Dystroglycan and Protein O-Mannosyltransferases 1 and 2 Are Required to Maintain Integrity of *Drosophila* Larval Muscles

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In vertebrates, mutations in Protein O-mannosyltransferase1 (POMT1) or POMT2 are associated with muscular dystrophy due to a requirement for O-linked mannose glycans on the Dystroglycan (Dg) protein. In this study we examine larval body wall muscles of *Drosophila* mutant for *Dg*, or RNA interference knockdown for *Dg* and find defects in muscle attachment, altered muscle contraction, and a change in muscle membrane resistance. To determine if POMTs are required for *Dg* function in *Drosophila*, we examine larvae mutant for genes encoding POMT1 or POMT2. Larvae mutant for either POMT, or doubly mutant for both, show muscle attachment and muscle contraction phenotypes identical to those associated with reduced *Dg* function, consistent with a requirement for O-linked mannose on *Drosophila* *Dg*. Together these data establish a central role for *Dg* in maintaining integrity in *Drosophila* larval muscles and demonstrate the importance of glycosylation to *Dg* function in *Drosophila*. This study opens the possibility of using *Drosophila* to investigate muscular dystrophy.

INTRODUCTION

Dystroglycan (Dg) is an extracellular matrix (ECM) receptor for a variety of ligands and it plays important roles in epithelial polarity, muscle viability, neural migration, and cancer progression (Winder, 2001). In skeletal muscle, as part of the dystrophin glycoprotein complex (DGC), *Dg* spans the sarcolemma and provides a link between the ECM and the cytoskeleton. Disruption of this complex leads to muscle dystrophy (Blake *et al.*, 2002; Deconinck and Dan, 2007). *Dg* protein is heavily glycosylated in its extracellular mucin-like domain with a variety of glycans, including O-linked mannose and mucin-type O-GalNAc structures (Ervasti and Campbell, 1993; Michele and Campbell, 2003; Combs and Ervasti, 2005). Mutations in glycosyltransferases that are involved in the posttranslational modification of *Dg* are likewise associated with muscular dystrophy and neural migration defects (Muntoni *et al.*, 2004; Kanagawa and Toda, 2006). These phenotypes seem to be directly due to the impact of these mutations on *Dg* and reflect the importance of glycosylation and posttranslational processing to *Dg* function.

The O-mannose glycans present on *Dg* have received particular attention because these play a role in ligand binding. In particular, *Dg* lacking O-mannose glycans show reduced binding to laminin (Lam; Chiba *et al.*, 1997; Michele *et al.*, 2002; Michele and Campbell, 2003; Muntoni *et al.*, 2004). The initial step in generating these glycans is the addition of mannose in an O-linkage onto serine or threonine residues of *Dg*. Biochemical data indicates that this activity is carried out by two proteins, POMT1 and POMT2, which together

form a biochemically active complex. Increased protein O-mannosylation in human cultured cells is only observed when both enzymes are transfected together (Manya *et al.*, 2004), and a further study demonstrated physical association between the two enzymes (Akasaka-Manya *et al.*, 2006). Genetic data demonstrates a requirement for both POMT1 and POMT2, as mutation of either protein results in muscular dystrophy and associated neural defects in humans and other mammals (Akasaka-Manya *et al.*, 2004; van Reeuwijk *et al.*, 2005).

Genetic studies in model invertebrates, where genes are often present in single copy and genetic studies are less complicated by redundancy, have proven useful for furthering our understanding of cellular mechanisms involved in complex disorders. The main components of the mammalian DGC are conserved in *Drosophila* (Greener and Roberts, 2000; Neuman *et al.*, 2001). Studies have identified a roles for *Dg* in epithelial and oocyte polarity (Deng *et al.*, 2003; Poulton and Deng, 2006; Schneider *et al.*, 2006) and recently age-dependent changes in adult muscles with reduced *Dg* levels have been identified (Shcherbata *et al.*, 2007). Interestingly, several *Drosophila* *Dg* isoforms are generated via alternative splicing (Deng *et al.*, 2003; Schneider *et al.*, 2006). Only one of these contains the full mucin-like domain, and it appears to be subjected to significant levels of glycosylation (Schneider *et al.*, 2006). *Dg* isoforms that lack the mucin-like domain are required to maintain polarity in the follicular epithelium (Schneider *et al.*, 2006), suggesting that the *Dg* isoforms can have different functional roles in *Drosophila*. POMTs are also found in *Drosophila* (Martin-Blanco and Garcia-Bellido, 1996; Ichimiya *et al.*, 2004; Lyalin *et al.*, 2006) and biochemical analysis of *Drosophila* POMT1, encoded by the gene *rotated abdomen* (*rt*; Martin-Blanco and Garcia-Bellido, 1996) and POMT2, encoded by *twisted* (*tw*; Lyalin *et al.*, 2006) has demonstrate that these enzymes possess POMT activity similar to their vertebrate homologues (Ichimiya *et al.*, 2004). When transfected together in insect cells they are able to

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transfer mannose to a GST- α -Dg substrate generating O-linked mannose (Ichimiya *et al.*, 2004). In addition, reducing the level of either enzyme *in vivo* by RNA interference (RNAi) resulted in reduced POMT activity of larval extracts toward the Dg substrate (Ichimiya *et al.*, 2004). Mutation of the *Drosophila* enzymes or reducing their function using RNAi results in rotation of adult abdominal muscles, suggesting the enzymes have some role in muscle function or development (Martin-Blanco and Garcia-Bellido, 1996; Ichimiya *et al.*, 2004; Lyalin *et al.*, 2006). However, it is unclear if these phenotypes are related to a role for the enzymes in Dg modification and function because such a phenotype has not been described in Dg mutants.

In this study we examined *Drosophila* larval body wall muscles and identified errors in muscle attachment, changes in muscle contraction, and reduced cellular integrity associated with Dg mutant alleles and RNAi-mediated reduction of Dg. We also find similar defects in larvae mutant for *rt* or *tw* or double mutant for both and show genetic interactions between *rt*, *tw*, and Dg, suggesting that the glycosyltransferases function in the same molecular pathway as Dg. These data are consistent with a nonredundant requirement for POMT enzymes and the O-linked mannose glycans they generate, for proper Dg function in *Drosophila*.

MATERIALS AND METHODS

Drosophila Stocks and Crosses

Dg²⁴⁸ and Dg³²³ are previously described P-element excision alleles: Dg²⁴⁸ results in reduced Dg expression, whereas Dg³²³ is null (Deng *et al.*, 2003; Shcherbata *et al.*, 2007). UAS-Dg-i, UAS-Dg-C, and UAS-Dg-cyt have been previously described (Deng *et al.*, 2003). *rt*² and *rt*^P are semilethal P-element insertion alleles as described by Martin-Blanco and Garcia-Bellido (1996). *tw*¹ is viable and carries an amino acid substitution in the conserved POM domain (Lyalin *et al.*, 2006). Lethal and semilethal alleles were balanced over green fluorescent (GFP)-marked balancers; absence of GFP was used to select homozygous mutant larvae. Double mutant stocks were also generated with GFP-marked balancers (*tw*¹/FM7 GFP; *rt*²/TM3 GFP, *tw*¹/FM7 GFP; *rt*^P/TM3 GFP, *tw*¹/FM7 GFP; Dg³²³/CyO GFP, Dg³²³/CyO GFP; *rt*²/TM3 GFP). The following Gal4 drivers used: 24B-Gal4, 69B-Gal4, and P-*tub*-Gal4. All crosses were maintained at 25°C. For 30°C experiments crosses were set up at 25°C, adults were allowed to lay eggs for 2 d, and vials were then transferred to 30°C.

Muscle Measurements

Larvae were dissected, fixed, and stained with phalloidin; muscles were imaged, and measurements obtained as described previously. All muscle measurements were from muscles 6/7 abdominal segments 3 or 4. Sarcomere measurements were from the central region of muscle 6 abdominal segments 3 or 4. No more than two muscles were measured from a single larvae. Sarcomeres were measured from gray scale intensity plots across phalloidin-stained sarcomeres (see Figures 2 and 3; Haines and Stewart, 2007), sarcomere size being the distance between adjacent peaks. In cases where the graph did not clearly show a single peak for a sarcomere, the center of the high-intensity region was selected by eye as the peak value.

Surface area of the 3rd instar larval muscles 6/7 from which sarcomere measurements were taken was as follows (μm^2 mean $\pm$ SEM, *n* = number muscles): in Figure 3B, 25°C *yw*: 76334 $\pm$ 2191, *n* = 22; UAS-Dg-i/+ : 76674 $\pm$ 2281, *n* = 21; 24B-Gal4/+ : 74794 $\pm$ 1579, *n* = 22; P-*tub*-Gal4/+ : 74398 $\pm$ 3260, *n* = 18; 24B-Gal4:UAS-Dg-i: 72544 $\pm$ 2006, *n* = 22; P-*tub*-Gal4:UAS-Dg-i: 71404 $\pm$ 2677, *n* = 14; 69B-Gal4:UAS-Dg-i: 79663 $\pm$ 2124, *n* = 12; in Figure 4B: 30°C *yw*: 71010 $\pm$ 2149, 24: UAS-Dg-i/+ : 77368 $\pm$ 1554, *n* = 20; 24B-Gal4/+ : 80932 $\pm$ 1478, *n* = 16; 24B-Gal4:UAS-Dg-i: 76610 $\pm$ 1973, *n* = 26; and in Figure 7F: *tw*¹: 77698 $\pm$ 1789, *n* = 50; *rt*² 64747 $\pm$ 1926, *n* = 19; *rt*²/*rt*^P: 70809 $\pm$ 3759, *n* = 22; *tw*¹/*rt*²: 60834 $\pm$ 1884, *n* = 29; *tw*¹/*rt*²/*rt*^P: 62877 $\pm$ 1725, *n* = 17.

Antibody Staining

Third instar larvae were dissected along the dorsal midline in zero-calcium HL3 saline (Stewart and McLean, 2004). On removal of the digestive tract, fat bodies, and main trachea, the preparations were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) for 10 min. The tissue was then washed in PBS, 0.1% triton, 0.5% bovine serum albumin (PBT), and stained in PBT + 5% normal goat serum, overnight at 4°C or for anti-Dlg and anti-horseradish peroxidase (HRP) at room temperature for 1 h. The following antibodies were used: rabbit anti-Dg (1:800; Deng *et al.*, 2003), guinea pig anti-Lam (1:1000; gift

T. Volk), rabbit anti-Dys (1:1000; Schneider *et al.*, 2006), mouse anti-Dlg (1:200; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), Texas Red goat anti-HRP (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:100, and rat anti- α -actinin (Babraham Bioscience Technologies, Cambridge, United Kingdom) at 1:10. Secondary antibodies were made in goat and purchased from Molecular Probes (Eugene, OR); anti-rabbit Alexafluor488, anti-guinea pig Alexafluor633, anti-mouse Alexafluor633, and anti-rat Alexafluor488. Antibody staining was, where appropriate, followed by lectin (see below) or phalloidin staining. Tetramethylrhodamine isothiocyanate-Phalloidin (Sigma, St. Louis, MO) or fluorescein isothiocyanate-phalloidin (Sigma) was used at 1 $\mu\text{g}/\text{ml}$ for 30 min.

Stained preparations were mounted in 90% glycerol in PBS, or where indicated Vectashield with propidium iodide (Vector Laboratories, Burlingame, CA). Images of antibody stained muscle sarcomeres were collected on a Zeiss LSM 510 confocal microscope (Thornwood, NY) using F-Fluor 40 $\times$ /1.3 oil lens with 2 $\times$ zoom. The pinhole was set to 70 μm , with a resulting confocal section thickness of 0.6 μm . Confocal images were exported to imageJ (NIH). Low-magnification images of whole muscles stained with antibodies or with phalloidin were taken with a 10 $\times$ lens on a Nikon E600FN microscope (Melville, NY) equipped with a Hamamatsu Orca ER CCD camera (Bridgeview, NJ). Images then were obtained from the camera with SimplePCI (Compix, Cranberry Township, PA) software and exported to imageJ. Where comparisons were made (Figures 4C, 5a, 6, a and b, and 6, c and d) samples were stained in the same tube, mounted together on a slide and imaged with the same settings.

Lectin Staining

Larvae were dissected, fixed and washed as for antibody staining and incubated with 4 $\mu\text{g}/\text{ml}$ lectin in PBT for 30 min at room temperature. Lectins used were as follows: fluorescein-*Vicia villosa* agglutinin (VVA), rhodamine-*Sophora japonica* agglutinin (SJA), fluorescein-*Griffonia simplicifolia* lectin II (GSL-II), rhodamine-wheat germ agglutinin (WGA), and fluorescein-*Sambucus nigra* lectin (SNL), all from Vector Laboratories.

Western Blot

Six 3rd instar larvae of each genotype were dissected along the dorsal midline in zero calcium HL3 saline. Digestive tract, fat bodies, main trachea, and CNS were removed. Tissue was transferred to 12 μl of buffer containing 50 mM Tris pH 7.4, and 1% Triton with complete proteases inhibitors (Roche, Indianapolis, IN). The sample was homogenized with a microtissue grinder and incubated at 4°C for 20 min. Twelve microliters of 2 $\times$ SDS loading buffer + dithiothreitol was added, and the samples were boiled for 5 min. Fifteen microliters of each sample was separated on a 6% SDS-polyacrylamide gel and Western-transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with rabbit anti-Dg (1:3000) and detected with an HRP-coupled secondary antibody, followed by chemiluminescent detection (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). Four microliters of each sample was loaded on 10% SDS-polyacrylamide gel, blotted, and incubated with β -tubulin (1:100 MabE7, Developmental Studies Hybridoma Bank) for loading control.

Physiology

Physiology assays were carried out in HL3 saline (Stewart *et al.*, 1994) with 0.75 mM calcium, as previously described (Stewart and McLean, 2004). Excitatory junctional potentials (EJPs) were analyzed using the cursor options of Clampfit 10.0 (Molecular Devices, Sunnyvale, CA), and miniature EJPs (mEJPs) were analyzed with the template search option. Sample size was 7 for each genotype.

Statistics

One-way ANOVA tests were used for statistical comparisons except where indicated. Tests were carried with Graphpad 4.0 (Prism Software, San Diego, CA), with *p* < 0.05 chosen as the level of significance.

RESULTS

Muscle Attachment Defects in Dystroglycan Mutants

To determine if Dg plays a role in *Drosophila* muscles, we examined the musculature of larvae carrying mutant alleles of Dg. Dg alleles have previously been generated and described (Deng *et al.*, 2003; Shcherbata *et al.*, 2007); we examined larvae homozygous for a null allele, Dg³²³, a hypomorph, Dg²⁴⁸, and Dg³²³/Dg²⁴⁸ larvae. These Dg mutants survive until early 3rd instar; we dissected late second instar larvae to reveal the neuromuscular system and stained it with fluorescently labeled phalloidin to visualize the muscles. In wild-type larvae, muscles are arranged in a stereo-

Figure 1. Dg mutants have defects in muscle attachment. Phalloidin-stained musculature of 2nd instar larvae. (A) *yw*, wild-type arrangement of muscles in part of a hemisegment, and muscles referred to in text are labeled by number according to standard nomenclature. (B–F) Examples of muscle attachment defects in Dg mutants. (B) Dg^{248} , muscle 5 normally located between the arrowheads, is missing. (C) Dg^{323}/Dg^{248} , muscle 5 is underneath, rather than above muscle 4, as indicated by arrowhead. Extra muscle tissue is also present (arrow). (D) Dg^{248} , muscles 6 and 7 cross over and swap their normal attachment sites. Arrowhead on the left side of the panel indicates a normal attachment site for muscle 6, and the muscle at this site is attachment to the normal muscle 7 location on the right-hand side of the panel (arrowhead on right). The muscle at the normal muscle 7 attachment site on the left (arrow) crosses under the other muscle to attach on the right-hand side, as indicated by the arrow. (E) Dg^{323} , muscle 5 attachment site is shifted slightly from its normal position indicated by arrow. (F) Dg^{323}/Dg^{248} , muscle 6 is absent, as indicated by arrowheads. All images are shown to scale. Scale bar, (A) 50 μ m.

typical pattern and are attached to the underlying epidermis via a link with tendon cells at defined locations (Bate, 1990; Prokop *et al.*, 1998; Volk, 1999; Figure 1A). In abdominal segments 2–7, 30 individual muscles are present in each hemisegment. We found that in Dg^{323} , Dg^{248} , or Dg^{323}/Dg^{248} larvae one or more muscles were frequently missing or mis-attached (Figure 1, B–F, and Table 1). In some larvae a single muscle from one hemisegment was affected, whereas in other cases more than one hemisegment was affected. No consistent pattern as to which hemisegment or muscle was affected could be determined. Occasionally additional muscle tissue was present (Figure 1C). Some examples of muscle mis-attachment occurred in hemisegments that lacked a muscle and may result from rearrangement of the remaining muscles. In other examples, mis-attachment was observed despite a normal number of muscles in the hemisegment. Together these results demonstrate that Dg plays a role in normal muscle attachment in *Drosophila*.

Dystroglycan Mutant Muscles Have Altered Sarcomere Size

In the course of examining the muscle attachment phenotypes we also noticed that some muscles appeared to be shorter and wider than normal (see below). To examine the underlying muscle sarcomeres we stained the muscles with phalloidin which labels the F-actin present in the muscle contractile machinery resulting in broad stripes along the

muscles (Figure 2). The center of each F-actin band, the Z-band, stains more intensely with phalloidin. The spacing of the F-actin bands can be analyzed by graphing gray scale intensity across phalloidin-stained sarcomeres, and Z-bands are represented as peaks. The distance between peaks can be used as a measure of sarcomere size, and the width of the curve can be used to assess the width of the F-actin band (Figure 2; Haines and Stewart, 2007). We examined the spacing of the F-actin bands along muscle 6, segments 3 or 4; when the banding pattern across the entire length of the muscle was intact (*yw*, $n = 8$; Dg^{248} , $n = 8$; Dg^{323}/Dg^{248} , $n = 8$; Dg^{323} , $n = 6$).

First, we found that *yw* larvae showed very consistent sarcomere sizes of $\sim 8 \mu$ m ($8.01 \pm 0.07 \mu$ m, $n = 40$ sarcomeres from eight muscle; Figure 2D). In contrast, Dg^{248} mutants the F-actin bands appeared closer together than in controls; on average Dg^{248} sarcomeres were $7.3 \pm 0.07 \mu$ m ($n = 53$ sarcomeres from eight muscles), but the width of the F-actin band was unaffected (Figure 2, B and B'). This could suggest the muscles are hypercontracted. We would expect hypercontracted muscles to be wider and shorter than controls, and so we measured the width and length of the muscles, together with overall muscle surface area. The width of muscle 6/7 combined from Dg^{248} mutants was larger than controls, whereas muscle length was smaller (width $\pm$ SEM in μ m; length $\pm$ SEM in μ m; $n =$ no. of muscles; *yw*: 61.3 ± 1.2 , 195.2 ± 6.3 , $n = 41$; Dg^{248} : 66.8 ± 1.6 , 166.8 ± 2.6 , $n = 27$). This resulted in a significantly different width/length ratios for the Dg^{248} mutants compared with controls ($p < 0.001$; width/length $\pm$ SEM; *yw*: 0.33 ± 0.011 , $n = 41$; Dg^{248} : 0.41 ± 0.014 , $n = 27$). No significant difference in surface area was found ($p > 0.05$; area $\pm$ SEM μ m², *yw*: 12844 ± 577 , $n = 41$; Dg^{248} : 11566 ± 239 , $n = 27$). This change in muscle shape, but no change in surface area is consistent with the Dg^{248} muscles being hypercontracted.

Further examination of the Dg^{248} data revealed that, along with the reduction in the mean value, the sarcomere measurements were more variable. For example, the coefficient of variation (SD/mean) was 5.6% for *yw* samples compared with 7.8% for Dg^{248} samples. This greater variability is also evident from the scatter plot shown in Figure 2D.

We next examined stronger Dg combinations, including Dg^{323}/Dg^{248} and Dg^{323} mutants. The mean value for all the data from Dg^{323}/Dg^{248} muscles was similar to *yw*: $8.26 \pm 0.15 \mu$ m, $n = 39$ sarcomeres from eight muscles. However the data were obviously more variable than in the *yw* sample, with a coefficient of variation of 12% and 14 sarcomeres

Table 1. Frequency of muscle attachment defects in 2nd instar Dg mutants

Muscle defect	<i>yw</i>	Dg^{248}	Dg^{323}/Dg^{248}	Dg^{323}
M5 or M8				
Abnormal attachment	1	5	2	1
Missing	0	2	0	0
Split or very thin	1	1	2	1
M6, 7, 12, and 13				
Abnormal attachment	0	2	0	2
Missing	0	2	3	0
n/a				
Extra muscle tissue	0	1	0	1
Total defects	2	13	7	5
Hemisegments examined	114	137	99	56
Defects/hemisegment	0.018	0.095	0.071	0.089

Figure 2. Sarcomere size is altered in *Dg* mutants. Phalloidin-stained sarcomeres from muscle 6 (A) *yw*, (B) *Dg²⁴⁸*, and (C) *Dg³²³*. The graphs (A'–C') show gray scale intensity across the dashed line drawn on the sarcomeres in A–C. Six sarcomeres are in the 48-μm-wide region in *yw* compared with seven in the *Dg²⁴⁸* mutant and five in *Dg³²³*. Red bars indicate one sarcomere. (D) Scatter plot of sarcomere size in the different genotypes, where the horizontal line represents the mean value of the data. *Dg²⁴⁸* sarcomeres are smaller than *yw* but more variable. *Dg³²³/Dg²⁴⁸* and *Dg³²³* show a large variation in sarcomere size and larger sarcomeres compared with *yw*.

larger than the largest measured from the *yw* sample. For *Dg³²³*, the mean value for all the data was increased to $8.7 \pm$

$0.12 \mu\text{m}$, $n = 35$ sarcomeres from six muscles, and the coefficient of variation was 8.4%. In *Dg³²³* samples there were 18 sarcomeres larger than the largest *yw* sarcomere.

Analysis of variance was performed on the sarcomere size data from the *yw*, *Dg²⁴⁸*, *Dg³²³/Dg²⁴⁸*, and *Dg³²³* groups to test for differences among the mean values. This revealed significant variation between the groups ($p < 0.0001$). Post-tests revealed that only *yw* compared with *Dg³²³/Dg²⁴⁸* was not different ($p > 0.05$), whereas all other combinations were different from each other ($p < 0.01$). Furthermore, Bartlett's test for equal variance revealed a significant ($p < 0.001$) difference in the sample variability between the genotypes.

Together these results show *Dg* alleles can significantly affect sarcomere size. The weakest allele causes sarcomeres to become smaller, whereas increasing allelic strength results in progressively larger sarcomeres, but also a higher degree of variability in the mutant lines. Because *Dg³²³* is a null allele, these data indicate the strongest loss-of-function phenotype is longer sarcomeres.

RNAi Knockdown of *Dg* Alters Sarcomere Size

To overcome the lethality associated with the *Dg* mutant alleles and to investigate the requirement for *Dg* in different tissues, we turned to an RNAi approach. We expressed a UAS-coupled RNAi construct against *Dg* with a mesoderm driver (24B-Gal4), a ubiquitous driver (*P-tub*-Gal4), and a pan-ectodermal driver (69B-Gal4). Although 24B-Gal4 UAS-*Dg-i* was viable, some lethality during larval stages was observed with 69B-Gal4 and *P-tub*-Gal4; however in each case adult flies could be obtained.

We examined 3rd instar larval muscles to determine if they showed a change in sarcomere size similar to those we had observed in the *Dg* mutants. Driving expression of the UAS-*Dg-i* construct with *P-tub*-Gal4 resulted in sarcomeres that were significantly smaller than controls ($p < 0.001$; Figure 3, A and B). With 24B-Gal4 we observed a more variable phenotype. Some muscles had small sarcomeres and others were large. Generally, individual larvae showed either small or large sarcomeres in each of its muscles. We did not observe both small and large sarcomeres in a single muscle. This variable phenotype resulted in the 24B-Gal4: UAS-*Dg-i* having a similar mean sarcomere size as controls but a larger variation (Figure 3B). This increased variation resulted in a coefficient of variation value of 14.35% for 24B-Gal4:UAS-*Dg-i* compared with 7.03% for *yw* and 5.21 and 6.21% for UAS-*Dg-i*/+ and 24B-Gal4/+, respectively. Furthermore, Bartlett's test for equal variance revealed a significant ($p < 0.001$) difference in the sample variability between the genotypes.

Expressing UAS-*Dg-i* with 69B-Gal4 did not affect sarcomeres size (Figure 3B). We did not observe a difference between the overall size of the muscles across the different genotypes, as measured by surface area of muscle 6 and 7 combined ($p > 0.05$). Together these results demonstrate that loss of *Dg* in muscle results in altered sarcomere size in 3rd instar larval muscles and that both smaller and larger sarcomeres can result from reduced *Dg* levels. This is consistent with our analysis of the 2nd instar *Dg* mutant muscles where we observed examples of smaller and larger sarcomeres. In the *Dg* mutants we had found that the hypercontraction phenotype was predominant among larvae homozygous for the *Dg* hypomorphic allele *Dg²⁴⁸*, whereas wider, more relaxed sarcomeres were more common in *Dg³²³/Dg²⁴⁸* or homozygous null *Dg³²³* individuals. This suggests that more severe loss of *Dg* may tend to result in larger sarcomeres, and weaker *Dg* knockdown could result in hypercontraction.

Figure 3. Sarcomere size changes associated with *Dg* RNAi knockdown. (A) Graph shows gray scale intensity of phalloidin-stained sarcomeres shown below. Sarcomeres are aligned on the left of the graph but rapidly become out of phase with each other because the *tub-Gal4::UAS-Dg-i* sarcomeres are smaller than *yw* controls. (B) Scatter plot displays sarcomeres size across the different genotypes, where the horizontal line represents the mean value of the data. Sarcomere size was measured as the peak-to-peak distance from graphs as shown in A. Note the increased variance in the *24B-Gal4::UAS-Dg-i* sample compared with controls and the decrease in size of the *tub-Gal4::UAS-Dg-i* sarcomeres. $n > 100$ sarcomeres of each genotype.

To test this idea we took advantage of the temperature dependency of the Gal4 system and enabled the *24B-Gal4::UAS-Dg-i* larvae to develop at 30°C, rather than at 25°C as in our experiments described above. This resulted in a stronger decrease of *Dg* protein on Western blots compared with *24B-Gal4::UAS-Dg-i* larvae raised at 25°C (Figure 4A). Although the *24B-Gal4::UAS-Dg-i* larvae raised at 25°C had shown a high degree of variance among sarcomeres the variance for the *24B-Gal4::UAS-Dg-i*, sarcomeres at 30°C were similar to controls (Figure 4B). The coefficient of variation value was 8.6% for *24B-Gal4::UAS-Dg-i* raised at 30°C compared with 8.0% for *yw*, 6.5% for *24B-Gal4/+*, and 5.2% for *UAS-Dg-i/+* when raised at 30°C. Although no significant change in variation between the samples was observed, the mean sarcomere size was significantly larger for the *24B-Gal4::UAS-Dg-i* compared with the control groups ($p < 0.001$; Figure 4B). Together these results indicate that strong *24B-Gal4*-driven *Dg* RNAi knockdown results in sarcomeres that are consistently larger than controls (Figure 4B) and together with our results from RNAi knockdown experiments at 25°C suggests that the larger sarcomere phenotype is the result of a more severe loss of *Dg* compared with the variable or hypercontracted phenotype.

As with the smaller sarcomeres, we did not observe a difference in the width of the phalloidin-stained band (Fig-

Figure 4. Strong knockdown of *Dg* in muscles results in large sarcomeres. (A) Western blot with *Dg* antibody detects several bands (marked with arrowheads) in *yw* (lane 1) larvae muscle preparation; these bands are reduced in *24B-Gal4::UAS-Dg-i* at 25°C (lane 2) and are further reduced when these larvae develop at 30°C (lane 3). Bottom panel, β -tubulin loading control. (B) Sarcomere size of controls and *24B-Gal4::UAS-Dg-i* all at 30°C. *24B-Gal4::UAS-Dg-i* 30°C are consistently larger than controls. $N > 100$ sarcomeres each genotype. $*p < 0.001$. (C) α -actinin (green)- and phalloidin (red)-stained sarcomeres. The pattern of c-actinin and F-actin are normal despite the larger sarcomeres in the *24B-Gal4::UAS-Dg-i* 30°C muscles.

ure 4C). To further examine the *24B-Gal4::UAS-Dg-i* 30°C muscles, we used an antibody against the Z-band marker α -actinin and found that this protein was localized normally. The apparently normal arrangement of F-actin and the Z-band marker in the *24B-Gal4::UAS-Dg-i* muscles suggests that despite the changes in sarcomere size the contractile machinery remains intact. Further the *Dg* RNAi knockdown and *Dg* mutant larvae appear to crawl normally, suggesting that the ability of the muscles to contract is not significantly compromised. We conclude the simplest explanation for the change in sarcomere size that we observe is altered muscle contraction.

The 3rd instar *UAS-Dg-i* larvae were also examined for muscle attachment defects; however, driving expression with *24B-Gal4*, *69B-Gal4*, or *P-tub-Gal4* did not result in a significant number of attachment defects when compared with the Gal4 drivers alone. This suggests that the temporal or spatial knockdown of *Dg* achieved with these drivers is insufficient to disrupt this aspect of *Dg* function.

Electrical Properties of *Dg* RNAi Muscles

In vertebrate muscles lacking components of the DGC membrane, fragility leading to microlesions in the membrane have been suggested as early events that begin a cycle of muscle damage (Deconinck and Dan, 2007). We used intracellular recording techniques to measure membrane resistance, membrane potential, and both spontaneous mEJPs and nerve-stimulated EJPs synaptic release to determine if the muscle membrane function or synaptic function was compromised in the *24B-Gal4::UAS-Dg-i* muscles. We found

Table 2. Membrane resistance is increased in *Dg* RNAi knockdown muscles

	25°C		30°C	
	<i>Dg-i/+</i>	24B-Gal4:: <i>Dg-i</i>	<i>Dg-i/+</i>	24B-Gal4:: <i>Dg-i</i>
Membrane potential (mV)	-69 ± 2.5	-67 ± 1.1	-65 ± 2.0	-67 ± 1.4
Membrane resistance (mΩ)	5.8 ± 0.45	8.3 ± 0.61 ^a	3.7 ± 0.89	5.8 ± 0.37 ^a
mEJP frequency (Hz)	2.62 ± 0.36	2.26 ± 0.42	1.13 ± 0.14	0.70 ± 0.05
mEJP amplitude (mV)	0.89 ± 0.05	1.1 ± 0.11	0.73 ± 0.10	0.85 ± 0.03
EJP amplitude (mV)	34.42 ± 4.2	44.41 ± 2.5 ^a	20.29 ± 2.3	32.86 ± 1.4 ^a
Quantal content	44.32 ± 5.7	46.46 ± 2.7	30.76 ± 5.1	44.11 ± 2.9

Each parameter was analyzed by two-way ANOVA (genotype × temperature); ^a *p* < 0.05; two-way ANOVA.

that the resting membrane potential was similar for all genotypes (Table 2), but that there was a significant difference in membrane resistance between the controls (*UAS-Dg-i/+*) and 24B-Gal4::*UAS-Dg-i* larvae that were raised at either 25°C and at 30°C (*p* < 0.05 for both comparison; Table 2). However, rather than decreased membrane resistance as we expected if membrane were more permeable because of lesions, we found a significantly higher resistance in 24B-Gal4::*UAS-Dg-i* muscles. In line with the increased membrane resistance, the EJP amplitude was also significantly increased. No change in mEJP amplitude was detected, although the mean value was increased in 24B-Gal4::*UAS-Dg-i* muscles at both temperatures. Together these results indicate a change in membrane function in the 24B-Gal4::*UAS-Dg-i* muscles compared with controls.

Dystroglycan Is Associated with the Transverse-Tubule Network in Larval Muscles

Dg expression has previously been reported in muscle fibers in *Drosophila* embryos by in situ hybridization (Dekkers *et al.*, 2004); however, its subcellular location has not been reported. Because we have identified phenotypes when *Dg* levels are reduced in muscles with RNAi, we sought to establish the subcellular localization of *Dg* in the *Drosophila* larval muscles using an anti-*Dg* antibody (Deng *et al.*, 2003). *Drosophila* *Dg* seems not to be cleaved into α and β subunits but remains as a single polypeptide (Deng *et al.*, 2003), and this antibody recognizes an epitope in the intracellular domain (Deng *et al.*, 2003). *Dg* staining was observed in puncta on the top of the muscle cells (Figure 5, A and B). In the internal area of the muscles *Dg* staining was present in repeating stripes through the sarcomeres (Figure 5D). Given the transmembrane domain in *Dg*, we reasoned that this

could represent *Dg* at the transverse (T)-tubule membrane. These invaginations of the plasma membrane penetrate deep into the muscle and are involved in excitation contraction coupling. In vertebrate cardiac muscle *Dg*, its associated protein Dystrophin (*Dys*), and *Lam* are found in regions where T-tubules are located (Klietsch *et al.*, 1993; Kaprielian *et al.*, 2000), and the staining pattern of *Dys* in *Drosophila* larval muscles has been suggested to be T-tubule associated (van der Plas *et al.*, 2006). We stained muscles with an antibody against the known T-tubules marker Discs Large (*Dlg*; Razzaq *et al.*, 2001) and found that the *Dlg* and *Dg* signals are localized in a similar pattern across the sarcomeres (Figure 5, C–F). The similar pattern but the lack of specific colocalization suggests that these proteins are localized to different regions of the T-tubule network.

N-terminal Dystroglycan Extends into the T-tubule Lumen

If *Dg* is associated with the T-tubule membrane, we would expect that its N-terminal domain would extend into the T-tubule lumen, allowing *Dg* to interact with extracellular ligands. As vertebrate α -*Dg* is heavily glycosylated in its mucin domain we investigated the possibility that lectins could be used to specifically recognize the extracellular region of *Dg* in *Drosophila* muscles via glycan structures. Therefore, we first needed to validate potential lectins as tools to probe *Dg* localization.

To identify potential *Dg* binding lectins we overexpressed a *UAS-Dg* (Deng *et al.*, 2003) construct using 24B-Gal4; this full-length construct encodes the *Dg-C* isoform that contains the entire mucin domain (Deng *et al.*, 2003; Schneider *et al.*, 2006). In this overexpression situation, anti-*Dg* antibody reveals high levels of *Dg* present in the sarcomeres and strong accumulation at the postsynaptic

Figure 5. Antibody against *Dg* reveals staining through the sarcomeres in larval muscles. (A) Antibody against the C-terminal *Dg* epitope reveals staining in larval muscles. Strong *Dg* puncta are present on the muscle, and staining is present at the sarcomeres. Staining is reduced in 24B-Gal4::*UAS-Dg-i* muscles. (B) An example image of *Dg* puncta (green) on the muscle surface, the muscle nuclei are stained in red with PI. (C–F) Z-stack of seven 0.6- μ m sections through sarcomeres. T-tubules marked with (C) *Dlg* (blue), (D) *Dg* (green), and E is merge of panels C and D. (F) Overlap showing location of F-actin bands of muscle contractile apparatus labeled with phalloidin (red).

Figure 6. Lectin that recognizes N-terminal Dg overlaps Laminin staining in the larval muscles. (A and B) Dg antibody (blue), VVA (green), and HRP (red). (A) 24B-Gal4-driven expression of a Dg-C construct leads to strong accumulation of Dg and VVA at postsynaptic regions. (B) 24B-Gal4-driven expression of a truncated Dg, which lacks the extracellular domain; Dg antibody reveals strong accumulation of Dg, but VVA staining is not detected. (C and D) Images are single confocal sections; VVA (green), anti-Lam (blue), and F-actin (red). (C) *yw* muscles Lam and VVA overlap at the sarcomeres. (D) 24B-Gal4::UAS-Dg-i 30°C muscle Lam staining appears normal, but VVA staining is strongly reduced compared with C.

side of the neuromuscular junction (Figure 6A). Therefore, lectins that bind Dg should also show ectopic postsynaptic staining in the UAS-Dg muscles. No change in staining pattern was observed with WGA, SJA, or GSL-II (data not shown); however, background staining may interfere with our ability to detect an increase in staining at the neuromuscular junction with these lectins. SNL, which has specificity toward sialic acid, showed some increase in postsynaptic staining, but *Vicia villosa* Agglutinin (VVA), which binds *N*-acetylgalactosamine-terminated glycoconjugates, showed a dramatic accumulation at postsynaptic sites in 24B-Gal4::UAS-Dg-C muscles, but no significant postsynaptic staining in *yw* controls (Figure 6A and data not shown; compare to Figure 6B).

To confirm that VVA was binding to the N-terminal region of Dg, we expressed a truncated Dg construct that lacks the entire extracellular domain, UAS-Dg-cyt (Deng *et al.*, 2003). The Dg antibody recognized this truncated protein, and the antibody showed strong staining in the sarcomeres and in postsynaptic regions, similar to overexpression of the full-length construct (Figure 6B); however, no VVA staining was detected at postsynaptic regions in these muscles. These results demonstrate that VVA can recognize the extracellular domain of Dg.

We next examined VVA staining in wild-type muscles and observed staining in broad stripes in the sarcomeres overlapping the phalloidin signal (Figure 6C). This staining is reduced in 24B-Gal4::UAS-Dg-i 30°C muscles (Figure 6D). Altogether these results demonstrate that Dg is a major

VVA-reactive protein in the larval muscles, and suggest that *Drosophila* Dg is modified with GalNAc-terminated glycans. The VVA staining pattern establishes the location of N-terminal Dg in broad stripes that appear to overlap phalloidin. This staining pattern likely represents N-terminal Dg projecting into the T-tubule lumen.

Laminin and Dystrophin are T-Tubule Associated

The N-terminal region of Dg interacts with a number of extracellular ligands and in muscles a major ligand is Lam. We sought to establish if Dg could potentially interact with Lam in *Drosophila* larval muscles. Using an antibody against Lam, we found that this protein is present in repeating stripes through the sarcomeres where it overlaps with the VVA signal (Figure 6C).

In vertebrates binding between Dg and Lam provides a link between the membrane and the extracellular matrix (Blake *et al.*, 2002); a second link between the intracellular C-terminal domain of Dg and the actin-binding protein Dys serves to anchor the complex to the cytoskeleton (Blake *et al.*, 2002). A previous study demonstrated that Dys is found in repeating stripes in the *Drosophila* larval muscles and suggested the possibility that Dys could be associated with T-tubules (van der Plas *et al.*, 2006). Altogether the location and proximity of the core DGC proteins, Dg and Dys, and the presence of Lam in the *Drosophila* muscles indicated that a T-tubule DGC-like complex is present in the larval muscles.

Mutants in POMTs Display Muscle Attachment Defects and Altered Sarcomere Size

Having established that Dg is required for normal muscle integrity and provided evidence that Dg is localized to T-tubules, along with the other known members of the DGC complex, we next examined the importance of glycosylation to Dg function. Mutations in POMT1 or POMT2 are associated with muscular dystrophy in vertebrates because of a requirement for O-linked mannose glycans on the Dg protein (Akasaka-Manya *et al.*, 2004; van Reeuwijk *et al.*, 2005). We investigated whether POMTs may be required for Dg function in *Drosophila* by examining larvae mutant for either *rt* (POMT1) or *tw* (POMT2) or doubly mutant for both. Muscle attachment defects have previously been reported in *rt* mutants (Martin-Blanco and Garcia-Bellido, 1996), and our analysis found muscle attachment errors in single *rt* and *tw* mutants and in double mutants (Figure 7, A–E). These defects occurred at a frequency similar to that seen in the Dg mutants (Table 3). Examples of muscle absence, mis-attachment, and extra or split muscle fibers were observed (Figure 7, A–E). To determine if these mutants showed changes in muscle contraction, sarcomere size was measured from 3rd instar larval muscles. *tw* and *rt* mutants and *tw;rt* double mutant sarcomeres were all larger than controls (Figure 7F) and were similar in size to the sarcomeres of 24B-Gal4::UAS-Dg-i larvae that had been raised at 30°C. Together these data are consistent with a requirement for O-linked mannose glycans on *Drosophila* Dg and support biochemical data that indicate a nonredundant role for these enzymes in modifying Dg (Ichimiya *et al.*, 2004).

Genetic Interactions between POMTs and Dystroglycan

To further establish a link between POMTs and Dg, we generated larvae that carried mutations in *tw* or *rt* in combination with Dg alleles and examined the musculature for the attachment and sarcomere size phenotypes. Both *tw* and *rt* interacted with Dg. *tw*/+; Dg²⁴⁸/+ 3rd instar larvae, showing muscle attachment defects (0.110/hemisegment, *n* = 82), but normal sized sarcomeres ($8.34 \pm 0.09 \mu\text{m}$, *n* =

Figure 7. *rt* and *tw* and double *tw;rt* mutants show defects in muscle attachment and large sarcomeres. (A) *tw*¹ larvae showing absence of muscle 5; arrowheads indicated normal location of this muscle. (B) *rt*² mutant showing absence of muscle 5 (arrowheads show normal location). (C) *rt*²/*rt*^P muscle 5 (arrowheads) is very thin. (D) *tw*¹, muscle 5 (arrowheads) is very thin and is attached at an abnormal location. Muscle 4 has split fibers (arrow). (E) *tw*¹; *rt*²/*rt*^P muscles 6 is missing (arrowheads). (F) *tw*, *rt*, and *tw;rt* double mutants all have large sarcomeres compared with *yw*. Images A–E are shown to scale. Scale bar, (A) 100 μm.

73). A strong interaction was observed between *Dg* and *rt*, the *Dg*³²³/*+*; *rt*^P/*+* combination was semilethal, and larvae that survived to 3rd instar showed muscle attachment defects (0.073/hemisegment, *n* = 82) and large sarcomeres (9.045 ± 0.07 μm, *n* = 47). These interactions support a role for the glycosyltransferases in the same molecular pathway as *Dg*.

We next examined double mutant combinations. We found that *tw*¹/*Y*; *Dg*³²³/*Dg*³²³ or *Dg*³²³/*Dg*³²³; *rt*²/*rt*² animals showed an identical phenotype to the *Dg*³²³/*Dg*³²³ single mutants. The larvae survived until early 3rd instar and *tw*¹/*Y*; *Dg*³²³/*Dg*³²³ (*n* = 4), and *Dg*³²³/*Dg*³²³; *rt*²/*rt*² (*n* = 6) larvae showed changes in sarcomere size and muscle attachment defects. Attachment defects were observed at a frequency of 0.094/hemisegment (*n* = 32) for *tw*¹/*Y*; *Dg*³²³/*Dg*³²³ and at 0.091/hemisegment (*n* = 33) for *Dg*³²³/*Dg*³²³; *rt*²/*rt*². The lack of additional phenotypes in these double mutant combinations, compared with the *Dg*³²³/*Dg*³²³ single mutants, argues that *Dg* is the major functional substrate for these glycosyltransferases in *Drosophila*.

DISCUSSION

The DGC is known to play a central role in maintaining integrity in a variety of different muscle types. In this study

we established that *Dg* is required in *Drosophila* larval muscles to maintain integrity. We have identified changes in muscle contraction associated with reduced *Dg* function. Second instar *Dg*²⁴⁸ larvae had short and wide muscles, and a decrease in sarcomere size, together indicating that the muscles are hypercontracted (Figure 2). Among larvae null for *Dg* we found that sarcomere size was more variable between individuals, with some muscles having smaller sarcomeres and others larger (Figure 2). Among 3rd instar larvae knockdown for *Dg* we also observed changes in sarcomere size. When an RNAi construct against *Dg* was driven with the ubiquitous driver *P-tub*-Gal4, we found that sarcomeres were consistently smaller than controls (Figure 3). Driving the RNAi construct with the mesoderm driver 24B-Gal4 resulted in a variable phenotype, with both small and large sarcomere being observed. This phenotype shifted to exclusively larger sarcomeres when these larvae developed at 30°C, likely due to a greater decrease of *Dg* protein (Figures 3B and 4). Because the large sarcomere phenotype was seen in null *Dg* larvae and with strong RNAi knockdown compared with smaller sarcomeres in the *Dg*²⁴⁸ individuals and with weaker RNAi knockdown of *Dg*, we conclude that the large sarcomere phenotype is related to more severe loss of *Dg* than with hypercontraction.

Table 3. Frequency of muscle attachment defects in 3rd instar *rt* and *tw* mutants

Muscle defect	<i>yw</i>	<i>tw</i> ¹	<i>rt</i> ²	<i>rt</i> ² / <i>rt</i> ^P	<i>tw</i> ¹ ; <i>rt</i> ²	<i>tw</i> ¹ ; <i>rt</i> ² / <i>rt</i> ^P
M5 or M8						
Abnormal attachment	0	5	5	0	3	2
Missing	0	5	1	1	3	1
Split or very thin	0	10	1	5	4	8
M6, 7, 12, and 13						
Abnormal attachment	0	2	0	0	1	0
Missing	1	2	0	0	3	2
n/a						
Extra muscle tissue	2	4	1	3	3	4
Total defects	3	28	8	9	17	17
Hemisegments examined	129	219	60	82	128	147
Defects/hemisegment	0.016	0.128	0.133	0.110	0.132	0.116

Because we observed changes in muscle sarcomere size with RNAi driven with the ubiquitous driver and the mesoderm driver but not with the pan-ectodermal driver, we conclude that the sarcomere size changes are due to loss of Dg in muscles. Together our results demonstrate that reduced levels of Dg alter muscle contraction and that Dg plays a role in *Drosophila* muscles.

Consistent with our findings is that both hypercontraction and overstretching of sarcomeres is seen in vertebrate dystrophic muscles before they progress to a more advanced degeneration phenotype (Cullen and Fulthorpe, 1975). A tendency to hypercontract is also associated with disruption of DGC proteins in *Caenorhabditis elegans* (Bessou *et al.*, 1998; Grisoni *et al.*, 2002). These findings suggest that contractile changes are a common early response to lack of Dg function in different types of muscle. Further studies will focus on understanding whether these changes in muscle contraction stem from developmental changes in the muscles or are the result of early changes associated with muscle degeneration.

In vertebrate muscles lacking DGC components, the mechanisms leading to muscle dystrophy remain unclear; however, membrane fragility is likely involved (Blake *et al.*, 2002; Deconinck and Dan, 2007). Mechanical stress from muscle contraction is thought to lead to membrane microlesions and compromised muscle membrane function. Our electrophysiological analysis showed an increase in membrane resistance and greater EJP amplitude in 24B-Gal4::UAS-Dg-*i* muscles. The increase in passive membrane resistance could be due to down-regulation of the leakage channel activity by the muscle. The increased resistance would help to maintain excitation-contraction coupling. Interestingly, a similar increase in muscle membrane resistance has been reported in mice mutant for *dystrophin* (*mdx*; De Luca *et al.*, 1997).

In vertebrate cardiac muscle T-tubule-associated Dys, Dg α and Lam have been reported (Klietsch *et al.*, 1993; Kaprielian *et al.*, 2000). This location differs from the sarcolemma localized DGC found in vertebrate skeletal muscle (Blake *et al.*, 2002). The inherited muscular dystrophies are associated with dilated cardiomyopathy, and differences in the degree of dysfunction between cardiac and skeletal muscle in individuals with muscular dystrophy has lead to the suggestion that the DGC may have alternative cellular roles in these muscle types (Lapidos *et al.*, 2004; Goodwin and Muntoni, 2005). Our finding that Dg and Lam are T-tubule associated in *Drosophila* larval muscles suggests these *Drosophila* muscles may provide a good model for investigating the cellular function of T-tubule-associated DGC. Studying the role of the DGC in different types of muscle will increase our overall understanding of how this complex functions at both a molecular and cell biological level.

Lam and adhesion molecules such as integrins play important roles in muscle attachment in *Drosophila* (Brown, 1994; Yarnitzky and Volk, 1995; Prokop *et al.*, 1998; Delon and Brown, 2007). Muscles detach from the epidermis and round up or stick nonspecifically to other muscles or the ECM in *Drosophila* carrying mutant alleles of these genes (Brown, 1994; Prokop *et al.*, 1998). Given the role of Dg in ECM adhesion in other cellular contexts (Winder, 2001) and the role of Lam in muscle attachment in *Drosophila* (Prokop *et al.*, 1998), the muscle attachment phenotypes we have observed in the Dg mutants most likely result from weakening of the connection between muscle and epidermal tendon cell to which it connects or these cells and the ECM. Such a weakness could explain the random nature of the phenotypes. Failure of a muscle to maintain its connection with the tendon cell could result in loss of the muscle or

result in the muscle forming a link with another muscle or tendon resulting in a mis-attachment phenotype.

In vertebrates, Dg function is regulated by glycosylation (Michele and Campbell, 2003; Muntoni *et al.*, 2004; Barresi and Campbell, 2006). We observed changes in sarcomere size and defects in muscle attachment in *Drosophila* mutant for genes encoding POMT1 and POMT2. We also found that both these mutants interact with Dg mutant alleles in transheterozygous combinations. The similarity of the *rt* and *tw* muscle phenotypes to those associated with reduced Dg function and the interactions between mutant alleles of *rt*, *tw*, and Dg provide strong evidence that *rt* and *tw* are required for Dg-dependent processes in *Drosophila*. Given the O-mannosyltransferase activity of the *rt* and *tw* gene products toward Dg (Ichimiya *et al.*, 2004) and the genetic evidence from vertebrates that loss of POMTs results in hypoglycosylation of Dg and subsequent disruption to Dg function, these data provide compelling evidence supporting a functional requirement for O-mannose glycans on Dg in *Drosophila*. The adult abdomen in *tw* and *rt* mutant flies is rotated. We have not observed this phenotype in our experiments with Gal4-driven Dg RNAi knockdown. Possibly the abdominal rotation is due to loss of Dg function in these mutants and the lack of this phenotype in the RNAi flies is due to insufficient knockdown of Dg. It also remains possible that *rt* and *tw* have another substrate, and loss of glycan structures on this protein results in the rotated abdomen phenotype.

Altogether the phenotypes we have identified by manipulating Dg, *tw*, and *rt* demonstrate that Dg plays a central role in maintaining cell integrity in the *Drosophila* larval muscles and that glycosylation of Dg is important to its function. This report therefore opens the possibility of using genetic analysis of the highly accessible neuromuscular system available in this model organism to analyze the mechanisms by which loss of DGC function leads to muscle dystrophy.

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