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Stress and muscular dystrophy: A genetic screen for Dystroglycan and Dystrophin interactors in *Drosophila* identifies cellular stress response components

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ABSTRACT

In Drosophila, like in humans, Dystrophin Glycoprotein Complex (DGC) deficiencies cause a life span shortening disease, associated with muscle dysfunction. We performed the first in vivo genetic interaction screen in ageing dystrophic muscles and identified genes that have not been shown before to have a role in the development of muscular dystrophy and interact with dystrophin and/or dystroglycan. Mutations in many of the found interacting genes cause age-dependent morphological and heat-induced physiological defects in muscles, suggesting their importance in the tissue. Majority of them is phylogenetically conserved and implicated in human disorders, mainly tumors and myopathies. Functionally they can be divided into three main categories: proteins involved in communication between muscle and neuron, and interestingly, in mechanical and cellular stress response pathways. Our data show that stress induces muscle degeneration and accelerates age-dependent muscular dystrophy. Dystrophic muscles are already compromised; and as a consequence they are less adaptive and more sensitive to energetic stress and to changes in the ambient temperature. However, only dystroglycan, but not dystrophin deficiency causes extreme myodegeneration induced by energetic stress suggesting that dystroglycan might be a component of the low-energy pathway and act as a transducer of energetic stress in normal and dystrophic muscles.

Introduction

Stress accelerates ageing and worsens pre-existing health conditions. Different tissues in the organism respond to unfavorable conditions via various mechanisms and have different thresholds of how much stress they can resist before damage becomes irreversible. Muscles are highly sensitive as well as quite resilient to stress and the signaling pathways involved in adaptive responses in normal muscles are comprehensively described (Palomero and Jackson, 2010). Normally muscles can withstand numerous rough situations induced by mechanical stress; however, dystrophic muscle cells that are present in Muscular Dystrophy (MD) patients are easily damaged and do not properly regenerate causing muscle tissue loss. Not only mechanical stress, but also other environmental stresses may affect muscle tissue welfare. It is not clear whether dystrophic cells just have an unstable membrane that is breaking upon stress or the signaling pathways that are required for proper cell homeostasis are disrupted causing necrotic processes to overtake the normal muscle regeneration.

Muscle degeneration is a hallmark of Muscular Dystrophies, a group of genetically inherited fatal diseases that are characterized by concomitant loss of muscular strength that ultimately leads to skeletal muscle deterioration and cardiac and/or respiratory failure (Batchelor and Winder, 2006; Durbeej and Campbell, 2002; Ervasti, 2003). MDs are mostly related to deficiencies in the Dystrophin Glycoprotein Complex (DGC), a membrane-associated multiprotein complex classically consisting of dystrophin, the dystroglycans (α and β), the sarcoglycans (α, β, γ and δ), sarcospan, the syntrophins (α1, β1, β2, γ1-, γ2) and α-dystrobrevin (Durbeej and Campbell, 2002).

In vertebrates the main component of the complex, Dystrophin (Dys) is expressed in skeletal and cardiac muscles and brain and consists of four structural domains, the N-terminal actin-binding domain, the spectrin-like rod domain, the cystein-rich domain, and the C-terminus with the Dystroglycan (Dg) interacting WW domain. Dg is the transmembrane anchor of the complex; it binds to the extracellular matrix (ECM) component laminin-2 at its N-terminal end and to the cytoskeleton via Dystrophin at its C-terminal end, providing a crucial link between the extracellular matrix and cytoskeletal network (Davies and Nowak, 2006).

In muscles the DGC is best envisioned as a mechanosignaling unit that has a dual role in muscle membrane stabilization: mechanical via anchoring the ECM to the cytoskeleton and non-mechanical as a signal-transducing module involved in cross talk between the internal and external environments of the muscle cell. The binding between the two main components of the DGC, Dg and Dys is mediated by the

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proline-rich motif of the Dg cytoplasmic tail and the WW domain of Dys, and the phosphorylation of Dg might act as a molecular switch between WW or SH3, and SH2 domains during cellular adhesion in the process of out–in signaling (Moore and Winder, 2010; Yatsenko et al., 2009). When Dg interaction with laminin is prevented, apoptosis is activated and the pro-cell survival signaling, PI3K/AKT is inhibited (Langenbach and Rando, 2002). Dg is also thought to modulate the MEK/ERK pathway and *c-jun* activity (Spence et al., 2004; Zhou et al., 2007) and recently it has been found in the nucleus of different cells, suggesting unknown nuclear functions (Fuentes-Mera et al., 2006).

The DGC helps muscles to withstand the rigors of contraction (cellular deformation and shortening) that requires the specific activity of both the nervous and somatic systems, from excitation of myofibers at the neuromuscular junction (NMJ) to the ATP-regulated power-stroke of myosin. The myofiber contractile machinery must remain intimately connected with the sarcolemma and the basement membrane of the ECM, upon which muscles depend for survival and function. This interaction is arbitrated primarily by the DGC complex and integrin receptors. α7 integrin is a muscle-expressed integrin that, like Dg connects laminin to the cytoskeleton contributing to the overall integrity of the sarcolemma (Burkin et al., 2001; Cote et al., 2002). Interestingly, increased amounts of α7 integrin are found in DMD patients and *mdx* mice, indicating that enhanced α7 integrin expression is a mechanism by which muscle can compensate for the loss of dystrophin, additionally upregulation of integrin α7 in the mdx background can ameliorate some aspects of muscular dystrophy (Burkin et al., 2001; Cote et al., 2002; Liu et al., 2008). Facts that integrins can compensate in mediating cell-extracellular matrix attachment but cannot fully rescue the dystrophic phenotype suggest that the DGC has additional roles in muscles than just being a structural link between the cell cytoskeleton and laminin in the basal lamina.

The DGC also has become known as a scaffold responsible for the membrane localization of signaling proteins (Pilgram et al., 2010). For example, neuronal nitric oxide synthase (nNOS) signaling, which regulates many signaling pathways and is responsible for the direct regulation of a subset of myo-specific microRNAs, is coordinated by the DGC (Adams et al., 2008; Cacchiarelli et al., 2010). Recently various kinases, channels, and other enzymes have been shown to associate with the DGC, although only a few of these interactions have been confirmed *in vivo* (Adams et al., 2008; Pilgram et al., 2010).

Despite the vast data about the functional diversity of DGC components, the exact mechanism of how dystrophic muscle cells degenerate is still elusive. Muscle contraction induces mechanical stress leading to muscle injury; however, the specialized repair system is rapidly activated in healthy muscle, while in dystrophic muscles necrosis is triggered (Jaalouk and Lammerding, 2009). There are several potential pathogenic mechanisms implicated in the initiation of muscle decay associated with insufficiency of the DGC, including the mechanical fragility of the sarcolemma, high calcium influx, aberrant cytoskeleton rearrangements, increased energetic stress and abnormal metabolic control and inappropriate cell signalling (Constantin et al., 2006; Vercherat et al., 2009; Wallace and McNally, 2009).

To address the question on what mechanisms contribute to dystrophic muscle degeneration we used a previously established genetically tractable *Drosophila* MD model (Shcherbata et al., 2007). First we show that in *Drosophila,* similarly to human, *Dystrophin* and *Dystroglycan* are localized in striated muscles and required for muscle maintenance. Second, we carried out the first *in vivo* genetic screen in the musculature of adult flies exhibiting muscular dystrophy and found new genetic components that are involved in the DGC signaling and regulation. The novelty of this work is that modifiers that have been examined have not been implicated in prior works to have muscle function and/or interact with the DGC. Third, we established that many of these genes are required for muscle integrity and physiological response to heat-induced stress. Most of them have human homologues that have been associated with different disorders and potentially can be used as easier drug targets for muscular dystrophy treatment. Finally, we found that unfavorable factors such as high temperature and oxidative stress cause myodegeneration regardless of the genetic background; in addition, in dystrophic muscle the damage is significantly amplified in response to low temperature, energetic stress and ageing. This shows that the adaptive reactions in *Dys* and *Dg* mutants are somewhat different and suggests that the DGC is not only required for muscle homeostasis and plasticity, but also plays a role in stress-response pathways. *Dys* and *Dg* mutants had rather distinctive response to different stresses, which shows that they not only act together as components of one complex, but also might interact with different partners to ensure proper perpetuation of muscle functioning.

Materials and methods

Fly strains and genetic screen

To identify heterozygous genetic interaction in muscles, loss-of-function *DysDf*, Dg^{O86} (Christoforou et al., 2008) and Dg^{323} (Deng et al., 2003) mutant females were crossed to males carrying the mutation of interest. The progeny heterozygous for *Dys* or *Dg* and the screened allele were collected for muscle analysis. Heterozygotes, in which one allele of each recessive gene that function in unrelated pathways is mutant, would show no phenotype. However, if the genes act in the same pathway, then mutations in two steps should enhance each other and cause a phenotype. To identify dominant suppressors/enhancers of the muscle degeneration phenotype, virgin females $Dys^{N\text{-}RNAi}$*:act-Gal4* and Dg^{RNAi}*:tub-Gal4* (Kucherenko et al., 2008) that have 2.5 and 6 fold mRNA downregulation, respectively (Supplementary Tables 1, 2) were crossed to males carrying the screened mutation. Alleles used for the screen were obtained from DGRC and BDSC. All crosses were kept at 25 °C. Flies with the correct genotype were aged for three weeks at 25 °C and subsequently analyzed for muscle degeneration. The data were statistically compared using the χ^2 test and p was calculated based on the critical value.

Other alleles used in this study are: Dg^{O55} (Christoforou et al., 2008), $CG7845^{EMS\text{-}Mod4}$ (Kucherenko et al., 2008), $SP1070^{Uif\text{-}E(br)155}$ and $SP1070^{Uif\text{-}2B7}$ (Zhang and Ward, 2009), *tub-Gal4* and *MHC-Gal4* enhancer trap lines (BDRC), all RNAi lines are from VDRC. For control crosses either *OregonR* or w^{1118} flies were used.

Histology

For analysis of indirect flight muscle (IFM) morphology 10 μm paraffin-embedded sections were cut from fly thoraxes. In order to prepare *Drosophila* muscle sections, the fly bodies were immobilized in collars in the required orientation and fixed in Carnoy fixative solution (6:3:1 = Ethanol:Chloroform:Acetic acid) at 4 °C overnight. Tissue dehydration and embedding in paraffin were performed as described previously (Kucherenko et al., 2010). Histological sections were prepared using a Hyrax M25 (Zeiss) microtome and stained with hematoxylin and eosin (H&E) or aniline blue (0.12 %). All chemicals for these procedures were obtained from Sigma Aldrich. Muscle analysis was done using a light microscope (Zeiss). The frequency of muscle degeneration was quantified as a ratio of degenerated muscles to the total number of analyzed muscles. The analyzed IFM sections were located at a position 200–250 μm to the posterior of the fly thorax.

To prepare *Drosophila* muscle cryosections flies were located in collars and immediately frozen in TissueTek® O.C.T. (Sacura) at about −40 °C. Frozen muscles were sectioned on a cryo-microtom Leica CM3050S (between −15 and −18 °C) with a section thickness of 15 μm. Fixation was carried out in 4% formaldehyde (Polyscience, Inc.) for 10 min at room temperature.

Lipid droplets were detected with oil red O stain on cryosections (Kucherenko et al., 2010). After tissue fixation, slides were washed with water twice for 5 min, equilibrated in propylene glycol for 10 min and stained for 3 h in oil red O stain at room temperature. Samples were washed 2 times for 5 min in propylene glycol and 30 min in 1× PBS. Nuclei were visualized with DAPI. Samples were mounted in 30% glycerol in 1× PBS.

Immunohistochemistry

Immunostaining was performed according to the previously described procedures (Shcherbata et al., 2007). After immunostaining tissue was mounted onto slides in 70% glycerol, 3% NPG, 1xPBS and analysed using a confocal microscope (Leica TCS SP5). The following antibodies were used: rabbit anti-Dg (Deng et al., 2003) and anti-Dys (Schneider et al., 2006) 1:500, rat anti-Kettin (1:200; Babraham Institute), Alexa 568 goat anti-rabbit, Alexa 488 goat anti-rat (1:500, Molecular Probes). Nuclei were visualized with DAPI.

Analysis of muscle degeneration in response to stress

Temperature, sugar-free food (energetic stress), Paraquat-containing food (oxidative stress) and ageing were used as stress conditions. Flies age and incubation time on stress conditions for different experimental groups are shown in Supplementary Table 5.

Energetic stress

All flies were kept on a standard corn-molasses medium with yeast, agar, propionic acid and nipagin. For energetic stress experiments animals were transferred to plates containing sugar-free food (2.12% agar–agar in dH_2O with 0.2 ml of yeast paste). The control plates contained medium with 2.12% agar–agar and 2.5% sugar in an apple juice:dH_2O solution (1:3) and 0.2 ml of yeast paste (apple juice plates).

Temperature conditions

For temperature condition experiments wild type and mutant flies hatched at 25 °C, then incubated at 18 °C or 33 °C on apple juice plates. The control animals were left at 25 °C for the same period of time.

Oxidative stress

Paraquat (N,N′-dimethyl-4,4′-bipyridinium dichloride) in apple juice medium was used to catalyze the formation of superoxide radicals, a major form of reactive oxygen species (Bus and Gibson, 1984). Wild type and mutant flies were hatched in normal food at 25 °C conditions and transferred as adults to 2.5 mM Paraquat-containing medium. The control experiment used same age animals on apple juice plates.

Data analysis

The muscle degeneration was counted as the percentage of muscles with signs of degeneration from total muscle number. Experiments were repeated at least 2 times for each genotype and 50–200 muscles were scored in each experiment. The “extreme” muscle degeneration (EMD, cases where all the muscle was deteriorated or substituted with non-muscle tissue) was included in percentage of total muscle degeneration but was treated as a separate group in statistical analysis. For statistics the One-Way ANOVA with post Dunnett's tests (Version SPSS 16.0) were used. For analysis of wild type muscle response to stress conditions the data for genotypes (*OregonR* and w^{1118}) within each of “experimental conditions” group were compared to each other and after data were proven not to be different they were treated as an individual data set. Data from “25 °C and normal food” conditions group were used as control. For analysis of dystrophic muscles response to stress conditions the data for genotypes within each of “experimental conditions” group (*DysDf* and $Dg^{O86/DgO55}$) were compared to control (*OregonR*), and then each genotype from every “experimental conditions” group was compared to the respective genotype from control group (25 °C and normal food). Taking into account the age of animals used in analysis all experiments were divided into experimental groups, which were compared to different controls. In each evaluation the total muscle degeneration and EMD were compared separately.

Climbing assay

The climbing assay was performed and the climbing index was calculated as described previously (Shcherbata et al., 2007). The climbing test was performed 3 times using 20–30 7–10 day old animals each trial.

Temperature-sensitive activity

This method was adapted from Montana and Littleton (2004). Flies were placed into a preheated vial at a temperature of 39 ± 1 °C. Temperature-sensitive behavioral defects were scored in 30 s intervals. The analysis was done with at least 5 repetitions for each genotype and each repetition contained an independent set of 6–18 flies that were 2–5 days old. For the data analysis the mobility index, which equals the minus logarithm of the absolute value of the slope (the slope equals change in ordinate divided by change in abscises) was calculated. The mean value and standard error of 3–5 trials were calculated and the Student's one-tailed *t*-test was used.

RNA preparation and real-time PCR

To determine the effect of the *Dg RNAi* transgene on the *Dg* expression levels quantitative reverse transcription (RT-qPCR) was performed on total RNA derived from whole adult animals. RNAs were extracted from flies with the RNeasy Mini kit (Qiagen), followed by reverse transcription using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocols. Dg was tested with RpL32 as an endogenous control for q-PCR using Fast SYBR® Green master mix on a Step One Plus 96 well system (Applied Systems). The reactions were incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 54 °C for 30 s. All reactions were run in triplicate with appropriate blank controls. The threshold cycle (C_T) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Primers were used as follows: RpL32 forward—AAGATGACCATCCGCCCAGC; RpL32 reverse—GTCGATACCCTTGGGCTTGC; Dg forward—ACTCAAGGACGAGAAGCCGC; Dg reverse—ATGGTGGTGGCACATAATCG; Dys forward—GTTGCAGACACTGACCGACG; Dys reverse—CGAGGGCTCTATGTTGGAGC. The ΔC_T value was determined by subtracting the average RpL32 C_T value from the average Dg C_T value. The $\Delta\Delta C_T$ value was calculated by subtracting the ΔC_T of the control sample (*tubGal4*/+) from the ΔC_T of the suspect sample (Dg^{RNAi}:*tubGal4*/+). The relative amount of mRNA was then determined using the expression $2^{-\Delta\Delta CT}$. Errors were determined starting with the standard deviation of the raw C_T values and performing appropriate regression analysis.

Metabolic rates

The production of CO_2 is correlated with oxygen consumption and reflects the metabolic rate. The respirometers were 1000 µl micropipette tips with a 50 µl capillary glued to the tip end. A piece of foam was placed into the pipette to keep flies from falling through to the end of the tip. Five flies were placed into the container and another piece of foam was fitted to the top portion of the tip. Soda lime, a CO_2 absorbent (Wako Chemicals, Japan) was added to the container and the top was sealed with parafilm and then dipped into liquid paraffin to seal the container. The containers were inserted tip down into a solution of eosin for color in a closed container at 25 °C and allowed to equilibrate for 15 min. The movement of the liquid up the capillary

was monitored over the next hour and the production of CO_2 was calculated per fly (on average 1 fly weighs 0.80 ± 0.11 mg ($n = 180$)). All flies were measured at the same time and a respirometer without flies was also measured to correct for variations in ambient temperature and pressure. Three to ten independent assays were performed for each genotype in each condition. The mean value and standard error of the replicates was calculated and the Student's *t*-test was performed to check for significant differences.

Homology prediction and interaction network

All homology predictions for candidate genes were made using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and STRING version 8.2 (www.string-db.org). The network for the DGC interactors was built by integrating obtained genetic interaction results to publically available interaction data. STRING version 8.2 (www.string-db.org) was used as the interaction data resource. The DGC-interacting members were classified into functional groups based on information obtained from FlyBase (http://flybase.org/) for *Drosophila* and the function of their orthologs. The association with disorders was identified using the GeneCard Human Gene Database version 3 (http://genecards.bioinformatics.nl).

Results

Like in vertebrates, Drosophila Dystroglycan and Dystrophin are expressed in adult muscles and their deficiencies cause age-dependent muscle degeneration

Adult *Drosophila* multi-fiber muscles structurally resemble the vertebrate striated muscles with a highly conserved basic patterning consisting of actin (Z-band) and myosin (M-band) containing myofibrils (Miller, 1950). We analyzed the tissue specific expression of Dys and Dg in several types of these multinucleated muscle cells: indirect flight muscles (IFM, Figs. 1A–H), leg (Figs. 1I–L), ovarian, gut and heart muscles (Supplementary Fig. 1). Consistent with Dg being a transmembrane protein, in *Drosophila* it is localized in the muscle sarcolemma (Figs. 1A, E, I, Supplementary Fig. 1). A punctate staining is seen in the regions that coincide with Z-bands, which resemble costameric regions of myofibril connections in vertebrates (Supplementary Figs. S1G, O, T). The presence of Dg in these bands suggests that it might connect *Drosophila* myofibrils to the extracellular matrix. In *Dg* loss-of-function mutant Dg^{O55}/Dg^{O86}, Dystroglycan staining is diminished (Figs. 1C, G, K, Supplementary Fig. 1). Dys is a cytoplasmic protein that connects to Dg via its N-terminus and to cortical actin via its C-terminal end. In *Drosophila*, the Dys protein is present in the muscle cytoplasm, and transverse sections show that Dys is enriched in close proximity to the sarcolemma where Dg is located (Figs. 1B, F, J, Supplementary Fig. 1). Dystrophin staining is no longer detected in *DysDf* loss-of-function mutant (Figs. 1D, H, L, Supplementary Fig. 1). These data show the similarity of Dys and Dg localization in *Drosophila* and invertebrate adult muscles (Cote et al., 2002; Ervasti, 2003) implying that in different species these proteins may have analogous roles.

Previously it has been shown that the *Dys* hypomorph and *Dys* and *Dg RNAi* mutants exhibit age-dependant muscle weakening and loss, climbing defects and reduced lifespan (Shcherbata et al., 2007; Taghli-Lamallem et al., 2008), now we confirmed the appearance of age-dependent MD in *Dg* and *Dys* loss-of-function mutants (Supplementary Fig. 2). *Dystrophin* mutants showed plenty of mildly degenerated muscles, while the extreme muscle degeneration phenotype was frequently seen in *Dys* and *Dg* old mutant flies (Supplementary Fig. 2). Mostly, myofibrils on the periphery of the muscle were extensively damaged (Supplementary Fig. 2H), the more central parts appeared to

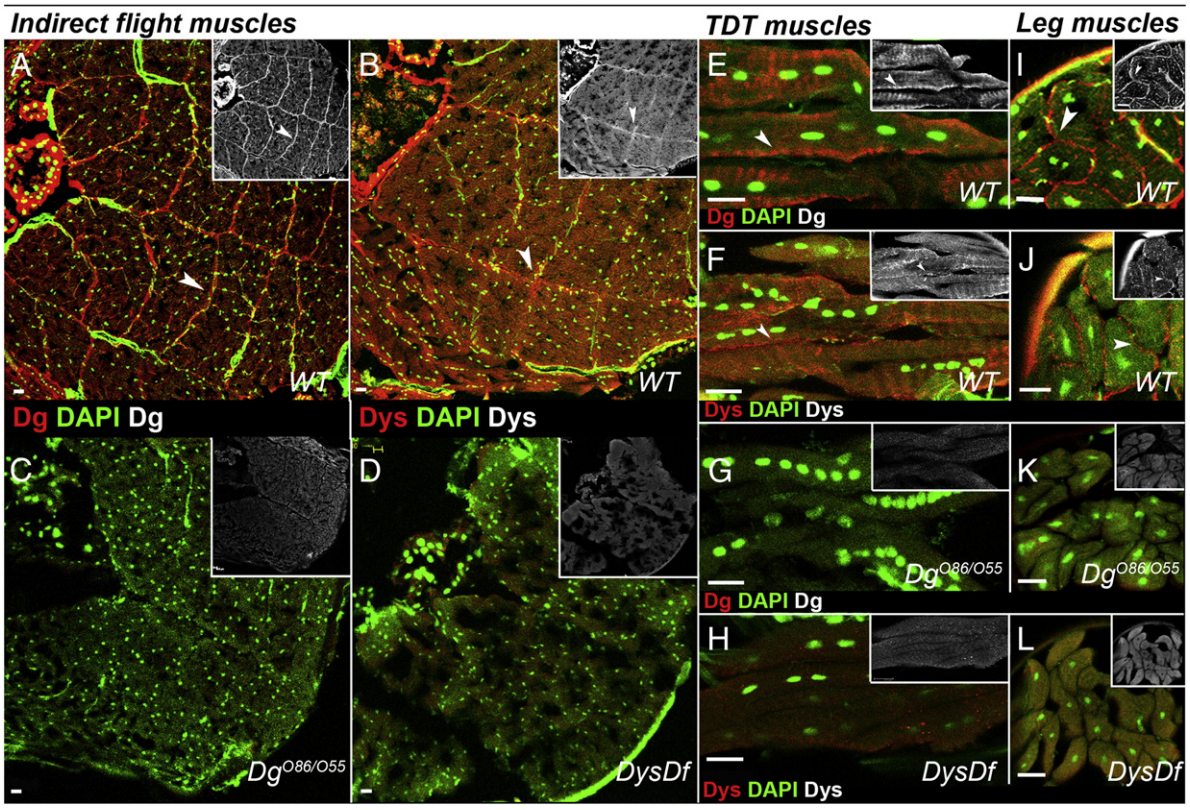

Fig. 1. *Drosophila* DCG is localized to the sarcolemma. Transverse sections of *Drosophila* IFM and leg muscles and longitudinal sections of trochanter (TDT) muscles stained with anti-Dg (A, E, I) and anti-Dys antibodies (B, F, J) (Dg, Dys—red and in separate channel, DAPI—green). Dystroglycan is localized to the muscle membrane and Dystrophin staining is seen in the cytoplasm with significant enrichment close to the membrane (indicated by arrowheads). Antibody staining is no longer seen in *Dg* (C, G, K) and *Dys* (D, H, L) mutant muscles.

be less vulnerable. Since distal myofibrils are subjected to greater levels of mechanical stress and one function of the DGC is to provide mechanical reinforcement to the sarcolemma, loss of Dys and Dg compromises sarcolemma integrity causing focal muscle deterioration. The healthy muscle cell actively regulates its metabolism, determined by substrate availability and energetic requirements. However, when muscle cell performance is jeopardized, metabolism regulation is disabled resulting in replacement of muscle by fatty and fibrous tissue. Since the prime source of energy during muscle contraction is fatty acids, we tested if *Dys* and *Dg* deficiencies lead to improper consumption of fat. We detected lipid droplets in *Dys* and *Dg* deficient muscles; however the amounts of intramuscular fat were not obviously different in control and mutant muscles (Supplementary Figs. 2L–M). Also they contained no extra quantities of collagen visualized by aniline blue staining (data not shown), instead, zones of abnormal muscle tissue showed signs of necrosis marked by pale H&E staining (Supplementary Fig. 2I).

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

The *Drosophila* MD model provides a unique possibility to screen for DGC interactors that are not necessarily biochemically linked but act transiently by testing for a trans-heterozygous interaction. Previously performed large-scale primary screens identified modulators of a wing-vein phenotype in *Dys* and *Dg* mutants (Kucherenko et al., 2008). Now we carried out the secondary screen to analyze if these modulators would specifically modify Dys/Dg age-dependent muscular dystrophy.

When *Dys* and *Dg* are reduced by one copy (*DysDf*/+, Dg^{323}/+ and Dg^{O86}/+), no obvious changes in muscle morphology are observed (Supplementary Table 3, Figs. 2A–B, F–G), therefore these mutants were used to identify trans-heterozygous interactions. Importantly, *Dys*/*Dg* trans-heterozygotes showed a genetic interaction, the occurrence of degenerated muscles significantly increased (*DysDf*/Dg^{O55} 17.7%, n = 69, *DysDf*/Dg^{O86} 19%, n = 97, *DysDf*/Dg^{323} 18%, n = 44, Figs. 2A–B, F, Supplementary Figs. 2H, J). Flies lacking one copy of the gene-candidate in the *Dys* and/or *Dg* heterozygous background were aged for three weeks and the frequency of degenerated muscles was quantified (Figs. 2A–B, F, H–J, Supplementary Tables 3 and 4). In addition, $Dys^{N\text{-}RNAi}$*:act-Gal4* and Dg^{RNAi}*:tub-Gal4* mutants showed a moderate muscle degeneration (Figs. 2C–D, Supplementary Table 3), which made them suitable for identifying dominant suppressors or enhancers (Figs. 2C–D, K–L, Supplementary Table 3). To confirm or disprove found interactions we used multiple alleles of the same gene; to avoid an additive effect control crosses to the w^{1118} line were made (Fig. 2E, Supplementary Tables 3 and 4). Some differences were noticed when different alleles were analyzed due to the diverse nature of mutations that can cause possible incongruent effects on protein structure and function, in turn causing a different pattern of interaction. In total we found 16 modifiers of *Dys* and/or *Dg*-dependent muscle degeneration (Supplementary Table 3) that have been sorted into five groups: (1) signal transduction and/or (2) cytoskeleton organization, (3) regulation of gene expression, (4) metabolism, and (5) genes with unknown function (Fig. 3A). Most interestingly many genes from these different groups could be labeled as the stress response genes that control cell adaptation to mechanical and cellular stress and factors involved in neuro-muscle communications (Fig. 3B).

We identified *Cam (Calmodulin), capt (capulet)* and *Lis-1 (Lissencephaly-1)* as Dys-interacting components (Fig. 3A). Reduction of *Cam* and *capt* by one copy showed heterozygous interaction with *DysDf* and $Dys^{N\text{-}RNAi}$*:act-Gal4* resulting in an increased frequency of muscle degeneration. Reduction of *Lis-1* also led to a heterozygous interaction with *DysDf* (~10–27%), and increased the Dg^{O86} phenotype, but since *Lis-1* heterozygotes also show some muscle degeneration, the *Dg*/*Lis-1* phenotype (~5–8%) was considered as additive (Fig. 2B, Supplementary Table 3).

Fkbp13, Pgk (Phosphoglycerate kinase), SP2353 and *vimar (visceral mesoderm armadillo repeats)* mutants showed strong interaction as trans-heterozygotes with *Dg,* but not *Dys* and were considered as Dg interacting components, *vimar* also strongly enhances the Dg^{RNAi}*:tub-Gal4* muscle degeneration phenotype (Fig. 2D, Supplementary Table 3).

Nine other DGC modifiers, *Nrk (Neuronal receptor kinase), Fhos, ßν-Integrin, robo (roundabout), chif (chiffon), mbl (muscleblind), Rack1 (Receptor of activated protein kinase C 1), CG7845* and *CG34400* interacted with both *Dys* and *Dg*. They increased the frequency of muscle degeneration of *Dys* and *Dg* loss-of-function heterozygous mutants and, mostly, enhanced *Dys* and *Dg RNAi* phenotypes (Fig. 3A, Supplementary Table 3). Intriguingly, reduction of *robo* by one copy suppresses muscle degeneration in $Dys^{N\text{-}RNAi}$*:act-Gal4* and Dg^{RNAi}*:tub-Gal4* mutants (Figs. 2C–D, Table S2). It has been shown that *D. melanogaster* cardiac lumen formation is dependent on interactions between the Slit/Robo pathway and Dg (Medioni et al., 2008) and the loss of Dys leads to an age-dependent disruption of the myocardium myofibrillar organization and alterations in cardiac performance (Taghli-Lamallem et al., 2008). Further studies of the DGC and Slit/Robo interactions are valuable since muscular dystrophies in humans cause progressive cardiomyocyte degeneration and fibrosis.

Since many genes that showed interaction with the DGC are not well characterized, we first examined if they are evolutionarily conserved and have homologs implicated in human diseases. Computational analysis of protein identity showed that all of the found genes have vertebrate homologs and many of them have a high degree of protein similarity and identity (Fig. 3C, Table 1). Data analysis showed that most of the vertebrate homologs could be classified by their association with two disorders: muscular dystrophies and/or tumors (Table 1). These findings are interesting due to the fact that the DGC is implicated not only in MD development, but also Dg is downregulated in a wide variety of tumors, with low levels of expression correlating with a poor prognosis (Muschler et al., 2002). Most tumor cells, like muscles, require a large amount of glucose and are likely to be subjected to energetic stress. Logically, both proteins involved in metabolic processes, Pgk and Vimar showed interactions with Dg only, suggesting that the low-energy pathway may play a role in tumor progression and muscle maintenance via Dg. This evidence provides good reason to study in more detail the effect of energy metabolism on dystrophic muscles.

Novel genes found in the DGC modifier screen have specific roles in muscle function

Next, we tested if mutations in found genes would affect muscle maintenance (Fig. 3, Table 1). Two of the genes found to interact with Dys, *Cam* and *capt* did not show considerable muscle degeneration when downregulated via *RNAi* driven by *tub-Gal4,* suggesting that both proteins may affect muscle only via Dys. Mutations in another Dys interactor *Lis-1*, both DGC-interacting genes that regulate gene expression: *mbl* and *chif,* a formin homology protein *Fhos,* and a transmembrane protein *CG34400* showed a significant frequency of defects in ageing muscles (Table 1, Figs. 4A, D–F). Consistent with the *Nrk* gene being expressed specifically in the nervous system (Oishi et al., 1997), downregulation of *Nrk* in motor neurons, but not in muscles, affected muscle maintenance (Table 1, Figs. 4A, G). The DGC-interacting mutants with unknown function: *CG7845, vimar, SP2353, Fkbp13* and non-DGC-interacting mutants: *nAcRalpha* and *SP1070* also showed significant muscle deterioration (Table 1, Figs. 3A, H–N).

Since it has been shown that high temperature stress causes behavioral dysfunction and noticeably exaggerates muscle electrical responses (Benshalom and Dagan, 1981; Montana and Littleton, 2004), we also tested if dystrophic muscle function is affected by heating flies (~39 °C) and calculated their mobility index. The high temperature induced mobility defects seen with *Dys* mutants are much more severe than those seen with *Dg* mutants, nonetheless both

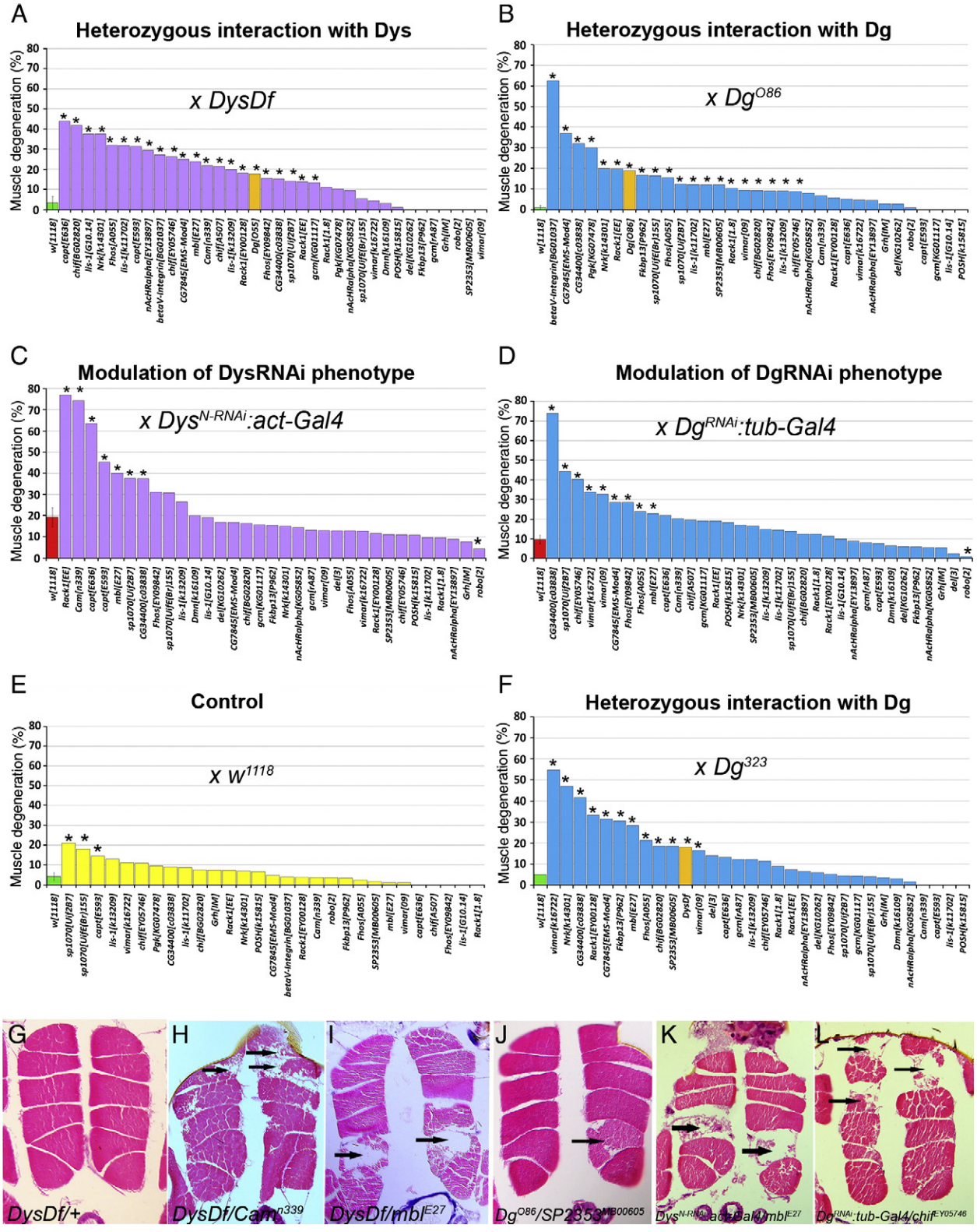

Fig. 2. In the genetic screen for components that genetically interact with *Dys* and *Dg* in muscle maintenance the frequency of muscle degeneration resulting from heterozygous interaction between *DysDf* (A), Dg^{O86} (B), Dg^{323} (F) and screened alleles was measured. (C, D) Modulation of $Dys^{N\text{-}RNAi}$*:act-Gal4* and Dg^{RNAi}*:tub-Gal4* muscle degeneration phenotype by one copy reduction of screened components. (E) The frequency of muscle degeneration phenotype by one copy reduction of screened components. Statistics were done using the χ^2-test with one degree of freedom and Yates's correction. Asterisks indicate statistically significant ($p \leq 0.05$) heterozygous interactions (A, B, F), enhancement or suppression of *Dys* and *Dg* RNAi phenotype (C, D), and dominant muscle degeneration phenotype in screened alleles (C). (H–L) Exemplary IFMs showing muscle degeneration from 3 weeks old animals lacking Dys or Dg and one copy of screened alleles in comparison to *DysDf/+* flies (G).

had a lower mobility index than wild type (Fig. 4B). In addition, under high temperature stress the majority of the screened mutants also have significantly reduced mobility indices (Table 1, Fig. 3B), suggesting their possible involvement in muscle stress responsive pathways. Since many of these genes have not been studied previously in *Drosophila*, further experiments are planned.

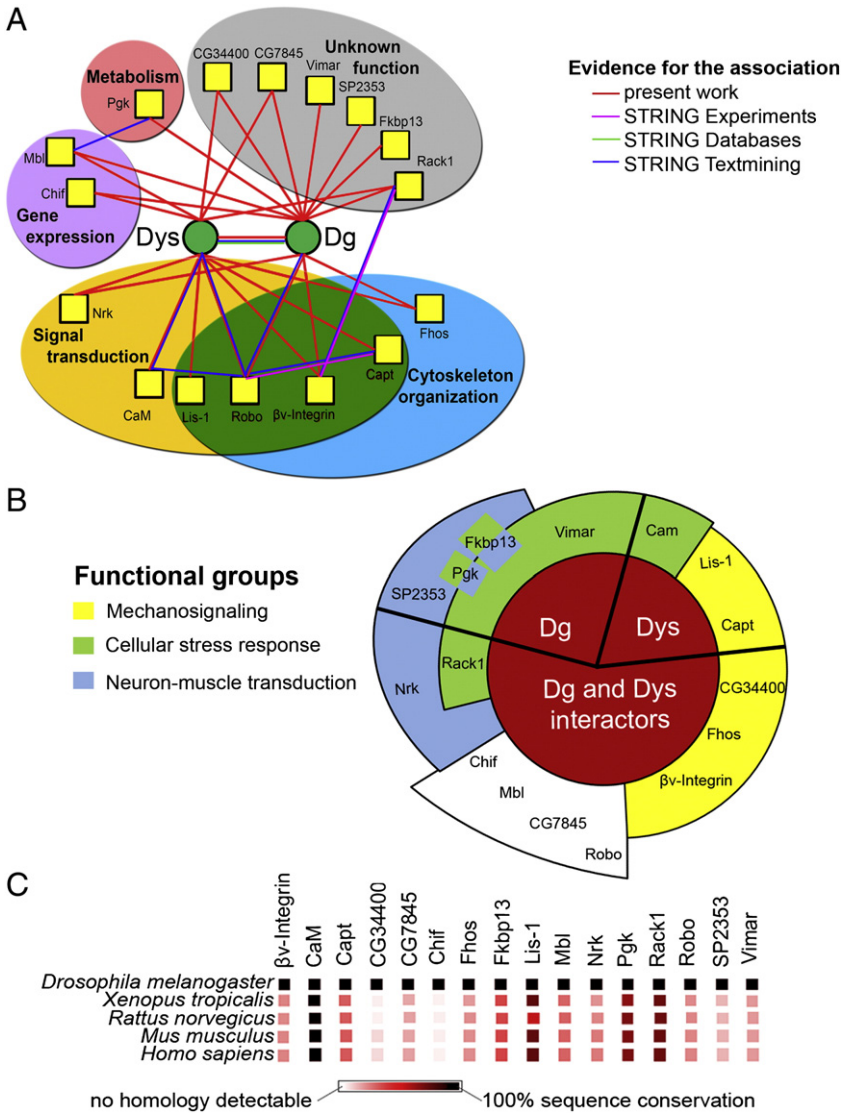

Fig. 3. DGC-interacting components. (A) An interaction network for the DGC. Proteins are shown as nodes and the relationship as edges (edge color indicates evidence for interaction). (B) Proportion of interacting factors divided into functional groups. (C) Homology of DGC-interacting components with vertebrates.

Drosophila muscles are susceptible to stress

Many genes found in our muscle screen have been shown previously to be involved in cellular adaptive responses. For example, *βν-integrin*, *Fhos*, *capt* and *CG34400* encode for proteins that bind to actin and control muscle cell cytoskeleton rearrangement in response to mechanical stress (Boehm et al., 2005; Gasteier et al., 2005; Mburu et al., 2003; Medina et al., 2008; Perkins et al., 2010). Mechanical stress is translated by the cell into biochemical signals such as changes in intracellular calcium levels leading to activation of diverse signaling pathways. Additionally, levels of Ca^{2+} can be regulated by Cam and FKBP13 and abnormal Ca^{2+} homeostasis leads to oxidative stress (Bellinger et al., 2009; Chakkalakal et al., 2006). The DGC interactors, Pgk and Vimar have been implicated in cellular homeostasis, and Rack1 modulates response to energetic stress (Qiu et al., 2010).

Since muscle is a highly sensitive tissue that responds to environmental stresses in its pattern of metabolic activity and tissue integrity, we decided to test first how *Drosophila* normal muscles respond to stress: suboptimal ambient temperatures, oxidative and energetic deficiency stress.

To begin with we evaluated the frequency of muscle degeneration in two control laboratory lines (*OregonR* and w^{1118}) kept at different temperatures. Muscle maintenance was not affected in flies residing at 18 °C and 25 °C and any significant difference was detected between these two groups. However, staying at higher temperature (33 °C) led to muscle maintenance defects. Not only was the frequency of muscle degeneration significantly increased (~4 times in comparison to 18 °C and 25 °C, Figs. 5A–D, Supplementary Table 5), extreme muscle degeneration was also observed (Fig. 5A, black bars).

It has been shown that hyperthermia-induced muscle degeneration is linked with oxidative stress (Mujahid et al., 2005). There is also increasing evidence that oxidative stress, due to reactive oxygen species (ROS) production overpowering the intracellular antioxidant systems, causes muscle wasting both during ageing and in chronic pathological states (Vercherat et al., 2009). Therefore we next attempted to amplify oxidative stress by culturing flies on food containing Paraquat, a superoxide radical generating agent. The occurrence of degenerated muscles was significantly higher than under normal conditions including extreme muscle degeneration (Figs. 5A, E–F, Supplementary Table 5).

Unexpectedly, in flies the frequency of abnormally maintained muscles did not increase with age, implying that healthy muscles did not show any dependence on animal's age (Fig. 5A, Supplementary Table 5).

Table 1
Components implicated in muscle maintenance.

Drosophila protein	Vertebrate homologs						*Drosophila* mutants					
							Muscle degeneration in 3 weeks old animals			Temperature-sensitive mobility defects in 1–5 day old animals		
	Human homolog	Identity, %	Positive, %	Gaps, %	Function	Involvement in disorders	Genotype	% [a]	n	Allele/+	Index [b]	n
Control	–	–	–	–	–	–	*Oregon R* *tub-Gal4/+* *MHC-Gal4/+* *D42-Gal4/+*	3.0 ± 3.0 0.8 2.4 4.0	n = 98 n = 81 n = 42 n = 97	*Oregon R*	1.00 ± 0.14	n = 118
βv-Integrin	ITGB5	31	48	7	Cell adhesion, signaling	Muscular dystrophy, cardiomyopathy, cancer	$βv\text{-}Integrin^{BG01037}$	0.0	n = 104	$βv\text{-}Integrin^{BG01037}$	0.68 ± 0.29	n = 65
Cam	CAM2	97	98	0	Ca^{2+} -dependant pathways regulation, interaction with DGC components	Enhances muscular dystrophy in mice	Cam^{RNAi}; *tub-Gal4*	7.8	n = 129	Cam^{n339}	1.31 ± 0.21	n = 46
capt	CAP1 CAP2	49 48	66 64	4 4	Actin polymerization, signaling, cell polarity, cell motility, CAP2 found in developing striated muscles	Cancer, metastasis	$capt^{RNAi}$; *tub-Gal4*	4.8	n = 84	$capt^{E636}$	0.86 ± 0.21	n = 42
CG34400	DFNB31	36	56	18	Actin cytoskeleton organization	Deafness	$CG34400^{c07121/c05107}$	32.0**	n = 50	$CG34400^{c05107}$	0.74 ± 0.52	n = 106
CG7845	WDR74	31	50	4	Unknown	Unknown	$CG34400^{RNAi}$; *tub-Gal4* $CG7845^{RNAi}$; *MHC-Gal4*	23.1** 20.0**	n = 65 n = 70	$CG7845^{EMS\text{-}Mod4}$	0.65 ± 0.10*	n = 61
chif	DBF4	25	42	23	Regulatory subunit of Cdc 7 kinase, DNA replication, cell cycle, integrin signaling	Cancer	$chif^{EY05746/A507}$ $chif^{EY05746/BG02820}$ $chif^{BG02820/A507}$	31.8** 22.6** 18.3**	n = 157 n = 53 n = 104	$chif^{BG02820}$	0.51 ± 0.03**	n = 41
Fhos	FHOD3	46	61	10	Actin polymerization, MTs organization, signaling, muscle function regulation	Unknown	$Fhos^{A055}$ $Fhos^{EY09842}$ $Fhos^{A055/EY09842}$	46.7** 17.1** 24.3**	n = 30 n = 117 n = 140	$Fhos^{A055}$	0.56 ± 0.04**	n = 57
Fkbp13	FKBP14	44	64	4	Unknown	Unknown	$Fkbp13^{RNAi}$; *tub-Gal4*	26.0**	n = 58	$Fkbp13^{P962}$	0.96 ± 0.12	n = 57
Lis-1	LIS1	70	87	0	Actin polymerization, dynein binding, cellular macromolecule localization, microtubule-based movement	Lissencephaly	$Lis\text{-}1^{RNAi}$; *MHC-Gal4*	34.0**	n = 94	NA	–	–
mbl	MBNL1	43	54	12	Regulation of splicing, muscle differentiation, recruitment of integrin to focal adhesions	Myotonic dystrophy	mbl^{RNAi}; *MHC-Gal4*	12.0*	n = 109	mbl^{E27}	0.57 ± 0.04**	n = 50
nAcRalph a-30D	CHRNA7	–	–	–	Acetylcholine receptor, Ca^{2+} transport, activation of MAPK	Schizophrenia, cognitive defects	$nAcRα\text{-}30D^{EY13897}$ $nAcRα\text{-}30D^{EY13897/KG05852}$	27.3** 16.1**	n = 44 n = 62	NA	–	–
Nrk	MUSK	60	75	5	Receptor for Agrin, MAPK signaling, transcription, protein phosphorylation, muscle development, function in NMJs	Congenital myasthenic syndrome	Nrk^{RNAi}; *MHC-Gal4* Nrk^{RNAi}; *D42-Gal4*	3.0 27.0**	n = 150 n = 18	Nrk^{k14302}	0.57 ± 0.04**	n = 51
Pgk	PGK1 PGK2	70 68	82 83	0 0	Glycolytic enzyme, electron carrier activity, transferase activity, functions in glycolysis, function in NMJs	Mental retardation, Menkes disease	Pgk^{RNAi}; *MHC-Gal4*	8.6	n = 85	$Pgk^{KG07478}$	1.23 ± 0.76	n = 52
Rack1	RACK1	77	87	0	Signaling	Cancer	$Rack1^{RNAi}$; *MHC-Gal4*	4.4	n = 90	$Rack1^{EY00128}$	0.66 ± 0.05*	n = 58
robo	ROBO2	38	55	4	Cytoskeleton rearrangement, axon guidance receptor, ureteric bud development	Multiple congenital abnormalities, vesicoureteral reflux	$robo^{RNAi}$; *tub-Gal4*	10.3	n = 58	$robo^{2}$	0.61 ± 0.06*	n = 40
SP1070	NOTCH1	38	51	7	Cell fate specification, aortic valve specification	Aortic valve disease	$SP1070^{Uif\text{E}(br)155}$/+ $SP1070^{Uif2B7}$/+	18.1** 21.1**	n = 105 n = 90	NA	–	–
SP2353	AGRNL AGRN	32	49	5	Dg ligand, photoreceptor ribbon synapse formation	Congenital muscular dystrophy (AGRN)	$SP2353^{RNAi}$; *tub-Gal4* $SP2353^{f01290/c05961}$	19.0** 30.7**	n = 21 n = 179	$SP2353^{MB00605}$	0.67 ± 0.05*	n = 37
vimar	RAP1	30	50	10	GTP–GDP dissociation stimulator , regulation of mitochondrial function	Cancer	$vimar^{RNAi}$; *MHC-Gal4*	81.0**	n = 32	$vimar^{EY09646}$	0.48 ± 0.01**	n = 37

[a] Muscle degeneration frequency results were statistically compared using χ^2 test with one degree of freedom and Yates's correction.

[b] Relative mobility results were statistically compared using *t* –test, *$p \leq 0.05$; **$p \leq 0.01$.

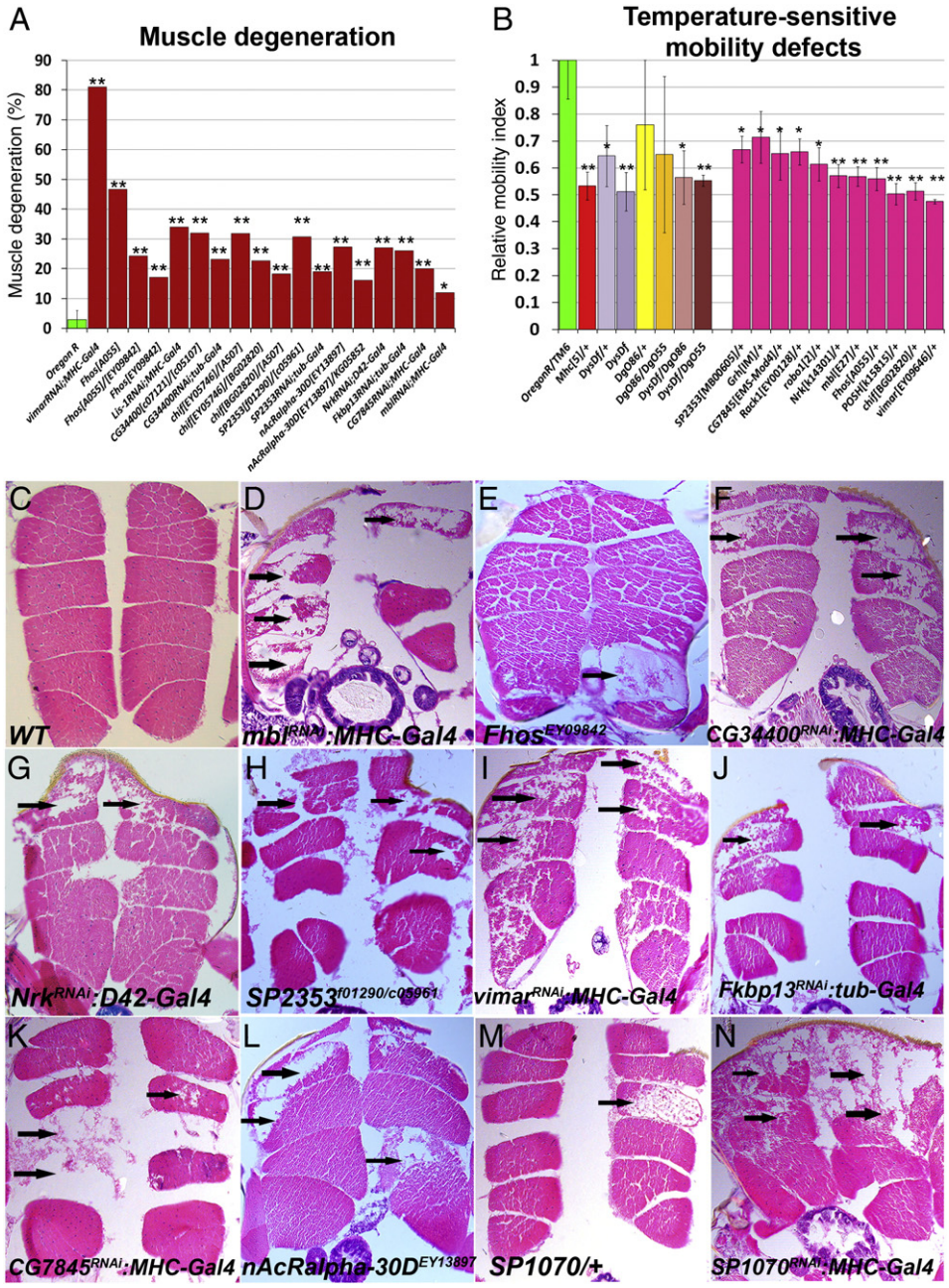

Fig. 4. Mutations in DGC modifiers result in muscle phenotypes. (A) Frequency of muscle degeneration in 3 week old mutant animals. Statistics were done using the χ^2-test with one degree of freedom and Yates`s correction. (B) Mutants exhibit dominant temperature-sensitive mobility defects. Statistics were done using the *t*-test. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$. (C–N) H&E-stained paraffin sections of exemplary IFMs, arrows indicate muscle degeneration.

Interestingly, sugar deprivation also had no effect on muscle welfare (Fig. 5A, Supplementary Table 5). Normally when flies are glucose-deprived, they adapt by changing their metabolism from glycolysis and glycogenolysis to lipolysis, which results in lower CO_2 production. Control flies lower their metabolic rate approximately 5 times as a reaction to change in food conditions measured here by the amount of produced CO_2 (Fig. 5G, Supplementary Table 6).

Adult Drosophila dystrophic muscles are more sensitive to stress

Now we showed that suboptimal conditions have an effect on normal muscle maintenance in *Drosophila*. Dystrophic muscles, however, may have a somewhat different response to stresses depending on the structural differences of muscle cells as well as changes in metabolic processes. To study the specificity of stress response in dystrophic muscles we applied different stresses to *Dys* and *Dg* mutants. Because of higher lethality of *Dys* and *Dg* mutants under the stress in comparison to control (*OregonR*) line, less amount of time was used to keep animals under experimental conditions (Supplementary Table 5, experimental groups 2 and 3).

As expected, high temperature and oxidative stress affected muscle welfare in all tested animals, wild type and mutants (Fig. 6A, Supplementary Table 5). Elevation of environmental temperature from 25 °C to 33 °C about two times increased the incidence of degenerated muscles in mutant animals, similarly to non-mutant. In all analyzed lines, the calculated phenotype included 20–30% of cases with extreme muscle degeneration (Fig. 6A, black bars, E–F, Supplementary Table 5). Also on Paraquat-containing food the ratio or degree of muscle degeneration was not increased in *Dys* and *Dg* mutants in comparison to control (Fig. 6A, Supplementary Table 5), suggesting

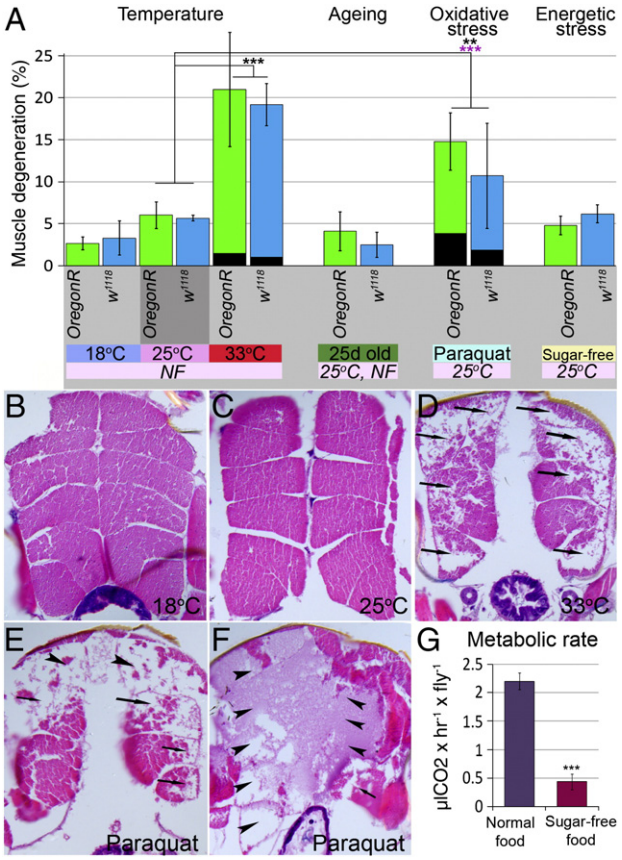

Fig. 5. Oxidative and temperature stress cause muscle degeneration in wild type *Drosophila*. (A) A bar graph shows frequency of muscle degeneration in *OregonR* and w^{1118} flies under influence of different conditions (temperature—18 °C, 25 °C, and 33 °C, ageing, oxidative stress induced by feeding Paraquat and energetic stress induced by sugar deficit, NF - normal food). Frequency of severe muscle degeneration is represented as black bars. Statistics were done using one-way ANOVA with post Dunnett's tests, where data from different "experimental conditions" groups (light grey box) were compared with control group (dark grey box). ** $p \leq 0.01$; *** $p \leq 0.001$. Black stars show comparison of total muscle degeneration, while pink stars represent comparison of extreme muscle degeneration. (B–F) Examples of *OregonR* IFMs at different conditions. Arrows indicate degenerated muscles and arrowheads point to muscles scored as extremely deteriorated. The metabolism response of wild type animals to loss of energy source is shown in (G). Statistics were done using the *t*-test. *** $p \leq 0.001$.

that oxidative stress is not the specific cause for dystrophic muscle degeneration in *Drosophila,* but muscle tissue in general is sensitive to oxidative stress.

Unlike in wild type animals, exposure of *Dys* and *Dg* mutants to lower temperature (18 °C) caused the appearance of severe progressive degeneration, followed by focal muscle loss (Fig. 6A, C–D, Supplementary Table 5). The muscle metabolism is temperature dependent and requires a coordinated system of metabolic control. Since *Dys* and *Dg* mutant muscles already are compromised, lowering the temperature in addition would accelerate degenerative processes. At 25 °C the frequency of muscle degeneration was the lowest, suggesting that 25 °C is an optimal temperature regime for dystrophic animals.

Interestingly, ageing appeared to cause muscle degeneration exclusively in dystrophic animals. Even though *Dys* and *Dg* mutant ageing animals both showed an increased number of myofibrils with moderately and extremely abnormal tissue structure, only mutation in *Dys* gene significantly amplified the amount of degenerated muscles (Fig. 6A, Supplementary Fig. 2, and Supplementary Table 5).

Sugar-free food conditions promoted severe muscle loss only in *Dg* mutants (Fig. 6A, black bar, G, Supplementary Table 5). Under normal conditions *OregonR* flies and *Dys* and *Dg* mutant animals showed similar metabolic rates (2.20 ± 0.15, 2.41 ± 0.09, 2.36 ± 0.18 respectively). Under glucose deprivation control flies produced five times less CO_2 indicating that their metabolic activity went down as expected (Fig. 6B). *Dys* mutants also significantly slowed down their metabolism; on sugar-free food they produced 2. 3 times less CO_2 than on normal food (Fig. 6B, Supplementary Table 6), while *Dg* flies continued to produce a fair amount of CO_2 (only 1. 6 times reduction, Fig. 6B, Supplementary Table 6) implying that the protective system required for lowering the metabolism in response to energetic stress is malfunctioning when Dg is absent. The *Dys* phenotype can be explained due to the fact that Dg localization is diminished in the absence of Dys (data not shown). These data suggest that Dg, but probably not Dys, plays a role in the pathway required to maintain muscle integrity under energetic stress and may be involved in the process of metabolic switch as an adaptive response to sugar shortfall.

Dg is implicated in control of muscle cell metabolism

One of the newly found DGC interactors involved in control of cellular metabolism is a phosphoglycerate kinase, PGK, an enzyme required for ATP generation in the terminal stage of the glycolytic pathway. Similar to the DGC, PGK has been localized in the *Drosophila* flight muscle cells to M-lines and Z-disks (Sullivan et al., 2003). *Drosophila Pgk* mutants display reduced lifespan, abnormal mobility, blocked synaptic transmission and heat-induced seizures (Wang et al., 2004). Our data also show strong genetic interaction of PGK with Dg, but not with Dys (Figs. 7A, C–D), suggesting that Dg together with PGK may have a role in regulation of cellular metabolism. To test this we analyzed the frequency of muscle degeneration in double trans-heterozygous mutants on sugar-free conditions. If PGK is involved in the control of glycolysis, then upon sugar deficit when animals transit their metabolism from glycolysis to fatty acids oxidation, the Dg and Pgk interaction should not be manifested. Indeed, on sugar-free food *Dg*/*Pgk* trans-heterozygous mutants did not display a muscle degeneration phenotype (Figs. 7A, E) showing that Dg has a role in regulation of the glycolytic pathway. The function of glycolysis and glycogenolysis in muscle is to provide ATP for myosin ATPase to enable contraction. Decrease in ATP levels should lead to the lowering of muscle contraction subsequently leading to abnormal motor behavior. Neither *Dg*/+ nor *Pgk*/+ mutants showed significantly decreased mobility upon heating (Table 1 for *Pgk*/+, Fig. 4B for *Dg*/+); however, *Dg*/*Pgk* heterozygotes significantly lessened their ability to move (Fig. 7B). Taken together these data show that Dg interacts with Pgk, a component of the glycolytic pathway that is essential to sustain energy for proper muscle functioning. This implies that Dg has a role in cellular response to energetic crisis.

Discussion

The DGC deficiencies lead to muscle degeneration and malfunction

Within the past couple of years different animal models for DGC-associated muscular dystrophy have significantly contributed to understanding the disease pathogenesis, but many questions about the mechanisms of these disorders remain unanswered. *Drosophila* has been shown to be an appropriate model to study the DGC since nearly all its known components are present and are evolutionarily conserved (Greener and Roberts, 2000), furthermore, mutations in its components, *Dystrophin*, *Dystroglycan* and *sarcoglycan* cause age-dependent progression of muscular dystrophy (Allikian et al., 2007; Shcherbata et al., 2007; Taghli-Lamallem et al., 2008).

We first show that like in vertebrates, *Drosophila* homologues of Dys and Dg are expressed in muscles. In multinucleated muscle cells Dg is present in the sarcolemma and is enriched around Z-bands that

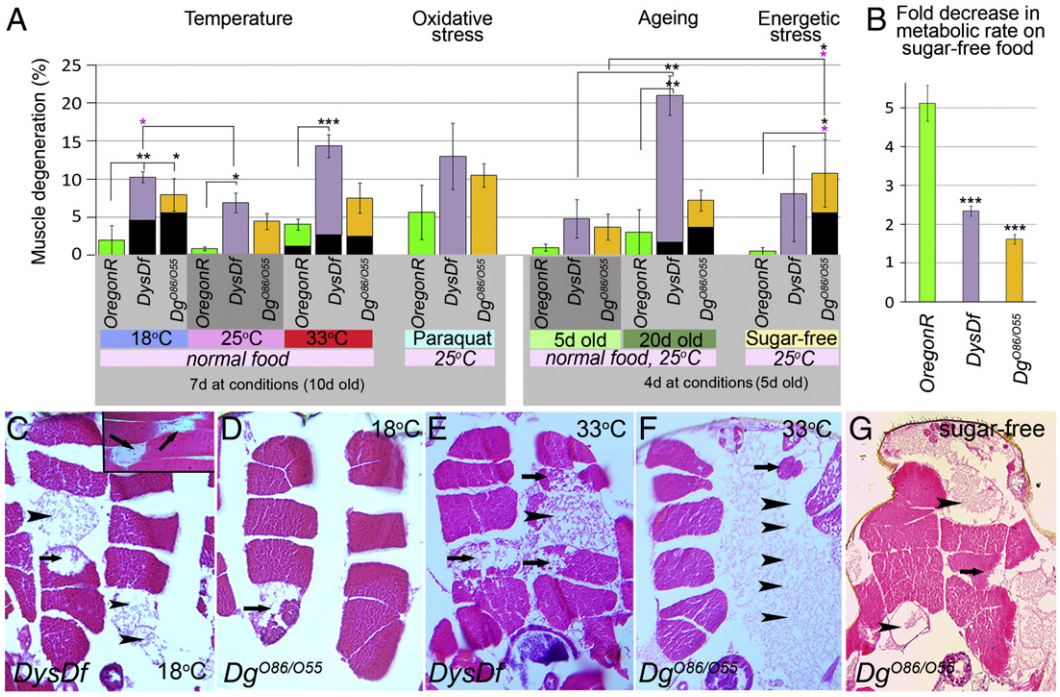

Fig. 6. Dystrophic muscles respond differently than wild type to stress. (A) A bar graph shows frequency of muscle degeneration in *OregonR* flies and *Dys* and *Dg* mutants under influence of different conditions (temperature—18 °C, 25 °C, and 33 °C, oxidative and energetic stress, ageing). Black bars represent the frequency of extreme muscle degeneration. Statistics were done using one-way ANOVA with post Dunnett's tests. First, data for *Dys* and *Dg* mutants were compared to *OregonR* within each "experimental conditions" group and then data from different groups (light grey box) were compared with control group (dark grey box). $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$. Black stars show comparison of total muscle degeneration, while pink stars represent comparison of extreme muscle degeneration. (B) Bar graph shows fold decrease in metabolic rate in dystrophic animals and *OregonR* line in response to loss of energy source. To determine the fold reduction in CO_2 production the amount of CO_2 generated under normal food conditions was divided by the amount of CO_2 generated under sugar-free food conditions for each genotype tested. The average value is reported with the error bars representing the standard error. Statistics were determined using a two-tailed Student's *t*-test, $^{***}p \leq 0.001$. (C–G) Exemplary *Dys* and *Dg* mutant IFMs at different conditions. Arrows indicate degenerated muscles and arrowheads point to muscles scored as extremely deteriorated.

correspond to costameres in vertebrates, while Dg's binding partner Dys is enriched in the muscle cytoplasm adjacent to the sarcolemma. Dys and Dg are also located postsynaptically in the larval NMJ, and both are believed to be involved in retrograde signaling to the presynapse (Bogdanik et al., 2008; van der Plas et al., 2006). Therefore the DGC controls not only cellular homeostasis of the muscle cell but also its ability to communicate with the motoneuron. Mutations in the DGC affect both, muscle tissue maintenance leading to myodegeneration and functioning causing heat-induced immobility.

Dys and Dg have different partners implying their involvement in different signalling

Even though Dys and Dg are biochemically linked, mutations in each of them cause partially distinct phenotypes, suggesting that they may act with various components regulating diverse processes required for maintaining muscle integrity. Dys mutants differ from Dg mutants in their behavior; while the first are not able to move, the second jump chaotically (based on observations). It has been noted in studies of the *Drosophila* larval NMJ that *Dys* and *Dg* mutants have opposite phenotypes, *Dg* mutants have shown a decrease in quantal content (Bogdanik et al., 2008), while *Dys* mutants have shown an increase in quantal content with an increase in spontaneous neurotransmitter release (van der Plas et al., 2006). When Dg was down regulated at the larval NMJ Dys expression was no longer localized (Bogdanik et al., 2008); however, when Dys was down regulated, Dg was still localized but to a lesser extent. The absence of Dg causes laminin to not be localized to the larval NMJ leading to disorganization of active zones (Bogdanik et al., 2008; Jacobson et al., 2001; Taniguchi et al., 2006; Tremblay and Carbonetto, 2006; van der Plas et al., 2006). This could lead to a severely mis-functioning NMJ that is not capable of transmitting a signal to the muscle to lead to a temperature-sensitive phenotype. Conversely, when Dys is absent, there is still the organization of the NMJ to lead to neurotransmission, but ultimately causing a failure of the contractile apparatus of the muscle possibly due to over excitation of neurotransmitter release. Therefore, we can propose that heat-induced immobility in *Dys* mutants is caused by improper retrograde signaling from muscle to the neuron, which causes constant muscle hypercontraction and subsequent degeneration of flight muscles. In *Dg* mutants shortage in NMJ functioning may cause faulty neurotransmission to the muscle leading to a loss of insufficiently innervated muscle fibers (manuscript in preparation).

Ageing also differentially affects muscle morphology: *Dys* mutants significantly increased the number of degenerated muscles, while *Dg* deficiency mainly promotes the severity of muscle deterioration. The similarity of phenotypes in *Dys* and *Dg* mutants is likely explained by the secondary destabilization of the entire DGC that results from deficiency of only one member. The differences in phenotypes are probably due to distinct roles of each protein, Dys being a cytosolic scaffold protein connecting the actin cytoskeleton to the plasmalemma, and a transmembrane Dg providing the link to the ECM.

Since both proteins may have different binding partners and be involved in different signaling pathways, we screened for unknown components that can influence Dys and Dg-dependent MD phenotypes. Our *in vivo* genetic screen in ageing dystrophic muscles revealed that the process of dystrophic muscle pathogenesis is multifunctional by nature, as is the DGC function. Dys and Dg genetically interact with multiple proteins that are involved in regulation of cell signalling, calcium homeostasis, cytoskeleton rearrangements, sarcolemma stability, energetic and oxidative stress, and cell polarity. Almost half of identified genes showed interaction with both proteins, and about one quarter specifically interacted with Dys or Dg (Fig. 3B). In general, all found interactors could be divided into three subgroups: genes that

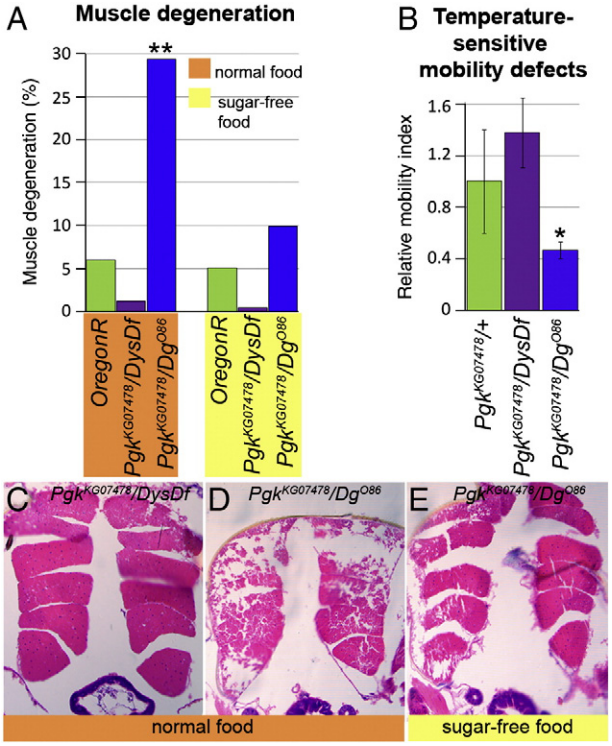

Fig. 7. Dg interacts with the glycolytic enzyme Pgk. (A) A bar graph shows frequency of muscle degeneration from genetic interaction of *Pgk* with *Dys* and *Dg* in 13-15d old animals kept for 10 days at normal or sugar-free food conditions in comparison with *OregonR*. Statistics were done using the χ^2-test with one degree of freedom and Yates`s correction. Percentage of degenerated muscles for $Pgk^{KG07478}/DysDf$ on normal food was 1% (n=72), sugar-free 0.5% (n=56) and $Pgk^{KG07478}/Dg^{O86}$ on normal food 33.8% (n=59), sugar-free food 10% (n=70), for *OregonR* control see Supplementary Fig. 5. (B) A bar graph represents temperature-sensitive mobility defects in *Pgk*/*Dys* and *Pgk*/*Dg* heterozygotes. Indexes for temperature-sensitive mobility in 1–5 day old animals is 1.23 ± 0.76 (n=52) for $Pgk^{KG07478}/+$, 1.68 ± 0.38 (n=58) $p=0.236$ for $Pgk^{KG07478}/DysDf$ and 0.57 ± 0.08 (n=58) $p=0.029$ for $Pgk^{KG07478}/Dg^{O86}$. Statistics were done using the *t*-test. $^*p\leq0.05$. (C–E) Exemplary IFMs showing muscle degeneration from 13–15 day old animals of the genotypes $Pgk^{KG07478}/DysDf$ and $Pgk^{KG07478}/Dg^{O86}$ kept at normal (C–D) and sugar-free (E) food.

are involved in mechanosignaling, cellular stress response factors, and genes that control neuron–muscle communication.

Muscle homeostasis depends not only on the cell autonomous DGC function, but also on neuron–muscle communication

Interestingly 4 out of 16 interactors (Nrk, Pgk, FKBP13 and SP2353) that have been found in our screen in ageing dystrophic muscles are supposedly involved in NMJ function and all of them, except for Nrk, genetically interact with Dg only. Disrupted glycosylation of α-DG in humans results in congenital muscular dystrophies that are associated with both progressive muscle degeneration and abnormal neuronal migration in the brain (Collins and Bonnemann, 2010). Disorders with the defects in NMJ transmission (congenital myasthenic syndromes, CMSs) also lead to muscle weakness (Massoulie and Millard, 2009). *Drosophila* Nrk (neurospecific receptor kinase) is highly homologous (60% identity) to human MuSK (muscle receptor kinase), which is essential for establishment and maintenance of the NMJs (Meriggioli and Sanders, 2009). Activation of MuSK by binding to agrin leads to clustering of acetylcholine receptors on the postsynaptic side of the NMJ (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 CMS development (Huze et al., 2009). We also found in the screen *Drosophila* SP2353, a novel agrin-like protein that contains a Laminin G domain, which makes it a potential new extracellular binding partner for Dystroglycan. The human ortholog for SP2353 (AGRN) is involved in congenital MD development and the mouse homolog, pikachurin has been shown to bind Dg in photoreceptor ribbon synapses (Huze et al., 2009; Sato et al., 2008). Supposedly, dystroglycan and MuSK (Nrk) could be two receptors and SP2353 a ligand 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.

FKBP13 is not extensively analyzed, however another member of the FK506 binding protein family, FKBP12 is believed to be important in regulating Ca^{2+} release through all ryanodine-receptor isoforms and the 1,4,5-inositoltriphosphate receptors. Recently it has been reported that FKBP12 is part of a macromolecular complex with RyR1 in mouse skeletal muscle. In *mdx* mice the associate of FKBP12 with RyR1 is reduced leading to sarcoplasmic calcium leakage (Bellinger et al., 2009). This dissociation is caused by increased RyR1 S-nitrosylation via inducible NOS (iNOS), which is up-regulated in dystrophic mouse (Bellinger et al., 2009). The DGC binds to neuronal NOS (nNOS) via α-syntrophin, where nNOS is the principle source of nitric oxide in skeletal muscle. When the DGC is disrupted via loss of dystrophin nNOS is downregulated and a compensatory mechanism causes the upregulation of iNOS, and consequently destabilization of RyR1 due to nitrosative stress.

The DGC may act as a sensor in the mechanical stress response pathway

Muscle mechanosensitivity modulates diverse cellular functions that ensure structural stability of muscle tissue in response to mechanical stresses. There is an interesting group of the genes, identified from our screen that play an important role in the assembly of the actin cytoskeleton architecture and signal transfer to cause cytoskeleton reconstruction. Dystrophic muscle stability is jeopardized upon mechanical stress (Petrof, 1998), which suggests misregulation of proteins that control cellular response to extracellular mechanics. This implies that the DGC can act as a mechanosensoring unit that transduces physical forces into biochemical information.

Actin cytoskeleton reorganization in response to mechanical tension is controlled via different signaling pathways and, interestingly Dys interactors Capt and Lis-1 are involved in this process (Moriyama and Yahara, 2002; Wynshaw-Boris, 2007). Capt (cyclase-associated protein) is a *Drosophila* homologue of human CAP1 (Table 1), which has been shown to play a key role in speeding up the turnover of actin filaments by effectively recycling cofilin and actin through its effect on both ends of actin filaments (Moriyama and Yahara, 2002). CAP1 has cofilin- and actin-binding domains, which makes it an attractive component to be involved in signal transduction and thereby links the cell signaling with actin polymerization. LIS1 is associated with Miller–Dieker (classical lissencephaly) syndrome; it interacts with the actin cytoskeleton and dynein activity (Gil-Krzewska et al., 2010; Wynshaw-Boris, 2007).

Found DGC interactor, RACK1 binds activated protein kinase C (aPKC) and anchors it to the cytoskeleton. It has not been shown yet that the *Drosophila* Rack1 gene can bind to aPKC, but it is 76% homologues to the mammalian Rack1 proteins (Table 1). In mouse skeletal muscle aPKC α associates with Annexin VI (Schmitz-Peiffer et al., 1998), which provides a possible structural link for Rack1 to the DGC through the hypothesized binding of Annexins to the TRPC channels TRPC1 and TRPC4 that bind with α-syntrophin (Sabourin et al., 2009). Rack1 also has a pro-apoptotic function by blocking Src activation of the Akt cell survival pathway (Mamidipudi and Cartwright, 2009). There has also been suggested a role for the DGC in inhibiting apoptosis by laminin binding to αDg which in turn activates the PI3K/Akt pathway (Langenbach and Rando, 2002).

An additional cytoskeleton-controlling mutant identified from the screen that exhibits muscle degeneration and temperature-sensitive mobility defects is *Fhos.* A microarray screen identified an up-

regulated Fhos transcript in *Drosophila Mhc* mutants and further *in situ* analysis revealed strong expression of Fhos in somatic muscles and putative midline mesodermal cell (Montana and Littleton, 2006). *Fhos* encodes for 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 has been identified (Prins et al., 2009). This proposes that the DGC and Fhos may act as a team in the MT organizing procedure.

Since skeletal muscle basal lamina is linked to the sarcolemma through transmembrane receptors, including integrins and dystroglycan, it is plausible that their function may be somewhat redundant. It had previously been shown that upregulation of integrin α_7 in the *mdx* background could ameliorate aspects of muscular dystrophy (Burkin et al., 2001). However, when functional dystrophin is absent, upregulation of integrin α_7 could only compensate in mediating cell-extracellular matrix attachment but cannot rescue the dystrophic phenotype (Cote et al., 2002). In addition, in *Drosophila*, integrin-mediated adhesion maintains sarcomeric integrity (Perkins et al., 2010). Our finding that one of the *Drosophila* integrins interacts with both Dys and Dg implies that the DGC is linked to the integrin signaling pathway and further studies of different components of the pathway may help to find a way how to strengthen the plasma membrane in dystrophic muscles. Additionally, mammalian homologs for *mbl* and *chif*, MBNL1 and DBF4 found in our screen have been implicated in integrin signaling (Chen et al., 2009; Vicente et al., 2007).

Since defects in mechanotransduction are linked with the development of various diseases, ranging from muscular dystrophies to cancer progression and metastasis, understanding the roles of the DGC and its interacting proteins in mechanical stress response is very important.

Stress influences the speed and the onset of muscle degeneration as dystrophic muscles have abnormal cellular metabolism

Although the genetic basis of many dystrophies is known, the exact processes by which muscles become progressively nonfunctional remain a mystery. Since genes found in our screen have been previously associated with cellular adaptive responses to stress, we first analyzed and compared the normal and dystrophic muscle stress responsiveness using different stress conditions. Our data show that muscle degeneration can be induced by stress. Furthermore, stress accelerates the onset and severity of age-dependent muscular dystrophy in *Dys* and *Dg* mutants. Normal and dystrophic muscles are similarly sensitive to elevated temperature and oxidative stress. Remarkably, cooler temperature amplified dystrophic muscle damage comparable to what was seen in aged mutant animals. Energetic stress had a large impact on the muscle structure of *Dg* mutants with severe muscle degeneration far exceeding that observed in *Dys* mutants. This finding is supported by previous studies, which also showed association of Dg with energy homeostasis (Mirouse et al., 2009; Takeuchi et al., 2009).

The muscle is the largest organ in the body that is required not only for movement, but also for heat production and cold tolerance, playing a crucial role in the overall energy balance. Calmodulin that plays a role in the oxidative stress response pathway, is an expected DGC interactor, since it binds to syntrophin (a component of the DGC) and CaM dependent kinase is involved in phosphorylation of dystrophin and syntrophin (Madhavan and Jarrett, 1999). In addition, the selective oxidation or nitration of CaM that occurs *in vivo* during ageing and under conditions of oxidative stress modulates signal transduction processes and intracellular energy metabolism (Squier, 2001). Additionally, the Ca^{2+}-buffering capacity of dystrophic muscles by Calmodulin and Calsequestrin also seems to be impaired due to a decrease in the levels of these proteins (Pertille et al., 2010); and targeted inhibition of CAM signaling worsens the dystrophic phenotype in *mdx* mouse muscle (Chakkalakal et al., 2006). Importantly, reduction of *Cam* by one copy, found as a Dys interactor in our screen rescues the Dys hypercontraction phenotype (manuscript in preparation).

Also we have determined here that in muscles Dg, but not Dys is required under conditions of energetic stress and both proteins involved in metabolic processes, PGK and Vimar, showed interactions with Dg only. Based on prior screen data, Vimar has been shown to regulate mitochondrial function via an increase in citrate synthase activity (Chen et al., 2008). Citrate synthase deficiency leads to a decrease in ATP levels consistent with disruption of mitochondrial energy production (Fergestad et al., 2006). Possibly, the involvement Vimar has with the muscular dystrophy phenotype could be due to its role in mitochondrial regulation. PGK is essential for the breakdown of glycogen, resulting in the release of energy (Das et al., 2010; Wang et al., 2004). In order to contract muscle cells needs ATP for myosin ATPase, which can be provided either via the glycolytic pathway or by mitochondrial oxidative phosphorylation. In *Drosophila*, a substantial fraction of the ATP for flight muscle contraction is provided through the glycolytic pathway (Leopold and Perrimon, 2007; Sullivan et al., 2003). In IFMs, glycolytic enzymes, including Pgk, are co-localized along sarcomeres at M-lines and Z-discs and this co-localization is required for normal muscle function (Sullivan et al., 2003; Wojtas et al., 1997). Similarly to *Dg* mutants, *Drosophila Pgk* mutants display reduced lifespan, abnormal motor behavior, altered synaptic structure, defective neurotransmitter release, and temperature-sensitive seizures (Wang et al., 2004). *Pgk* deficiency in humans is a rare inherited metabolic disorder sometimes associated with myopathies (Das et al., 2010) and it would be thought-provoking to study in more detail the effect of energy metabolism on dystrophic muscles.

Taken together our data demonstrate that the DGC is involved in the muscle stress response pathway. Understanding the differences between healthy and dystrophic adaptive reactions can lead to new approaches for dystrophic muscle metabolism manipulation to prevent progressive muscle loss. Further analysis of found Dys and Dg specific interactions will allow for new opportunities for easier drug targets in muscular dystrophy therapeutics and a better understanding of muscular dystrophy dynamics.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.01.013.

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