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Dissecting muscle and neuronal disorders in a *Drosophila* model of muscular dystrophy

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Perturbation in the Dystroglycan (Dg)–Dystrophin (Dys) complex results in muscular dystrophies and brain abnormalities in human. Here we report that *Drosophila* is an excellent genetically tractable model to study muscular dystrophies and neuronal abnormalities caused by defects in this complex. Using a fluorescence polarization assay, we show a high conservation in Dg–Dys interaction between human and *Drosophila*. Genetic and RNAi-induced perturbations of *Dg* and *Dys* in *Drosophila* cause cell polarity and muscular dystrophy phenotypes: decreased mobility, age-dependent muscle degeneration and defective photoreceptor path-finding. Dg and Dys are required in targeting glial cells and neurons for correct neuronal migration. Importantly, we now report that Dg interacts with insulin receptor and Nck/Dock SH2/SH3-adaptor molecule in photoreceptor path-finding. This is the first demonstration of a genetic interaction between Dg and InR.
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Introduction

The transmembrane protein Dystroglycan (Dg) is part of a complex that links the extracellular matrix (ECM) to cytoskeletal actin via the cytoplasmic protein Dystrophin (Dys). The Dys contains an actin binding domain on its N-terminus and the Dg interacting WW + EF hand-domain on its C-terminus (Hoffman *et al*, 1987; Koenig *et al*, 1987; Winder, 2001). These linkages are vital and disruption of any component or the interaction between them can cause muscular dystrophy and brain defects in humans (Campbell, 1995; Cohn and Campbell, 2000; Michele *et al*, 2002; Moore *et al*, 2002; Montanaro and Carbonetto, 2003; Cohn, 2005).

Mutations in Dystrophin glycoprotein complex (DGC) in vertebrates lead to muscle degeneration as well as phenotypes in many other cell types (Durbeej and Campbell, 2002; Cohn, 2005). For example, several muscular dystrophies exhibit neuronal migration disorders (Muntoni *et al*, 2002; Qu and Smith, 2004), showing that Dg interactions are essential for normal neuron migration. However, the mechanism of action and regulation of this complex are not fully understood in any cell type. Multiple proteins interacting with Dg have been identified through biochemical assays resulting in the hypothesis that Dg is involved in regulation of the actin cytoskeleton, signal transduction and cell morphology (Yang *et al*, 1995; Sotgia *et al*, 2001; Spence *et al*, 2002, 2004a, b).

It is now critical to analyze which of these biochemical interactions are required for Dg–Dys function and regulation and in which cell types do these interactions take place. Model organisms are essential for these functional studies and a few of such models exist and have been analyzed. For example, Dys is defective in Duchenne Muscular Dystrophy (DMD) patients as well as in mdx mice, the highly studied mouse model for DMD. However, in mdx mice, a compensating process limits muscular necrosis during most of the animal's life (Durbeej and Campbell, 2002; Michele *et al*, 2002; Moore *et al*, 2002). In addition, *Caenorhabditis elegans* and zebrafish have recently been used to model muscular dystrophies (Gieseler *et al*, 2000; Parsons *et al*, 2002; Bassett and Currie, 2003).

Dys is a 427 kDa rod-shaped protein that is defective in DMD. The huge gene encodes for three full-length dystrophin isoforms and four shorter, truncated products, controlled by different internal promoters. The complex structure of the gene is highly conserved during evolution. Similarly to the mammalian dystrophin gene, the fly gene encodes three full-length dystrophin-like products (DLPs) and three truncated products consisting of the C-terminal and cysteine-rich domains with various extensions into the spectrin-like repeats domain of DLP. Like the human gene products, the *Drosophila* gene products are expressed in a tissue-specific manner (Neuman *et al*, 2001, 2005; Figure 1A).

We now report that *Drosophila Dg* and *Dys* mutants develop age-dependent muscle degeneration and mobility defects, indicating that this easy to genetically manipulate organism serves as a remarkably good model for muscular dystrophy. Using this model, we demonstrate that Dg–Dys complex is required in brain in the photoreceptor neurons and in the targeting glial cells for proper axon path-finding, suggesting that ECM-based process regulated both from neuronal and glial side contribute to axon migration. Furthermore, the loss-of-function-mutant analysis and genetic interactions suggest that Dg and Dys act in similar axon path-finding processes as Insulin Receptor (InR) and the adaptor protein Nck/Dock.

Results

Dg and Dys are both required for cellular polarity in Drosophila

A gain of function screen for mutants defective in polarity in *Drosophila* oogenesis resulted in the finding of *Drosophila*

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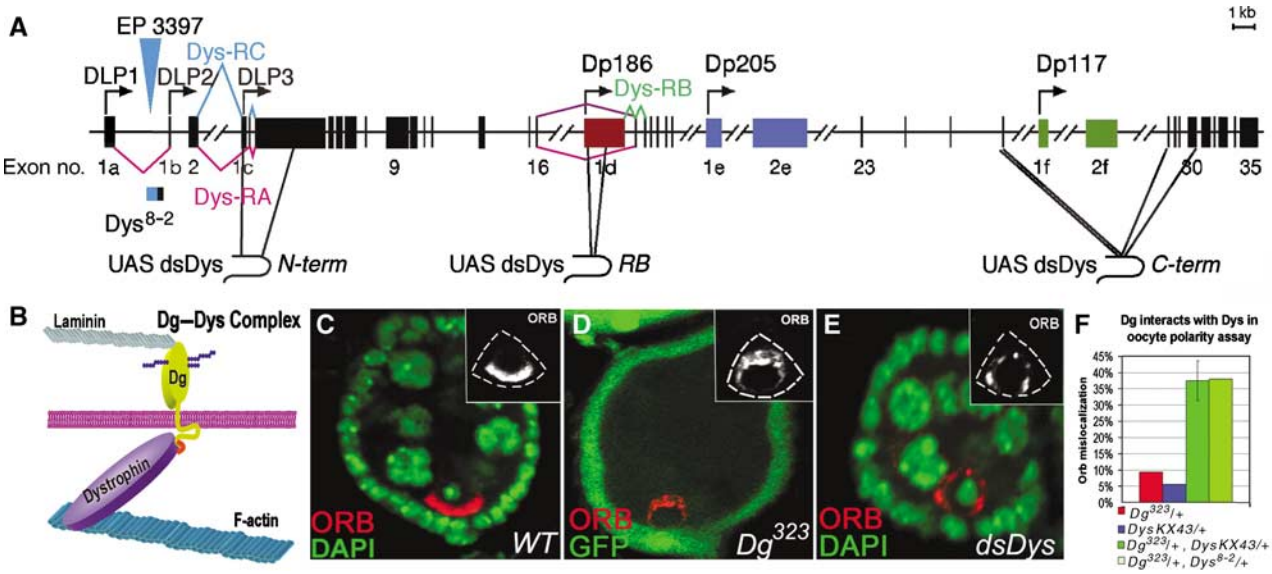


Figure 1 Dg and Dys interact *in vivo*, setting up the anterior–posterior polarity of the oocyte. (**A**) The *Drosophila Dys* gene structure. Bars represent exons, horizontal bold lines—introns, bent arrows—translation start sites. *Dys* genetic deletion *8-2* and *UAS RNAi Dys* constructs are shown. *UAS dsDys C-term* will affect all known Dys transcripts while *UAS dsDys RB* is specific for the short transcript RB and *UAS dsDys N-term* for the transcripts RA and RC (additional transcripts have recently been isolated (Neuman *et al*, 2005)). (**B**) A cartoon of *Drosophila* Dg–Dys complex is shown. The transmembrane protein Dg provides a link between Laminin in the extracellular matrix and Dys that is attached to the intracellular cytoskeleton. Dg C-terminal Dys-binding peptide shown in Figure 2C is marked in red. (**C–E**) The oocyte polarity marker Orb, which colocalizes with MTOC, is mislocalized in the oocytes of *Dg* mutants (D, *hsFLP; FRT42D* Dg^{323}*/FRT42D Ubi-GFP), Dys* mutant *(*$Dys^{8\text{-}2}$*/DysKX43;* Table I) and transgenic *dsDys* flies (E, *dsDys N-term/MatTub-GAl4;* Table I). Instead of being localized to the posterior of the developing oocyte at stages 4–6, Orb surrounds the oocyte in a circle or accumulates in a clump at one side of the oocyte. Red = Orb, Green = DAPI (C, E) or GFP (D). In the right top corners: mislocalization of MTOC in stage 6 oocytes marked by Orb. (**F**) A bar graph showing that Dg interacts with Dys in the oocyte polarity assay. Transheterozygous $Dg^{323}/+$*; DysKX43/+* and $Dg^{323}/+$*;* $Dys^{8\text{-}2}/+$ mutants show oocyte polarity defects with increased frequency in comparison to control $Dg^{323}/+$ or *Dys KX43/+* flies ($Dg^{323}/+$ 9.4%, $n = 117$; *Dys KX43/+* 5.5%, $n = 163$; $Dg^{323}/+$*; DysKX43/+* 37.4±6.1%, $n = 309$ and $Dg^{323}/+$*;* $Dys^{8\text{-}2}/+$ 37.9%, $n = 124$).

homologs of components in the Dg complex: *Drosophila* Dg, LamininA and Dys (Deng and Ruohola-Baker, 2000; Deng *et al*, 2003; Figure 1B). Further analysis revealed that the *Drosophila* genome has all the known components of the Dg complex (Greener and Roberts, 2000; Dekkers *et al*, 2004). While vertebrates have two closely related proteins, dystrophin and utrophin, encoded by two different genes, *Drosophila* has only one gene encoding *Dystrophin*. The expression of *Drosophila* Dys overlaps with Dg in adult and embryonic tissues (Supplementary Figures 1–3).

To test whether Dg and Dys act in the same cell types and interact genetically in *Drosophila*, we isolated a mutant allele for *Dystrophin* and generated *dsRNA* constructs (Figure 1A). We have analyzed the phenotypes associated with two *Dys* deletion mutants *(*$Dys^{8\text{-}2}$ and Dys^{e6}) and three different *dsRNA* constructs (Figure 1A; *dsDysN-term* targets the long, *dsDysRB* the short and *dsDysC-term* all *Dys* isoforms) and compared these to the phenotypes of previously isolated Dg mutants (Deng *et al*, 2003). A significant reduction of Dys was observed with all mutants analyzed (Supplementary Figure 3C–I, M and N; Supplementary Table I). Specifically, while the genetic loss-of-function mutant *(*$Dys^{8\text{-}2}$*/DysKX43)* showed a 149-fold reduction, N- and C-terminal *dsDys* constructs showed 6–18-fold reduction of the transcript DLP2 (Supplementary Table I).

Dg is required for cellular polarity: in *Dg* germline clones, the early oocyte polarity marker Orb fails to show the normal posterior localization in stage 4–6 oocytes (Deng *et al*, 2003; Table I; Figure 1C and D). This and the accompanied growth defect of the egg chamber can be partially rescued by germ line expression of full-length Dg protein (Yatsenko *et al*, 2006). To analyze whether Dys is also required in the germline for oocyte polarity, we examined Orb localization in *Dys* mutant ovaries (*$Dys^{8\text{-}2}$*/DysKX43*, *Dys^{e6}*/DysKX43* or *pUASPdsDys N-term/MatTub-Gal4*). Reduction of Dys function in the germline resulted in an Orb mislocalization phenotype reminiscent of the phenotype seen in *Dg* mutant; Orb surrounds the entire oocyte in a circle, or it accumulates in a clump at the sides of the oocyte (Figure 1E; Table I). Therefore, Dys, like Dg, is required in germ line for establishment of early oocyte polarity. We also analyzed Dys function in another cell type, follicle epithelial cells, and observed that reduction of *Dys* results in polarity defects in this cell type as well (Supplementary Figure 3J–L).

To test whether Dg and Dys act in the same process in the germ line, we tested to see if Dg and Dys showed genetic interactions in the oocyte polarity assay; the polarity of $Dg^{323}/+$*; DysKX43/+* and $Dg^{323}/+$*;* $Dys^{8\text{-}2}/+$ oocytes was analyzed. The double heterozygous animals showed significant polarity defects indistinguishable from the homozygous Dg^{323} or $Dys^{8\text{-}2}$*/DysKX43* mutants, suggesting that Dg and Dys interact in this process (Figure 1F). Thus, both Dg and Dys are required in the germ line and interact in the establishment of cellular polarity during oogenesis.

The Dys–Dg interaction is conserved from human to flies

While the crystal structure of the human Dys–Dg complex has been solved (Huang *et al*, 2000), the binding affinity for this interaction in human or *Drosophila* has not been ana-

Table I *Dg* and *Dys* mutations cause similar developmental phenotypes in *Drosophila*

	Control	*Dg^{323}*	*$Dys^{8\text{-}2}$*	Control		*RNAi mutants*		
	OR	*$FRT42DDg^{323}$/ $FRT42DDg^{323}$*	*$Dys^{8\text{-}2}$/Def KX43*	*UAS GFP*	*UAS dsDg*	*UAS dsDys*		
Phenotypes						*N-term*	*RB*	*C-term*
Oocyte polarity	10%, $n=50$	96%[a], $n=26$	41%[b], $n=69$	5%, $n=64$	—[c]	× *MatTub-Gal4* 49%, $n=324$	—[c]	—[c]
Mobility ($T_{1/2}$, days)	24, $n=114$	—	12, $n=108$	22, $n=91$	10, $n=79$	× *tubP-Gal4* 12, $n=74$	14, $n=95$	13, $n=83$
Muscle degeneration	3 days, 20(0)%[d], $n=10$	—	3 days, 35(0)%, $n=34$	17(0)%, $n=23$	24(8)%, $n=103$	× *tubP-Gal4 (3 days old)* 22(0)%, $n=103$	27(0)%, $n=30$	—
				24(0)%, $n=54$	62(48)%, $n=65$	× *tubP-Gal4 (12 days old)* 58(24)%, $n=113$	58(48)%, $n=36$	—
	12 days, 25(0)%, $n=57$		12 days, 66(22)%, $n=110$	18(0)%, $n=110$	32(8)%, $n=119$	× *24B-Gal4 (3 days old)* 21(4)%, $n=159$	—	25(4)%, $n=81$
				11(0)%, $n=47$	73(33)%, $n=108$	× *24B-Gal4 (20days old)* 69(57)%, $n=124$	—	—
Axon path-finding	11%, $n=18$	85%[e], $n=33$	67%, $n=27$	29%, $n=17$	74%, $n=19$	× *GMR Gal4* 74%, $n=32$	57%, $n=26$	61%, $n=36$
				29%, $n=67$	71%, $n=80$	× *repo-Gal4* 60%, $n=27$	55%, $n=18$	76%, $n=58$

n = number of analyzed egg chambers in polarity analysis, flies in mobility and longevity analyses, individual thoracic muscles in muscle degeneration or brain hemispheres in axon path-finding analyses.

[a]*hsFLP; FRT42D Dg^{323}/FRT42D Ubi-GFP* (only germ line clones analyzed).

[b]The frequency of oocyte polarity defects in an independent loss-of-function mutant *Dys^{e6}/DefKX43* is 40.5% ($n=84$).

[c]The construct that allows germline expression *(pUASp dsDg* or *dsDys)* does not yet exist.

[d]In parentheses is shown the percentage of extreme muscle degeneration phenotypes (loss of muscle fibers or vacuolization of muscle tissue). Independent indirect flight muscles were calculated.

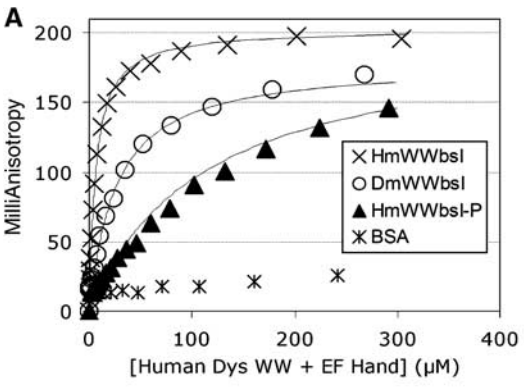
[e]eyFLP, GMR-lacZ; FRT42D Dg^{323}/FRT42D l(2)cl-R11[1].

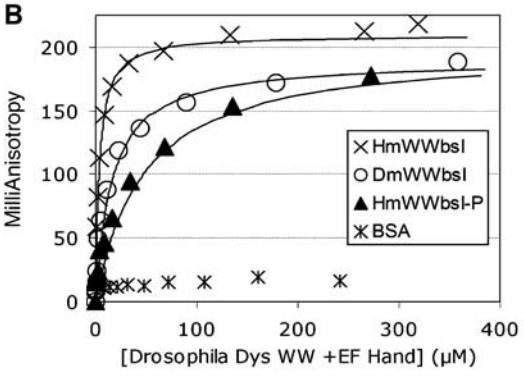
lyzed. We developed a fluorescence polarization assay to determine the binding dissociation constants (K_d) for both the human and *Drosophila* complexes (Figure 2). In this assay, human or *Drosophila* Dys (WW + EF hand domains) was titrated into buffer containing fluorescent-labeled Dg peptide. Human Dys binds human Dg peptide (HmWWbsI) with a K_d of $7.6 \pm 1.6\,\mu M$. While *Drosophila* Dys binds *Drosophila* Dg peptide (DmWWbsI) with a K_d of $16 \pm 4\,\mu M$ (Figure 2A, B and D). To verify that we were measuring binding at the same interface elucidated by the crystal structure, we tested human Dys binding with a mutant Dg peptide in which the tyrosine of the PPPY motif (Tyr 892; Figure 2C) was mutated to a proline (HmWWbsI-P: KNMTPYRSPPPPVSP). This tyrosine contributes two hydrogen bonds to the binding interface and forms van der Waals contacts to a hydrophobic pocket on the dystrophin WW domain (Huang *et al*, 2000), all of which are expected to be lost upon mutation to proline. As expected, this titration showed reduced affinity (K_d $172 \pm 39\,\mu M$) indicating that the assay measures the correct interaction (Figure 2A and D).

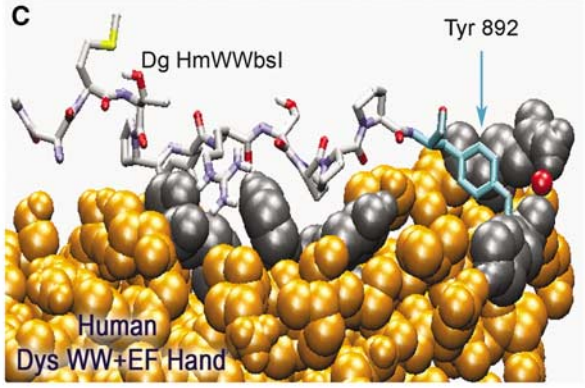
In addition, we tested whether human Dg can interact with *Drosophila* Dys and vice versa. Both these cross species interactions were in the same range as the within species interactions with binding affinities of 24 and 3.7 μM, respectively (Figure 2D). The affinity measured for Dg–Dys interactions is in the expected range for previously analyzed WW-interactions (Kato *et al*, 2002). These data show that the Dys–Dg protein interface is highly conserved from humans to flies, suggesting that insights from *Drosophila* should be transferable to humans.

Dys and Dg mutants show mobility defects

Defects in the Dg complex in human cause muscular dystrophies, which are associated with muscle weakening and degeneration (Cohn and Campbell, 2000). To test whether the Dys–Dg complex plays a similar role in *Drosophila* muscle function, we first analyzed the mobility of the *Drosophila Dg* and *Dys* mutants by measuring their climbing capability (Benzer, 1967) using *dsDg* and *dsDys* constructs driven by *P-tub-Gal4* and the *Dys* loss-of-function mutant $Dys^{8\text{-}2}$/*DysKX43*. This rate of climbing decay in *Dg* and *Dys* mutants was significantly faster than in wild-type flies, suggesting that Dg and Dys might be required in the musculature (Figure 3A and B; Table I, $T_{1/2}$(mobility): control 22–24 days, $Dys^{8\text{-}2}$/*DysKX43* 12 days).







D Table 2. Dissociation constants (K_d) for dystrophin–Dystroglycan interaction in *Human and Drosophila*

Dystroglycan WWbsI[a]	Sequence	Dystrophin WW + EF Hand (μM)	
		Human	*Drosophila*
HmWWbsI	KNMTPYRSPPPYVPP	7.6 ± 1.6	3.7 ± 0.3
HmWWbsI-P	KNMTPYRSPPPPVPP	172 ± 39	47 ± 10
DmWWbsI	GKSPATPSYRKPPPYVSP	24 ± 8	16 ± 4

[a]WWbsI – Dystrophin WW domain binding site I

Figure 2 Dg and Dys interact *in vitro*; fluorescence polarization assay reveals that Dys binding to Dg is highly conserved from human to flies. (**A**) MilliAnisotropy values of human Dys WW + EF hand titrated into buffer containing fluorescently labeled Dg peptides show that human Dys can bind both human (HmWWbsI) and fly (DmWWbsI) Dg peptides. BSA titrated with 200 nM HmWWbsI peptide serves as a negative control. (**B**) MilliAnisotropy value of *Drosophila* Dys WW + EF hand titrated into buffer containing fluorescently labeled Dg peptides indicates that *Drosophila* Dys can also bind both *Drosophila* (DmWWbsI) and human (HmWWbsI) Dg peptides. BSA is used as the negative control. Binding affinity is reduced in both human and *Drosophila* models when the wild-type human peptide is substituted with a mutated peptide (HmWWbsI-P) in which the terminal tyrosine of the PPxY motif is mutated to a proline. (**C**) Space filling model of the interaction surface between human Dys WW + EF hand and human Dg peptide HmWWbsI. Human Dys residues that directly contact the HmWWbsI are colored gray. The tyrosine of the PPxY motif, mutated to proline in the HmWWbsI-P peptide is colored in cyan (arrow). (**D**) Dissociation constants of human and *Drosophila* Dys–Dg interaction. Data indicate that this interaction is highly conserved from fly to man: human Dg can interact with *Drosophila* Dys (WW + EF hand) and vice versa with similar K_d.

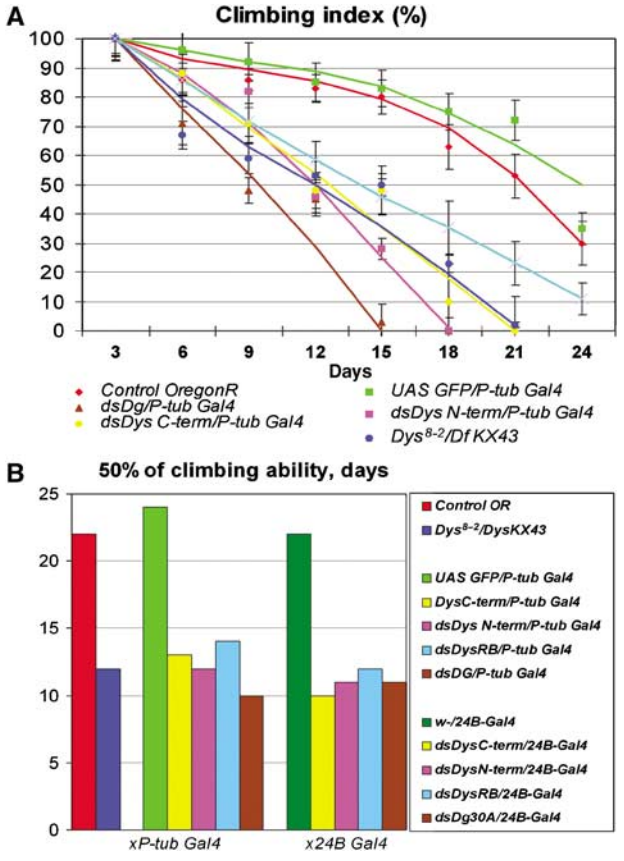


Figure 3 Mutations in *Dg* and *Dys* cause decreased mobility. (**A**) Dys and Dg function is required for normal locomotion. *Dys* and *Dg* mutants exhibit impaired climbing ability relative to control flies. They begin adult life with normal mobility, but the climbing decay rate is faster in mutants than in wild-type flies. This indicates that *Dg* and *Dys* defects cause age-dependent climbing disability. (**B**) A bar-graph showing that *Dys* mutant *8-2* and transgenic ubiquitous *Dg* and *Dys RNAi* animals (*dsDg* and *dsDys/P-tub Gal4*) and muscle-specific *dsDg* and *dsDys/24B-Gal4* mutants have lost 50% of their climbing ability in 10–14 days after eclosion in comparison to 22–24 days in control.

To test whether the climbing defects in DGC mutant animals were due to Dg and Dys function in muscle tissue, we analyzed mobility of *Dys* and *Dg* mutants using *dsDg* and *dsDys* constructs driven by mesodermal driver *24B-Gal4*. The speed of climbing decay in *dsDg* and *dsDys/24B Gal4* mutants was similar to what was observed for *dsDg* and *dsDys/P-tub Gal4* mutants (Figure 3B, $T_{1/2}$(mobility): control 22–24 days, *dsDg/P-tub-Gal4* 10 days, *dsDg/24B-Gal4* 11 days, *dsDysC-term/P-tub-Gal4* 13 days, *dsDysC-term/24B-Gal4* 10 days). These results indicate that Dg–Dys complex is required in the mesoderm.

Age dependent degeneration of Dys and Dg mutant muscles

To understand the cell biological basis for the observed mobility defects in *Dg* and *Dys* mutants, we analyzed their muscle morphology (*dsDg* and *dsDys/P-tub-Gal4, Dys^{8-2}/DysKX43)*. Histological analysis of the major thoracic muscles showed age-dependent muscle degeneration in *Dg* and *Dys* mutants, consistent with the mobility dysfunction in these mutants (Figure 4A–G; Table I). Confocal and light micrographs of histological sections revealed that the cellular appearance of muscle in *Dg* and *Dys* mutants was less organized than in control flies, numerous lesions within the muscular tissue were observed. In 12-day-old control flies the indirect flight muscles (IFM) have well structured muscle fibers with peripherally located nuclei (Figure 4A and D). Twelve days after eclosion, animals expressing *dsDg* and *dsDys* or Dys^{8-2}/*DysKX43* mutants show loss of muscle fiber organization, vacuolization (Figure 4B, C and G) and absence of some muscles (Figure 4F). These phenotypes became much more pronounced in older mutant flies; the frequency of muscle degeneration increased six-fold in the mutants (Dys^{8-2}/*DysKX43* or *dsDg* and *dsDys* crossed to *P-tub Gal4*) compared to the controls during a 9-day period (Figure 4H; Table I). Similar phenotypes have been observed before in *Drosophila parkin* and *pink1* mutants (Pesah *et al*, 2004; Yang *et al*, 2006).

To determine whether this age-dependent muscle degeneration phenotype is due to a requirement of Dg–Dys complex in muscle tissue, we used a mesoderm specific *24B-Gal4*-driver to express the *Dg* and *Dys RNAi* constructs. Severe muscle degeneration phenotypes, accompanied with extensive vacuolization of muscle tissue and muscle fiber loss were observed when the *Dg* and *Dys RNAi* were directed in the mesoderm (Figure 4H–K; Table I). We further showed that, similar to what was observed in ubiquitous *Dg* and *Dys RNAi* animals (*dsDg* and *dsDys/P-tub Gal4*), in muscle-specific *dsDg* and *dsDys/24B-Gal4* mutants the muscle deterioration process has an age-dependent character (Figure 4H–K; Table I). Taken together, these results suggest that, similar to human, Dg and Dys are required for muscle maintenance throughout the lifetime of *Drosophila*.

Dg and Dys are required for proper photoreceptor axon path-finding

Brain-selective deletion of Dg in mice is sufficient to cause congenital muscular dystrophy-like brain malformations, including disarray of cerebral cortical layering and aberrant migration of granule neuronal cells (Michele *et al*, 2002; Moore *et al*, 2002; Qu and Smith, 2004). Within the cortex, however, it is not clear whether the Dg–Dys complex is required in neurons, glia, or both for proper neuronal migrations. To better understand the function of the Dg complex in the brain, we analyzed potential brain defects in the *Drosophila Dg* and *Dys* mutants.

Dg is expressed in the *Drosophila* adult eye, brain, and the developing larval brain and visual system, especially in optic lobes and photoreceptors (Figure 5B; Supplementary Figure 4D). In the optic lobe, Dg is present both on photoreceptor axons (in the optic stalk, lamina plexus and medulla neuropil) and the Repo-expressing brain glial cells (Figure 5B). Dys shows similar expression patterns in the optic lobes.

To examine the role of Dg–Dys complex in the *Drosophila* brain, we analyzed frontal sections of adult heads from mutant *Dys* and *Dg* adult flies and observed abnormalities in the formation of retina: retinal photoreceptor cells were not elongated in *Dg* or *Dys* mutants (*eyFLP; Dg^{323}FRT 42D/FRT 42D l(2)cl-R11* 100%, Dys^{8-2}/*DysKX43* 88%, *dsDg30A* and *dsDg33A/P-tubGal4* 92%, *dsDysC-term/P-tubGal4* 100%, control *UAS GFP/P-tubGal4* 0%; Supplementary Figure 4), suggesting that the Dg–Dys complex is required in these photoreceptor sensory neurons.

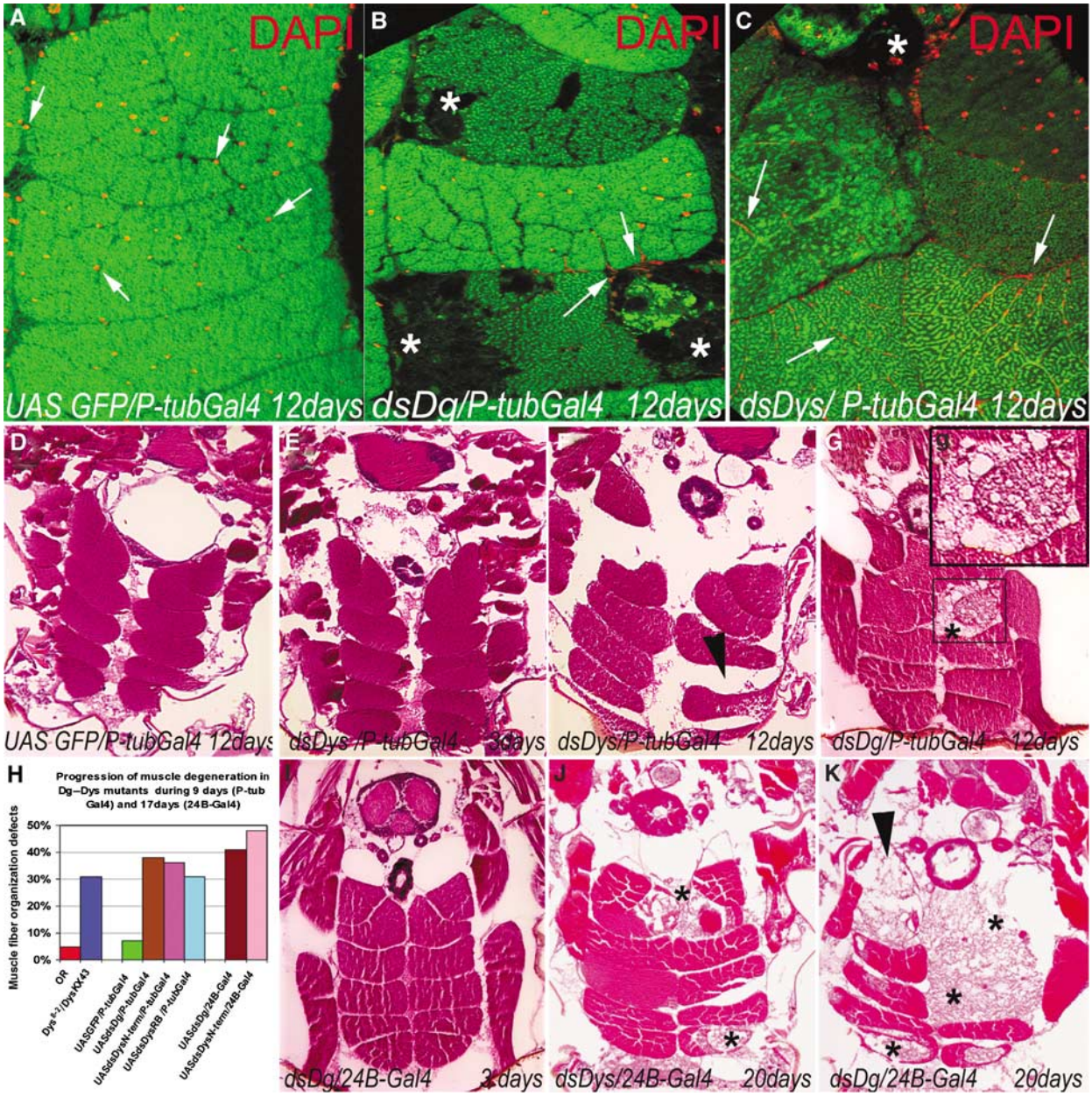


Figure 4 *Dg* and *Dys* mutants manifest age-dependent muscle degeneration. (**A–C**) Confocal analysis of histological transverse sections of IFM of 12 days old adult flies stained with a nuclear marker DAPI in red. (A) Control flies (*UASGFP/P-tub-Gal4)* show normal organization of the IFM and the muscle fibers are well structured with the nuclei located at the periphery (arrows). (B, C) *Dg* and *Dys* mutants (*dsDg30A* and *dsDysN-term/P-tub-Gal4)* show severe muscle degeneration: wasting and loss of muscle tissue, vacuolization (asterisks), the integrity of subsets of muscle cells is disrupted and the nuclei appear to be dispersed between fibers (arrows). (**D–G, I–K**) Light microscopy of histological transverse sections of IFMs stained with H&E. (D) Control (*UASGFP/P-tub-Gal4*, 12 days after eclosion). (E–G) *Dys* and *Dg* mutants (*dsDys N-term/P-tub-Gal4, dsDg30A/P-tub-Gal4*) exhibit mainly normal muscle architecture at 3 days after eclosion, but at 12 days in most of the cases the muscle degeneration progresses, the density of myofibrils per muscle decreases and some muscles are absent (arrowhead) or vacuolized (G, asterisk). (**H**) Bar graph represents increase in frequency of muscle fiber organization defects in 9 days in $Dys^{8\text{-}2}$*and Dg–Dys* transgenic animals (*dsDg* and *dsDys/P-tub-Gal4*) and in 17 days in flies with directed knockout of *Dg* and *Dys* in muscle (*dsDg* and *dsDys/24B-Gal4*), which suggest that muscle degeneration has an age-dependent character. Independent IFMs were calculated (Table I). (I–K) The mesoderm-specific RNAi-based reduction of *Dg* and *Dys* (*dsDys C-term* and *dsDg30A/24B-Gal4*) at 20 days after eclosion, but not at 3 days after eclosion (I) show obvious IFM muscle pathology: the loss of fiber density and vacuolization (asterisks).

The *Drosophila* compound eye consists of ~800 ommatidia, each containing eight different photoreceptor sensory neurons, R cell subtypes that project axons into one of two optic ganglia layers in the brain during late larval development. R1–R6 axons innervate the most superficial layer, the lamina, generating a smooth lamina plexus, whereas R7 and R8 project axons through the lamina into the deeper medulla layer (Figure 5A and D) (Perez and Steller, 1996; Tessier-Lavigne and Goodman, 1996; Clandinin and Zipursky, 2002; Ruan *et al*, 2002). The patterning of the R-cell subtypes in eye discs and the extension of their axons to the optic lobes of the developing brains occur by late third instar larvae, while the elongation of the retinal cell body takes place at pupal stage (Izaddoost *et al*, 2002).

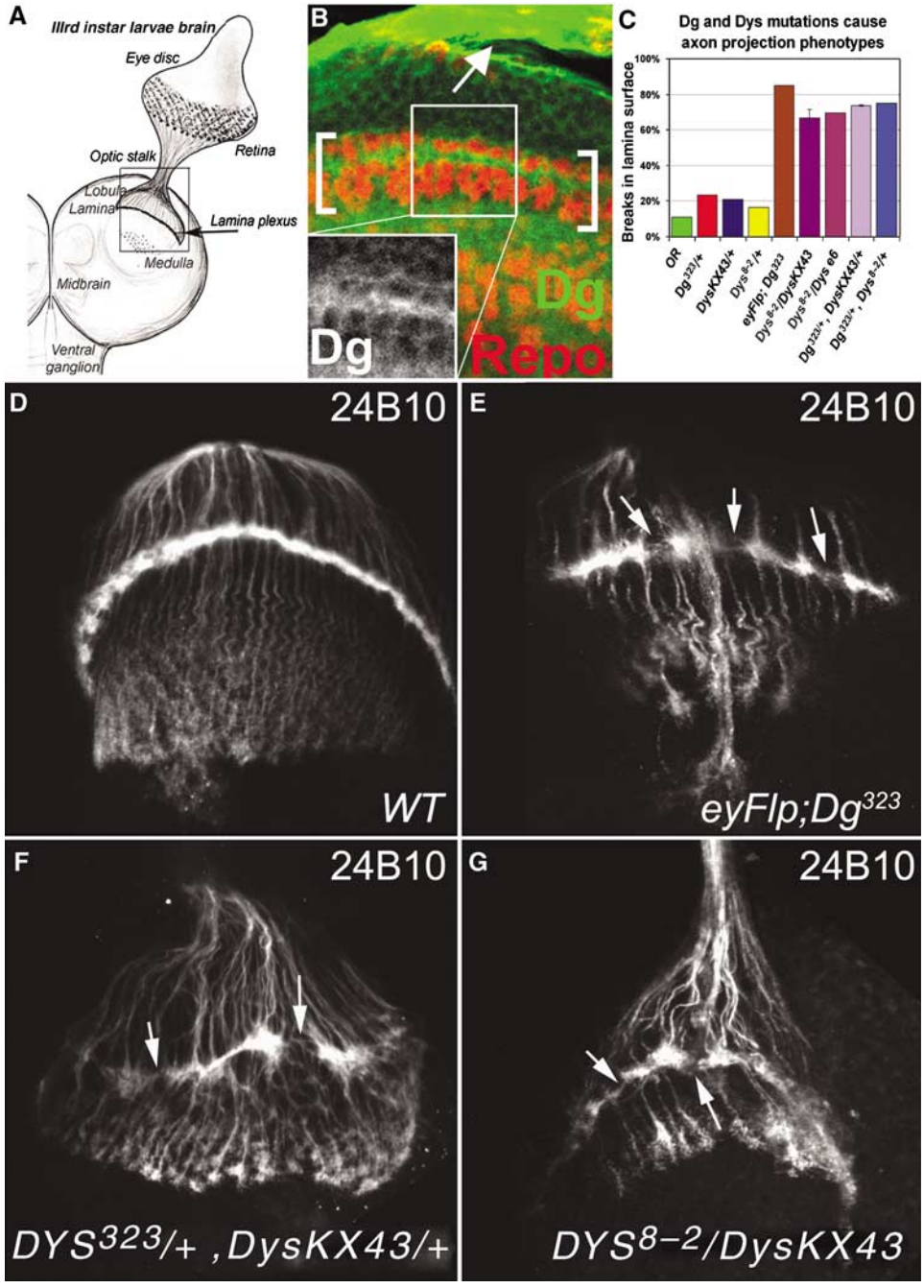


Figure 5 Dg–Dys complex is required for proper axon path-finding in *Drosophila* brain. (**A**) Schematic drawing of developing *Drosophila* third instar larval brain. Boxed area indicates the lamina plexus and the medulla. (**B**) Dg antibody staining shows that Dg protein is expressed in neurons and glia in larval *Drosophila* brain. High levels of Dg in larval *Drosophila* brain are detected on axons of photoreceptor sensory neurons in the optic stalk (yellow arrow) in addition to glial cells in the optic lobes. Red = Repo, Green = Dystroglycan. (**C**) A bar graph represents the frequency of axon path-finding defects caused by mutations in Dg–Dys complex. Both *Dg* and *Dys* loss-of-function mutants *(eyFLP; Dg^{323}FRT42D/FRT42D l(2)cl-R11* and *$Dys^{8\text{-}2}$/DysKX43*; Table I) as well as *Dg/Dys* transheterozygous mutants (*Dg^{323}/+; DysKX43/+*, $73.5 \pm 0.4\%$ $n = 95$, s.d. is from three independent experiments) show photoreceptor axon projection phenotypes. (**D–G**) Photoreceptor axonal projection patterns in third-instar larvae visualized with 24B10 antibody, the lamina plexus is indicated with brackets. (D) Wild-type pattern of photoreceptor neuron projection in the lamina plexus. In *Dg* loss-of-function mutants (E, *eyFLP; Dg^{323}FRT 42D/FRT 42D l(2)cl-R11*), *Dg/Dys* transheterozygous mutants (F, *Dg^{323}/+; DysKX43/+*) and *Dys* loss-of-function mutants (G, *$Dys^{8\text{-}2}$/DysKX43*) photoreceptor axons are clumping at the lamina and stop irregularly making gaps in the normal termination zone of the lamina plexus (arrows).

To determine at which stage Dg is required in photoreceptor neuron development, we induced eye-specific mutant clones *(eyFLP; Dg^{323}FRT 42D/FRT 42D l(2)cl-R11)* and analyzed the developing neurons in late third instar larvae using a photoreceptor-specific monoclonal antibody 24B10. The patterning of the *Dg* mutant ommatidia was normal, suggesting that Dg is not required for the determination and differentiation of the R-cells. However, the axonal projections of these sensory neurons to the brain optic lobes were disturbed due to the lack of Dg, most of the axons

migrate to the correct termination zone in lamina, but formed abnormal patches in the lamina plexus. Similar axonal problems were observed in *Dys* mutants. In the normal wild-type brain the photoreceptor axons terminate in a stereotypic fashion producing a fan-like structure in the lamina plexus (Figure 5A, B and D). However, in 85% of *Dg* loss-of-function (*eyFLP; Dg^{323}FRT 42D/FRT 42D l(2)cl-R11*) and 67–75% of *Dys* loss-of-function (*$Dys^{8\text{-}2}$/DfKX43* and *Dys^{e6}/$Dys^{8\text{-}2}$*) mutant third-instar larvae optic lobes the lamina plexus is irregular; photoreceptor axons stop irregularly making gaps in the normal termination zone of the lamina plexus, deviate from the path and bundle aberrantly (Figure 5C, E and G; Table I).

Importantly, Dg and Dys proteins interact in controlling the photoreceptor axon path-finding since simultaneous reduction of the level of both genes (*Dg^{323}/+; DysKX43/+* and *Dg^{323}/+; $Dys^{8\text{-}2}$/+*) results in a high percentage of the axon projection phenotypes while reduction of each gene independently (*Dg^{323}/+*, *DysKX43/+ or $Dys^{8\text{-}2}$/+*) does not (Figure 5F and C).

Dg and Dys are required both in neurons and glia for regular lamina plexus formation

Photoreceptor axon guidance requires correct photoreceptor specification as well as proper function of brain glia and neurons; the axons extend along glial cells, stop in response to signals produced by marginal glial cells, and establish synaptic connections with lamina neurons (Perez and Steller, 1996; Tessier-Lavigne and Goodman, 1996; Poeck *et al*, 2001; Clandinin and Zipursky, 2002; Ruan *et al*, 2002). Previous studies demonstrate complex interactions between R-cell axons and laminal glial cells: R-cell axons induce the differentiation and migration of laminal glial cells (Perez and Steller, 1996), and conversely laminal glial cells present a stop signal for terminating R1–R6 axons within the lamina (Poeck *et al*, 2001).We tested whether axon path-finding defects in *Dg* or *Dys* mutants were caused by loss of Dg-complex function in extending neurons or supportive glial cells by using eye- and glia-specific drivers (*GMR-Gal4* and *repo-Gal4*).

We first showed that in the majority of *Dg* and *Dys RNAi* mutants driven by *P-tub-Gal4*, the photoreceptor axons exhibited targeting phenotypes similar to *Dg* clonal phenotypes, they bundled and/or terminated irregularly in the normal termination zone of the lamina plexus. When these *Dys* and *Dg* RNAi constructs were expressed in eye disks, photoreceptor axons similarly terminated irregularly in the lamina region of the brain and formed uneven lamina neuropil with gaps and abnormally densely packed regions (Figure 6A and B; Table I, *dsDg* and *dsDys/GMR-Gal4* 74 and 61%). When *Dys* and *Dg* RNAi constructs were expressed in all glial cells, including eye disk and lamina glia, but not neurons, axons of the photoreceptor sensory neurons also showed bundling and irregular termination (Figure 6A and C; Table I, *dsDg* and *dsDys/repo-Gal4* 71 and 76%). To test whether the obtained axon path-finding phenotype is specific to DGC function in neurons and glia, we knocked-down Dg and Dys in mesodermal tissue and observed no effect on the axon termination process above control samples (Figure 6A and D; *dsDg* and *dsDys/24B-Gal4*). To determine the potential effect of DGC mutations on the development of laminal glial cells, we stained the third-instar optic lobe using a monoclonal antibody that recognizes the glial-specific nuclear protein Repo (Perez and Steller, 1996; Poeck *et al*, 2001). In wild type (Figure 6E and G), differentiating glial cells migrate into the lamina forming two clearly separated layers of glial cells (i.e., epithelial and marginal glia), which in turn present a stop signal for terminating R1–R6 growth cones in the lamina (Poeck *et al*, 2001). In *Dg* and *Dys* mutants, although glial cells migrated correctly into the lamina, they appeared less organized lacking the clear separation of epithelial and marginal glial layers (Figure 6F and H). We also used the MARCM technique in order to generate marked photoreceptor neurons and/or glial cells mutant for *Dg^{323} (elav-Gal4 hsFLP;FRT42B tubGal80/FRT42BDg^{323};UAS GFP act<CD2<Gal4)*. The termination zone observed for mutant photoreceptors was irregular; clumping of axons at the lamina and lamina breaks were associated with the presence of *Dg* mutant glial cells as well as mutant photoreceptor axons (Supplementary Figure 5A and B). In contrast to the wild-type regular axon/glia/axon pattern, in *Dg* mutant lamina the gaps were occupied by mislocalized glial cells (Supplementary Figure 5B). These data suggest that Dg acts autonomously and non-autonomously for correct axon path-finding; Dg–Dys complex is required both in neurons and in glial cells for proper neuron axonal growth and targeting.

As discussed, several congenital muscular dystrophies exhibit neuronal migration disorders (Michele *et al*, 2002; Moore *et al*, 2002). The mediations of axon path-finding and neuronal migration require similar processes including supportive glial cells (Bloch-Gallego *et al*, 2005). In the vertebrate brain, Dg is required for granule neuron migration (Michele *et al*, 2002; Moore *et al*, 2002; Qu and Smith, 2004). It will be interesting to see in the future if similar to *Drosophila* axon path-finding, the Dg–Dys complex in vertebrates acts both in neurons and glial cells for this process. Indeed, Dg function has been demonstrated in a support cell type in peripheral nervous system, Schwann cells for neuronal connectivity (Saito *et al*, 2003).

Dg interacts with Nck/Dock SH2/SH3 adaptor protein and InR to regulate axon guidance in Drosophila brain

The phenotypes we have observed in Dg and Dys mutant photoreceptor axon path-finding are reminiscent of phenotypes observed before with Nck/Dock SH2/SH3 adaptor protein (Garrity *et al*, 1996) and InR (Song *et al*, 2003) mutants. To test whether Dock and InR might act in concert with Dg and Dys in this process, we analyzed whether they genetically interact with Dys and Dg. Importantly, Dg shows a strong interaction with InR and Dock, while Dys does not; *Dg, Dys, InR* and *Dock* heterozygous mutants (*Dg^{323}/+*, *DysKX43/+*, *$Dys^{8\text{-}2}$/+*, *$InR^{ex52.1}$/+*, *InR^{34}/+ $Dock^{P1}$/+*, *$Dock^{P2}$/+*) and double heterozygous animals *Dys/InR* and *Dys/Dock* (*DysKX43/$InR^{ex52.1}$*, *DysKX43/InR^{34}*, *$Dys^{8\text{-}2}$/$InR^{ex52.1}$*, *$Dys^{8\text{-}2}$/InR^{34}*, *$Dock^{P1}$/+; DysKX43/+*, *$Dock^{P2}$/+; DysKX43/+*, *$Dock^{P1}$/+; $Dys^{8\text{-}2}$/+*, *$Dock^{P2}$/+; $Dys^{8\text{-}2}$/+*) mostly had regular termination zone in the lamina plexus, while *Dg^{323}/$Dock^{P1}$*, *Dg^{323}/$Dock^{P2}$*, *Dg^{323}/+; $InR^{ex52.1}$/+* and *Dg^{323}/+; InR^{34}/+* double transheterozygous mutants showed a significantly increased frequency of axon projection defects (Figure 7A). Previous genetic and biochemical work showed that InR can function as a guidance receptor for Dock. However, this InR function is independent of Chico, the *Drosophila* insulin receptor substrate homolog (Song *et al*,

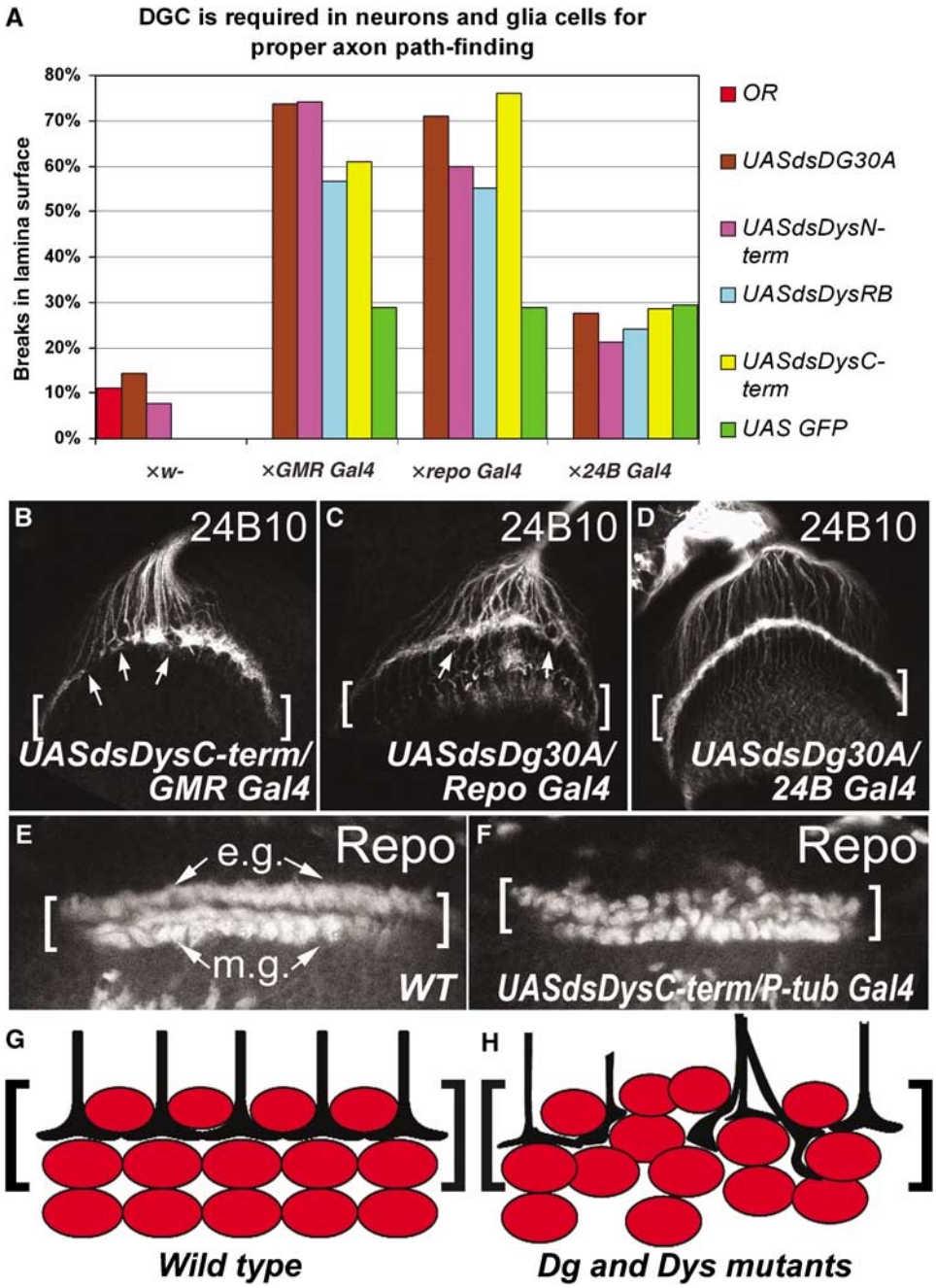


Figure 6 Dg–Dys complex is required in both neurons and glial cells for proper axon path-finding in *Drosophila*. (**A**) A bar graph represents the frequency of irregular and uneven lamina layer in *Dg* and *Dys* mutants. Analysis of axon path-finding phenotypes using photoreceptor *(GMR-Gal4)* and glia specific *(repo-Gal4)* drivers suggests that Dg–Dys complex is required in neurons and glial cells; lack of Dg–Dys complex in either cell type results in axonal mistargeting. Knocking out of DGC in the mesoderm *(24B-Gal4)* does not affect axon path-finding over control levels. (**B**) A representative image of the majority of preparations showing the clumping and uneven lamina plexus phenotype (indicated by arrows) in photoreceptor specific *Dg* and *Dys* mutants *(dsDg* and *dsDys/GMR-Gal4)*. (**C**) Similar phenotypes observed when *Dys* and *Dg* RNAi constructs were expressed in all glial cells, including eye disk and lamina glia, but not neurons *(dsDg* and *dsDys/Repo-Gal4)*. (**D**) Axons of photoreceptor sensory neurons in *dsDg* and *dsDys* crossed to a muscle-specific *24B-Gal4* driver showed regular lamina layer, indistinguishable from control (Figure 5D). (**E, F**) In wild-type (E) glial cells (marked with Repo) migrate from progenitor regions into the lamina where they are organized into two layers, the epithelial (e.g.) and marginal glia (m.g.), presenting a stop signal for the termination of R1–R6 growth cones at the lamina plexus (brackets). (F) In DGC mutants, although glial cells migrated correctly into the lamina, they appeared less organized. (**G**) In wild-type *Drosophila* brain the termination zone is organized stereotypically: each axon terminates between glial cells resulting in a regular axon/glia/axon pattern. (**H**) In *Dg* or *Dys* mutants the termination zone is disorganized: glial cells are irregularly positioned and photoreceptor axons bundle causing gaps and densely packed regions in the lamina.

2003). Similarly, while Dg interacts with InR, it does not interact with the substrate protein Chico. Double heterozygous $Dg^{323}/chico^{1}$ R-cell projection patterns were indistinguishable from wild-type (Figure 7A). These observations demonstrate that InR and the adaptor protein Nck/Dock can genetically interact with Dg but not Dys. Furthermore, since previous work has revealed that InR and Dock show genetic interactions in this process (Song *et al*, 2003), these data

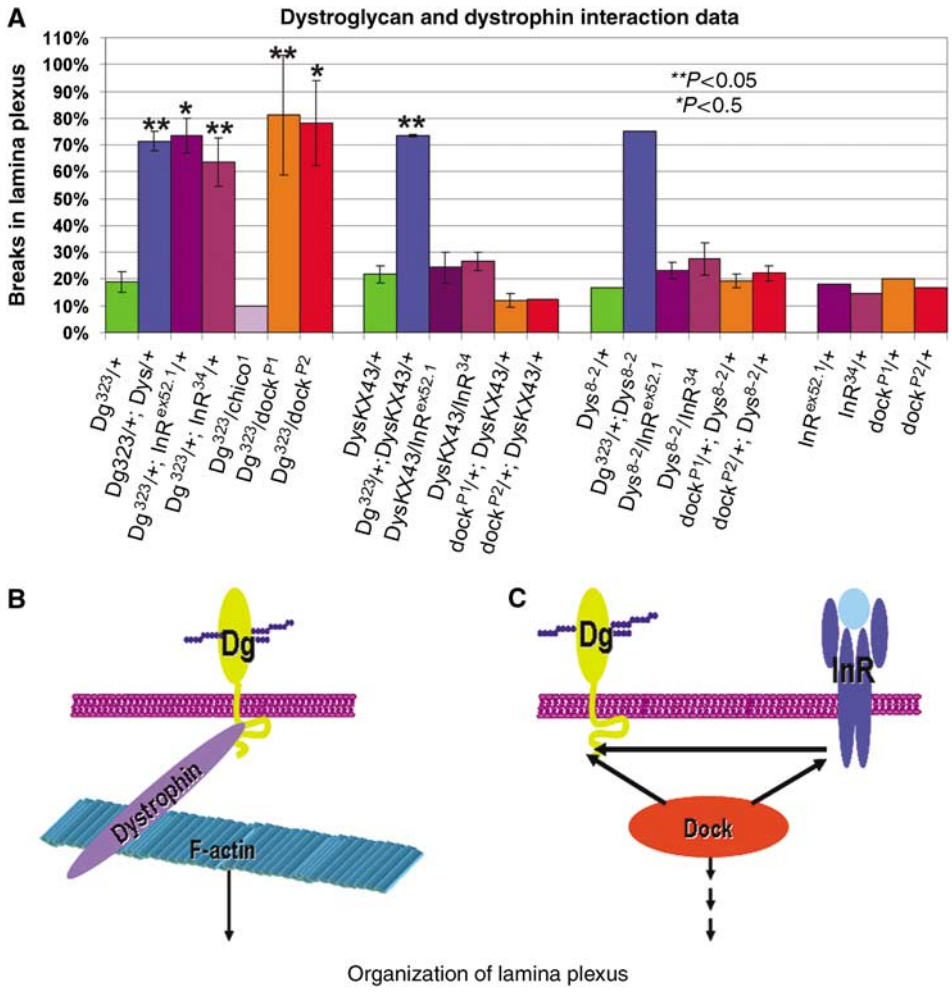


Figure 7 Dg interacts with Dock and InR pathways to regulate axon guidance in *Drosophila* brain. (**A**) A bar graph showing that Dg but not Dys interacts with Dock and InR pathways. The $Dg^{323}/+$, *DysKX43/+*, $Dock^{P1}/+$; *DysKX43/+* and $DysKX43/InR^{ex52.1}$ animals mostly displayed normal projection patterns, while $Dg^{323}/Dock^{P1}$ and $Dg^{323}/+$; $InR^{ex52.1}/+$ double transheterozygous mutants showed increased frequency of axon mistargeting (**$P<0.05$, *$P<0.5$ value calculated from two to four independent experiments). Similar results were obtained by using independent alleles for InR, Dock or Dys (InR^{34}, $dock^{2}$, Dys^{8-2}). Dg interacts with tyrosine kinase protein InR, but not with its substrate protein Chico. (**B**) Model showing that Dg connects to actin cytoskeleton through Dys during axon guidance, Dg interaction with Dock and/or InR could abolish Dg–Dys binding allowing cytoskeletal rearrangements (**C**). In this case, Dg might participate in Dock and InR mediated signal transduction.

suggest that Dg, InR and Dock interact in axon path-finding (Figure 7B and C).

Discussion

The fly genome contains many highly conserved orthologues to human disease genes (Reiter *et al*, 2001; Bier, 2005), including neurological, cardiovascular, endocrine and metabolic disease-genes. Among these, nearly all components of the Dg–Dys complex, which is involved in muscular dystrophies, are present in flies (Deng and Ruohola-Baker, 2000; Greener and Roberts, 2000; Deng *et al*, 2003). We now show that Dys and Dg interact genetically and biochemically and are required in the same cell types in *Drosophila*. A fluorescence polarization assay revealed that the Dg–Dys binding interface is highly conserved in humans and *Drosophila* (Figure 2). Both proteins are required for oocyte cellular polarity and interact in this process (Figure 1). Futhermore, mutants of both *Dg* and *Dys* genes show symptoms observed in muscular dystrophy. Reduction of Dg and Dys proteins results in age-dependent mobility defects (Figure 3). Eliminating Dg and Dys specifically in mesoderm derived tissues reveals that these proteins are required for muscle maintenance in adult flies: age-dependent muscle degeneration was observed in mutant tissues (Figure 4). Dg–Dys complex is also required for neuron path-finding and has both cell autonomous and non-cell autonomous functions for this process (Figures 5 and 6). Further, we have now shown that in neuronal path-finding process Dg interacts with InR and an SH2/SH3-domain adapter molecule Nck/Dock (Figure 7).

Drosophila as a muscular dystrophy model

Animal models have been used efficiently in muscular dystrophy studies. Some of the models are naturally occurring mutations (mdx-mouse, muscular dystrophy dog, cat and hamster), others have been generated by gene targeting (Watchko *et al*, 2002). However, the regulation and the control of Dg–Dys complex are not understood, and no successful therapeutics exist yet for muscular dystrophies

(however, systemic delivery-studies using adeno-associated viral vectors show promise (Gregorevic *et al*, 2004)). Studies in new model organisms with easy-to-manipulate genetics might reveal the mode of regulation of the complex by identifying key regulatory components through suppressor screens. In addition, careful functional analysis of the complex in different cell types in model organisms might result in a unifying theme that will reveal its molecular mechanism of function. Such recently developed models for muscular dystrophy exist in *C. elegans* and zebrafish (Gieseler *et al*, 2000; Parsons *et al*, 2002; Bassett and Currie, 2003). In *C. elegans Dys* mutant, the transporter *snf-6* that normally participates in eliminating acetylcholine from the cholinergic synapses, is not properly localized, resulting in an increased acetylcholine concentration at the neuromuscular junction and muscle wasting (Kim *et al*, 2004). The function of Dys in neuromuscular junctions has also been recently addressed in *Drosophila* (van der Plas *et al*, 2006). These results bring up the possibility that muscular dystrophies in humans might also at least partly be attributed to the altered kinetics of acetylcholine transmission through neuromuscular junctions.

We have now shown that *Drosophila melanogaster* acts as a remarkably good model for age-dependent progression of muscular dystrophy. Dg and Dys reduction in *Drosophila* show age-dependent muscle degeneration and lack of climbing ability. It is tempting to speculate that the common denominator between different defects observed in *Dg–Dys* mutants in *Drosophila* and *C. elegans* is defective cellular polarity. The defects observed in *C. elegans* could be due to a defect in polarization of a cell, which will generate a neuromuscular junction that leads to miss-targeted *snf-6*. Similarly, we have shown that *Drosophila* Dg–Dys complex is required for cellular polarity in the oocyte. In addition, neural defects observed are plausibly due to polarity defects in the growing axon.

Dg–Dys complex in axon path-finding

Similar to neuronal defects observed in human muscular dystrophy patients, neuronal defects were also found in *Drosophila Dg* and *Dys* mutant brains. In vertebrate brains, Dg affects neuronal migration (Montanaro and Carbonetto, 2003; Qu and Smith, 2004) possibly through interaction of neurons with their glial guides. The neuronal migration and process outgrowth have been shown to require supportive input from glial cells and involve the formation of adhesion junctions along the length of the soma. Also, the outgrowth of the leading process involves rapid extension and contraction over the length of the glial fiber (Rivas and Hatten, 1995; Shaham, 2005). Disruption of the cytoskeletal organization within the neuron, either of actin filaments (Rivas and Hatten, 1995) or microtubule interactions (Vallee *et al*, 2000), has been shown to inhibit glial-mediated neuronal migration. The glial function in this process is less well studied.

Drosophila photoreceptor path-finding provides an excellent system for genetic dissection of neuronal outgrowth and target recognition (Dickson, 2002). During the formation of the nervous system, newly born neurons send out axons to find their targets. Each axon is led by a growth cone that responds to extracellular axon guidance cues and chooses between different extracellular substrates upon which to migrate. Recent work has also identified a variety of intracellular signaling pathways by which these cues induce cytoskeletal rearrangements (Guan *et al*, 1996; Rao, 2005), but the proteins connecting signals from cell surface receptors to actin cytoskeleton have not been clearly determined. Dg is a good candidate for linking receptor signaling to the remodeling of the actin cytoskeleton and thereby polarizing the growth cone. We have now shown that perturbation of Dg–Dys complex causes phenotypes that resemble Nck/Dock-Pak-Trio axon path-finding phenotypes (Figure 5) (Rao, 2005), suggesting that Dg may be one of the key players in Nck/Dock signaling pathway for axon guidance and target recognition in *Drosophila*.

Interestingly, Insulin receptor-tyrosine kinase (InR) mutants also show similar phenotypes to those of Nck/Dock signaling in photoreceptor axon path-finding and these two proteins show genetic and biochemical interactions (Song *et al*, 2003). These data have led to speculations of mammalian InR acting in conjunction with Nck/Dock pathway in learning, memory and eating behavior (Dickson, 2003; Song *et al*, 2003). Our data now add Dg–Dys complex to this pathway; similar to what is seen in the case of Dg and Dys photoreceptor mutants, InR mutants show no obvious defects in patterning of the photoreceptors. However, the guidance of photoreceptor cell axons from the retina to the brain is aberrant (Song *et al*, 2003; Figures 5 and 6). Furthermore, genetic and biochemical evidence suggests that InR function in axon guidance involves the Dock-Pak pathway rather than the PI3K-Akt/PKB pathway. Independently, biochemical interaction between Nck/Dock and Dg has been reported (Sotgia *et al*, 2001) supporting the hypothesis that InR, Dg and Nck/Dock interaction regulates Dg–Dys complex. Furthermore, we have now shown that Dg interacts genetically with InR and Dock in photoreceptor axon path-finding. Since Dys interacts with Dg but not with InR and Dock, it is tempting to speculate that Dg can selectively interact with either Dys or InR and Dock (Figure 7). One possibility is that the tyrosine kinase activity of InR could regulate the Dg–Dys interaction by tyrosine phosphorylation in the Dg–Dys binding interphase (Figure 2). This tyrosine phosphorylation could prohibit the Dg–Dys interaction and thereby result in rearrangements in the actin cytoskeleton. Alternatively, other components observed in Dg–Dys complex might be involved in this regulation (Zhan *et al*, 2005). However, it is also possible that potential polarity defects in the Dg mutant axons result in defective InR membrane localization. Interestingly, in another cell type, the *Drosophila* oocyte, InR, Dg and Dys also show similar phenotypes (Deng *et al*, 2003; LaFever and Drummond-Barbosa, 2005; Figure 1). In addition, insulin-like growth factors (IGF) and InR are important in maintaining muscle mass in vertebrates (Singleton and Feldman, 2001). Further connection of InR to Dg–Dys complex comes from experiments showing that muscle specific expression of IGF counters muscle decline in mdx-mice (Barton *et al*, 2002; Shavlakadze *et al*, 2004; Dobrowolny *et al*, 2005). The work presented in this study is the first demonstration of genetic interaction between Dg and InR. Future biochemical studies should unravel the molecular mechanism of this interaction.

Furthermore, we have now shown that Dg–Dys complex is required both in neural and in targeting glial cells for correct neuronal axon path-finding in *Drosophila* brain. These data reveal that Dg–Dys complex also has a non-cell autonomous

effect on axon path-finding and suggest that Dg–Dys-controlled ECM both from neuron and glial cells regulate neuronal axon path-finding. Further experiments are required to reveal whether long-range Laminin fibers are involved in this process, as has been shown in epithelial planar polarity (Bateman *et al*, 2001; Deng *et al*, 2003), or whether glial processes are observed in close proximity to the neural growth cone (Georges-Labouesse *et al*, 1998). Interestingly, similar phenotypes are observed with Integrin mutants (Tanaka and Sabry, 1995; Campos, 2005; Curtin *et al*, 2005), suggesting that, as in planar polarity (Bateman *et al*, 2001; Deng *et al*, 2003), Integrin and Dg–Dys complex might act in concert to regulate the process of ECM organization that will regulate the cytoskeleton of the cells involved.

Taken together, the phenotypes caused by *Drosophila Dg* and *Dys* mutations are remarkably similar to phenotypes observed in human muscular dystrophy patients, and therefore suggest that functional dissection of Dg–Dys complex in *Drosophila* should provide new insights into the origin and potential treatment of these fatal neuromuscular diseases. As a proof of principle, using *Drosophila* as a model we have now identified InR as a signaling pathway that genetically interacts with Dg. Future studies are directed to unravel the molecular mechanism of Dg and InR–Dock interactions in invertebrates as well as vertebrates.

Materials and methods

Fly stocks

FRT42D Dg^{323}*/Cyo* and *FRT42B* Dg^{323}*/CyO (Dg* null allele), *UASdsDg* (*dsDg30A* and *dsDg33A* (Deng *et al*, 2003), *Df(3R)Dl-X43* (referred as *DysKX43*), EP(3)3397*(Dys)* (Bloomington Stock Center), the deletion mutant $Dys^{8\text{-}2}$ in *Dys* gene that was generated by inducing transposition of the EP(3)3397 P-element insertion (http://engels.genetics.wisc.edu//Pelements/index.html), Dys^{e6} deletion mutant (van der Plas *et al*, 2006), three *dsRNA* constructs were created to knock out the different Dys transcripts: *UASdsDysN-term (dsDys N-term)* knocks out the three long forms (DLPs), *UASdsDysRB (dsDys RB)* the short form (Dp186), and *UASdsDysC-term (dsDys C-term)* targets the common C-terminus, thereby knocking down all transcripts (see Supplementary Materials and Methods), *yw;FRT82BpM88C* InR^{34}*/TM6, FRT82B* $InR^{ex52.1}$*/TM6* (gifts from B Edgar), $dock^{P1}$*FRT40A/CyOGFP, yw;eyFlpgl-lacZ;Trio*^{1}*FRT80B/TM6, yw;eyFlpgl-lacZ;* Pak^{14}*FRT82B/TM6* (gifts from N Harden), *hsFLP; FRT42DUbi-GFP/Cyo* and *eyFLPGMR-lacZ; FRT42D l(2)cl-*$R11^{1}$*/CyO, Gal4-elav hsFLP; FRT42B tubGal80/*CyO^{3}*; act-GFP* and *P-tub-Gal4* (ubiquitous expression), *w;MatTub-Gal4/CyO* (germline expression), *GMR-Gal4* (eye expression), w^{-}*;;24B-Gal4* (mesoderm, muscle expression), w^{-}*;;repo-Gal4/TM3,Sb* (glial expression) from Bloomington Stock Center.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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