domain binding sites in Dystroglycan C-terminus are essential but partially redundant fur Dystroglycan function // BMC Developmental Biology, February 2009, 9:18.
- **Mariya M. Kucherenko***., Mario Pantoja*., Andriy S. Yatsenko, Halyna R. Shcherbata, Karin A. Fischer, Dariya V. Maksymiv, Yaroslava I. Chernyk, Hannele Ruohola-Baker // Genetic Modifier Screens Reveal New Components that Interact with the Drosophila Dystroglycan- Dystrophin Complex // PLoS ONE, June 2008, Volume 3, Issue 6, e2418.
- Andriy S. Yatsenko, Elizabeth E. Gray, Halyna R. Shcherbata, Larissa B. Patterson, Vanita D. Sood, **Mariya M. Kucherenko**, David Baker, Hannele Ruohola-Baker // A putative Src homology 3 domain binding motif but not the C-terminal Dystrophin WW-domain binding motif is required for Dystroglycan function in cellular polarity in Drosophila // Journal of Biological Chemistry,- vol282, № 20, P.- 15159-15169,- 2007.
- **Kucherenko M**., Obnyavka M., Chernyk Ya., Comparative investigation of different concentrations of neuroprotectors "Cerebral" and "Cerebrolisine" on degenerative processes

in *Drosophila melanogaster* brain // Visnyk of Lviv University, Vol.41. –P.21-25,-2006 (Ukrainian).

- Radysh V., Stupnytska I., **Kucherenko M**., Plahta O., Makarenko O., Hasul E., Maksymiv D., Chernyk Ya. The influence of "Adement" and its low molecular fractions on neurodegenerative processes development in *Drosophila melanogaster* mutants // Cytology and Genetics, - Vol 49, - №4,- P.45-52,- 2005 (Ukrainian).
- G.R.Shcherbata, N.P.Matiytsiv, Ya.I.Chernik, A.S.Yatsenko, V.V.Radysh, **M.M.Kucherenko**, D.V.Maksymiv. Genetic Analysis of X-chromosome *Drosophila melanogaster* Neurodegenerative Mutants Induced by Ethyl Methanesulphonate and Nitrosoethylurea // Genetica. 2004. V.40. № 9. P.1286-1292 (Russian).

*****equal author contribution