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Complex interaction of *Drosophila* GRIP PDZ domains and Echinoid during muscle morphogenesis

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Glutamate receptor interacting protein (GRIP) homologues, initially characterized in synaptic glutamate receptor trafficking, consist of seven PDZ domains (PDZDs), whose conserved arrangement is of unknown significance. The *Drosophila* GRIP homologue (DGrip) is needed for proper guidance of embryonic somatic muscles towards epidermal attachment sites, with both excessive and reduced DGrip activity producing specific phenotypes in separate muscle groups. These phenotypes were utilized to analyze the molecular architecture underlying DGrip signaling function *in vivo*. Surprisingly, removing PDZDs 1–3 (DGripΔ1–3) or deleting ligand binding in PDZDs 1 or 2 convert DGrip to excessive *in vivo* activity mediated by ligand binding to PDZD 7. Yeast two-hybrid screening identifies the cell adhesion protein Echinoid's (Ed) type II PDZD-interaction motif as binding PDZDs 1, 2 and 7 of DGrip. *ed* loss-of-function alleles exhibit muscle defects, enhance defects caused by reduced DGrip activity and suppress the dominant DGripΔ1–3 effect during embryonic muscle formation. We propose that Ed and DGrip form a signaling complex, where competition between N-terminal and the C-terminal PDZDs of DGrip for Ed binding controls signaling function.

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Introduction

Nascent somatic muscles use growth-cone-like projections to navigate towards specialized epidermal cells (tendon cells) during mid-embryonic development of *Drosophila*. Distinct cell guidance systems have been suggested to control targeting to tendon cells by specific muscle groups. The molecular apparatus sending and interpreting muscle guidance cues are only partially known (Volk and VijayRaghavan, 1994; Frommer *et al*, 1996; Kramer *et al*, 2001; Schnorrer and Dickson, 2004; Steigemann *et al*, 2004; Swan *et al*, 2004). Some of the best characterized players in this process are the Robo-Slit guidance system proteins (Kramer *et al*, 2001), which act in specific subsets of somatic muscles to selectively adhere to certain target tendon cells via PS-integrins (Fernandes *et al*, 1996). Other highly conserved signaling systems such as the EGF receptor pathway (Yarnitzky *et al*, 1998; Volk, 1999) and Wnt signaling (Volk and VijayRaghavan, 1994; Ghazi *et al*, 2003) also operate during muscle guidance in both embryos and pupae to select and reinforce developmentally programmed signaling between the muscle cell and its target tendon cell.

We recently found that the glutamate receptor interacting protein (DGrip) is also required for proper targeting of nascent muscles towards an attractive signal expressed at segment borders of *Drosophila* embryos (Swan *et al*, 2004), with genetic elimination of *dgrip* resulting in specific defects in patterning of ventro-lateral muscles (VLM, see also Figure 1A). In contrast, lateral transverse muscles (LTMs), which attach within segments, appeared unaffected in *dgrip* mutants (Figure 1A). Conversely, strong, pan-muscular overexpression of DGrip causes LTMs to produce projections forming ectopic attachment sites, while VLMs appeared unaffected (Swan *et al*, 2004).

DGrip consists of seven PSD-95/Discs-large/ZO-1 domains (PDZDs) but no other known protein–protein interaction motifs. The ~100–150 predicted PDZ proteins in the human genome are thought to direct the polarized localization of many developmentally and physiologically important membrane proteins. Indeed, in recent years, interaction screens have resulted in an explosion in the number of mammalian PDZ proteins identified as binding partners for growth factor receptors, G-protein-coupled receptors, neurotransmitter receptors, ion channels and adhesion molecules (Ranganathan and Ross, 1997; El Far and Betz, 2002).

Mammalian GRIP and GRIP2/ABP were identified by their interaction with AMPA-type glutamate receptors GluRs 2 and 3, and implicated in activity-dependent and subunit-specific GluR trafficking (Dong *et al*, 1997; O'Brien *et al*, 1998; Srivastava *et al*, 1998; Wyszynski *et al*, 1999, 2002; Liu and Cull-Candy, 2005). While genetic analysis has not yet shown an essential function for GRIP proteins in AMPA receptor clustering, GRIPs are meanwhile thought to participate in numerous cellular functions. GRIP1 mutant mice display

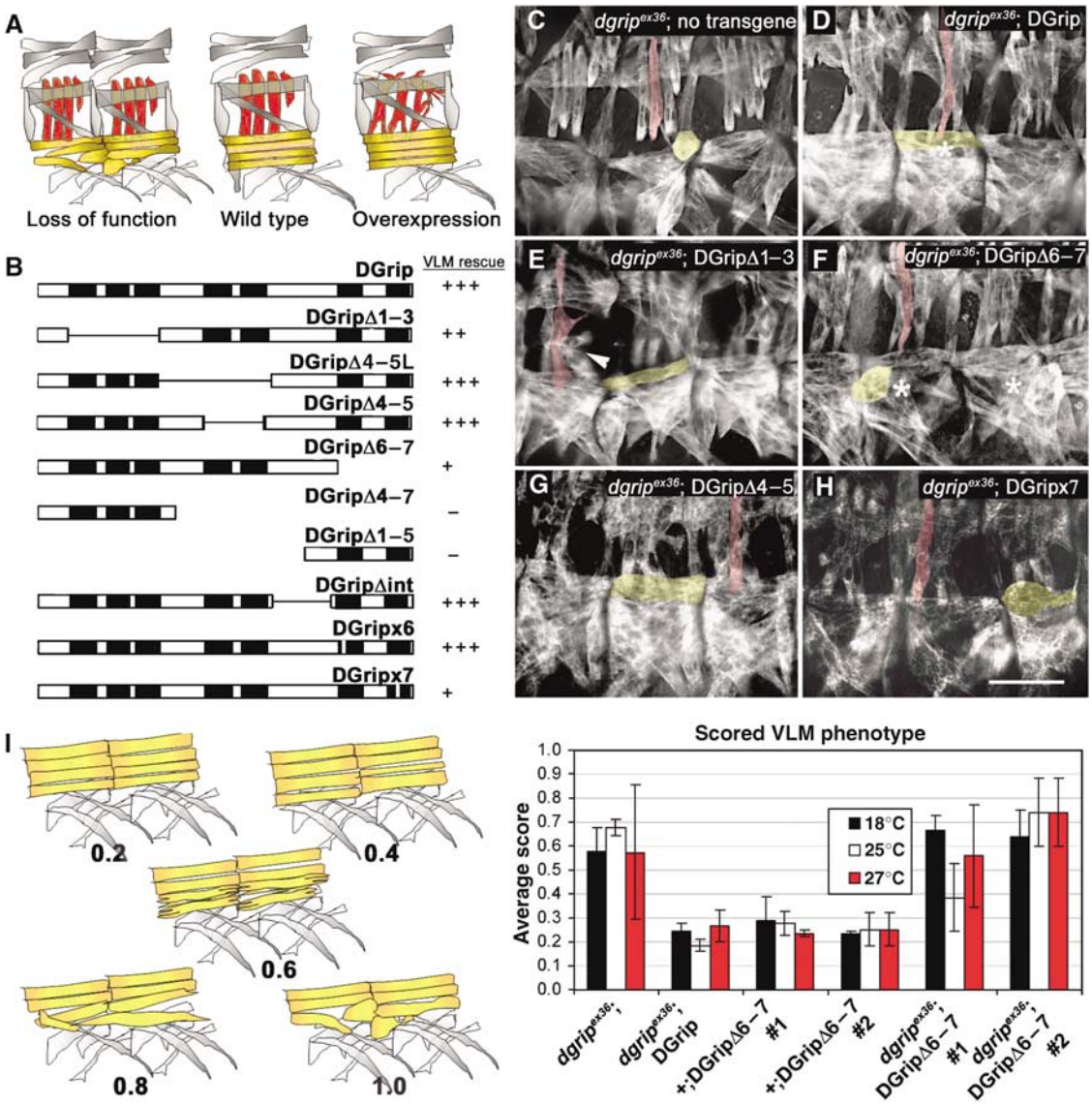


Figure 1 A structure–function map for DGrip in morphogenesis of VLM. (**A**) Scheme of muscle phenotypes evoked by DGrip loss of function and overexpression. Loss of DGrip function primarily affects VLM (yellow, see (C)) whereas LTM (red) remain unaffected. Strong overexpression of DGrip using *24B-gal4* disturbs LTM morphology (Swan *et al*, 2004). (**B**) Scheme of rescue activities of indicated DGrip constructs. Rescue of VLM morphology in *dgrip*^{ex36} mutant background is shown. Pan-muscular expression of wild-type DGrip using *twist-gal4* fully rescues the *dgrip*^{ex36} VLM phenotype (+ + +), while other constructs have a reduced rescue ability (+ +, +) or exert no effect (−) on muscle rescue. All constructs were characterized in at least two independent lines. (C–H) Muscle myosin stainings in stage 16 embryos. (**C**) *dgrip*^{ex36}, *twist-gal4* muscles show typical defects in VLM morphology. One VLM (yellow) and one LTM (red) are labeled for ease of identification. (**D**) Re-expression of wild-type DGrip using *twist-gal4* fully rescues these defects, whereas expression of DGripΔ1–3 in the *dgrip*^{ex36}, *twist-gal4* background (**E**) provokes strong dominant LTM (arrowhead) and slight VLM morphology defects. (F, H) Expression of DGrip missing the C-terminal PDZDs (DGripΔ6–7 (**F**) and DGripx7 (**H**)) results in only partial rescue of *dgrip*^{ex36} VLMs, with many VLMs appearing atypically round (asterisk in (F) compare to asterisk in (D)). (**G**) Constructs missing PDZDs 4 and 5 (DGripΔ4–5) behave like wild-type DGrip, fully rescuing the *dgrip*^{ex36} defect, without provoking LTM defects. Scale bar in (H): 30 μm. (**I**) Quantification of rescue activity for DGripΔ6–7. Left: scheme of VLM defects used as 'clinical score' (between 0.2 and 1) for quantification. Right: Average scores from over 30 larval hemisegments per condition are plotted, raising temperatures used indicated in colors. While DGripΔ6–7 hardly rescues *dgrip*^{ex36} VLM defects, no dominant effects of DGripΔ6–7 expression are observed in *dgrip*^{ex36}/+ heterozygous background.

kidney agenesis, polydactyly, syndactyly and gross morphological defects of the brain, a phenotype comparable to the human Fraser syndrome (Takamiya *et al*, 2004). GRIP has also been shown to interact with members of several signaling pathways including ephrins (Bruckner *et al*, 1999; Lin *et al*, 1999; Contractor *et al*, 2002; Hoogenraad *et al*, 2005) and liprins (Baran and Jin, 2002; Wyszynski *et al*, 2002; Dunah *et al*, 2005).

A functional insight into the biological significance of PDZD-ligand interactions has generally been limited by a lack of readily observable phenotypes. We set out to study the functional logic of DGrip, using its penetrant and easily scoreable phenotypes in *Drosophila* muscle guidance. Mutation and deletion analysis of PDZDs within DGrip strongly suggested that DGrip is indeed an integrative molecule, where PDZD-mediated interactions distributed over DGrip can have positive and negative influence on guidance function. We provide evidence that one particular PDZD ligand—the cell adhesion molecule Echinoid (Ed)—executes *both* positive and negative interactions on DGrip for muscle

guidance. We speculate that a complex interaction between DGrip PDZDs and Ed may spatio-temporally fine tune muscle guidance.

Results

Structure–function analysis for Drosophila Grip using loss- and gain-of-function phenotypes

GRIP proteins are evolutionarily conserved as a string of seven PDZDs, whose functional significance is so far unknown. Genetic elimination of *dgrip* results in defects of VLM (Swan *et al*, 2004), which are schematized in yellow in Figure 1A. Instead of forming a single polarized muscle projection, $dgrip^{-}$ VLMs frequently send out two or more projections in essentially randomized directions. When the muscle guidance period ceases, $dgrip^{-}$ VLMs typically appear 'frozen' in ball-like VLMs extending over only about half of a hemisegment without reaching their target tendon cell. In result, *dgrip* VLMs form ectopic, integrin-positive adhesion points on the epidermis and other muscles, away from tendon cells (Figure 1A; Swan *et al*, 2004). In contrast, LTMs (red in Figure 1A), which attach within segments, appear unaffected in *dgrip* mutants. Complementarily, strong pan-muscular overexpression of DGrip (using the driver *24B-gal4* together with two copies of *UAS-dgrip*) causes LTMs to produce projections that ectopically attach at segment borders (Swan *et al*, 2004). In this study, these two phenotypes formed a basis to study the function of individual DGrip PDZDs *in vivo*.

Muscle-specific re-expression of full-length DGrip cDNA fully rescues the VLM defect (Swan *et al*, 2004) in embryos hemizygous for *dgrip* null allele $dgrip^{ex36}$ (denoted $dgrip^{ex36}$). A structure–function map for DGrip was established by expressing at least two independent transgenic lines of DGrip variants in *dgrip* background using the muscle-specific *twist-gal4* driver (Figure 1B). Muscle defects were directly scored in late stage embryos. In addition, larval muscles, which due to their larger size allow reliable identification of more subtle defects, were analyzed as well. This was possible as the *twist-gal4* driver used in this study does not express in larval somatic muscles, restricting effects on muscle morphogenesis to embryonic stages. To test for the influence of expression strength on phenotypes, the *Gal4/UAS* expression system (Brand and Perrimon, 1993) temperature dependence was utilized by rearing animals at 18, 25 or 29°C to evoke successively higher levels of expression. When necessary, muscle morphogenetic defects were quantified by assigning scores to progressively more severe muscle defects, with more than 30 individual hemisegments being evaluated per genotype (Figures 1I and 2D).

A transgene with PDZDs 4 and 5 deleted (DGripΔ4–5; Figure 1B and G) rescued the $dgrip^{ex36}$ VLM misguidance phenotype to wild-type using *twist-gal4* even at 18°C (minimal expression conditions), as does the full-length DGrip cDNA (Figure 1D). Thus, PDZDs 4 and 5 appear not to have a determining role in DGrip function. Similarly, DGripΔ4–5L, which additionally removed a region between PDZDs 3 and 4 (Figure 1B) allowed full rescue of $dgrip^{ex36}$ muscles.

In contrast, removal of PDZDs 6 and 7 (DGripΔ6–7, Figure 1B and F) produced a protein that could scarcely rescue the $dgrip^{ex36}$ muscle phenotype (for detailed analysis see Figure 1I). Thus, PDZDs 6 or 7 of DGrip appear be to involved in DGrip's function in muscle morphogenesis. For this reason, we tested the functional role of ligand binding to these domains. Point mutations disrupting the PDZD 6 or PDZD 7 ligand binding surfaces (see Supplementary data; Daniels *et al*, 1998; Edwards and Gill, 1999; Lou *et al*, 2001) were introduced, giving DGripx6 and DGripx7. DGripx6 was able to fully rescue $dgrip^{ex36}$ VLMs, suggesting that PDZD 6 is not important for DGrip-dependent muscle function. DGripx7, however, showed impaired ability to rescue $dgrip^{ex36}$ VLMs (Figure 1H), very similar to the reduced rescue function of DGripΔ6–7. Thus, ligand binding to PDZD 7, but not to 4, 5 or 6 was found to be important for DGrip function within muscle morphogenesis.

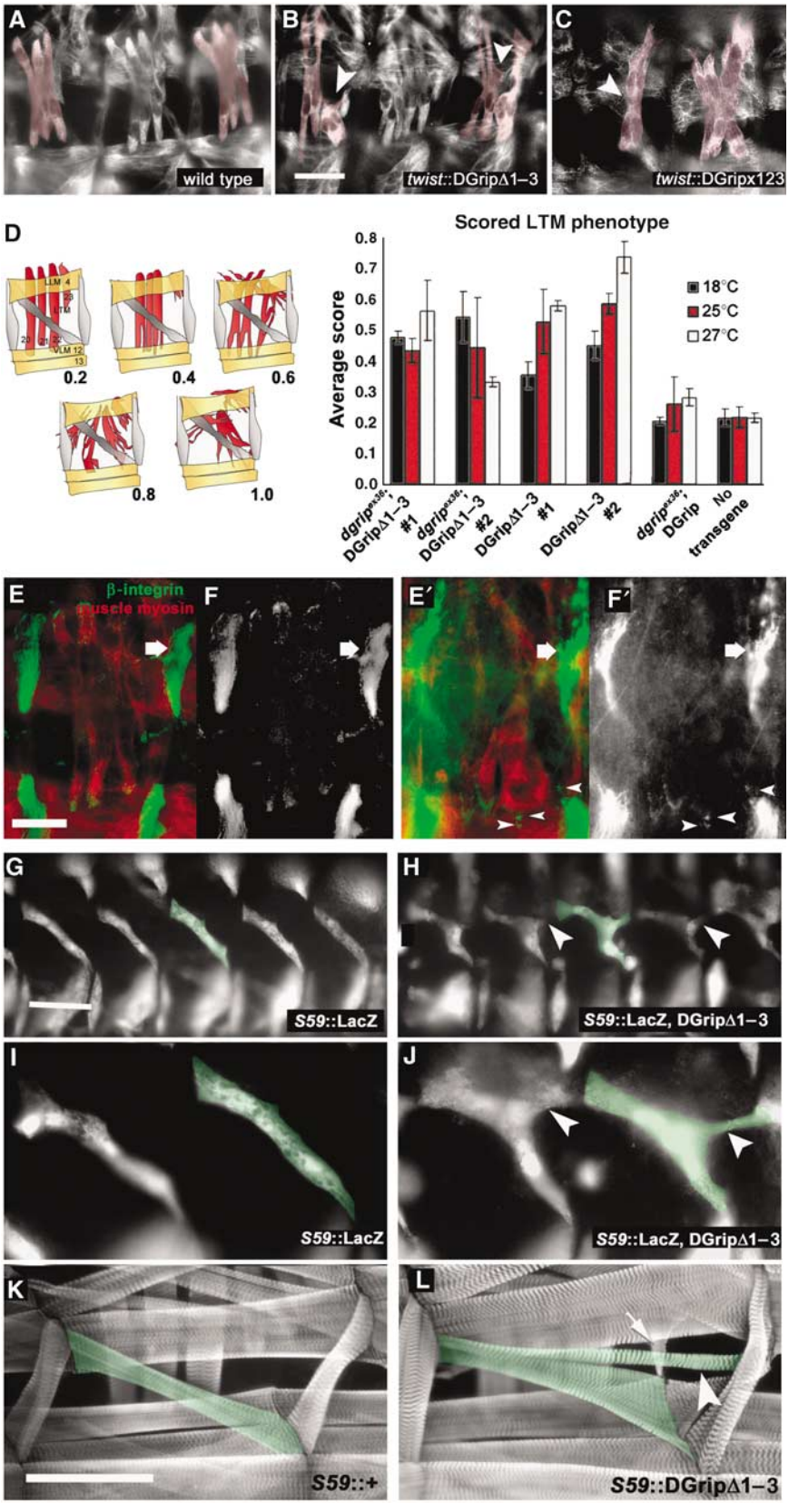
DGripΔ1–3 is an overactive species provoking ectopic projections during muscle guidance

Next, the functional importance of the N-terminal PDZDs was investigated. To this end, the first three PDZDs were deleted and the resulting DGripΔ1–3 expressed in $dgrip^{ex36}$ background. DGripΔ1–3 expression clearly restored VLM morphology to a level closer to wild-type when compared with $dgrip^{ex36}$ controls, with some minor defects still detectable (Figure 1E, yellow marked VLM). However, DGripΔ1–3 expression also evoked the same slight VLM defects in the $dgrip^{ex36}/+$ background, that is, in the presence of one wild-type *dgrip* gene copy.

VLM defects were never observed when expressing full-length DGrip using *twist-gal4,* despite the RT–PCR levels of both transgenes being very similar (Supplementary data, for brevity, all subsequent experiments were performed utilizing the *twist-gal4* driver at 25°C). This suggested that DGripΔ1–3 was a dominant, overactive DGrip species. In contrast to VLMs, LTMs consistently exhibited very strong defects when

Figure 2 DGripΔ1–3 provokes defects during embryonic muscle guidance. (A–C) Muscle myosin stainings in stage 16 embryos, some LTMs colored in red. (**A**) wild type; (**B**) DGripΔ1–3- and (**C**) DGripx123-expression in wild-type background with the pan-muscular driver *twist-gal4* causes LTM defects (arrowheads). LTM defects include splitting into multiple projections and bending away from their target tendon cells. (**D**) Quantification of LTM morphology defects after DGripΔ1–3 expression scored by average clinical score ($n > 30$, larval hemisegments per condition). At different expression levels (controlled by raising temperature) and with two independent transgenes, DGripΔ1–3 provokes LTM defects neither present in animals comparably expressing wild-type DGrip nor in *dgrip* mutants. (E, F′) Wild-type embryo (**E, F**) and *twist*::*dgripΔ1–3* (**E′, F′**) embryos costained for integrin (green) and muscle myosin (red) (E, E′), (F and F′) show integrin channel only. In *twist-gal4*::*dgripΔ1–3* embryos, LTMs form aberrant, integrin positive attachments in ectopic positions (arrowheads). The large integrin-positive attachment sites of segment border attaching muscles are labeled by arrows. (**G–I**) Genetically labeling embryonic muscle 5 (individual muscle 5 labeled in green) with the *S59-gal4* driver reveals guidance defects in DGripΔ1–3 expressing muscles. (H, **J**) *S59-gal4*::GripΔ1–3, lacZ muscles shortly after guidance process showing extra filopodia-like projections (arrowheads) not present in wild-type muscle 5 (G, I). (**K, L**) Ectopic projections (arrowheads) of muscle 5 (green) established in embryogenesis are still present in larval stages of *S59-gal4*::DGripΔ1–3 larvae (L, labeled in green) but not in *S59-gal4/+* controls (K). In addition, other muscles can ectopically adhere to DGripΔ1–3 expressing muscles (L, arrow). Scale bar in (B): 20 μm; scale bar in (E): 15 μm; scale bar in (G): 30 μm in (K): 150 μm.

expressing DGripΔ1–3 in the $dgrip^{ex36}$, $dgrip^{ex36}/+$ or wild-type backgrounds: formation and stabilization of multiple projections with splits of embryonic muscles (Figures 1E, arrows, 2B, D and 3B). Defects appeared qualitatively very similar when scored in embryos (Figure 2) or in larvae (Figure 3). When quantified in larval stages, *twist-gal4*::DGripΔ1–3 provoked muscle defects were fully evident already at 18°C, expression conditions under which full-



length DGrip was unable to provoke any LTM defects (Figure 2D). Thus, DGripΔ1–3 apparently was an overactive DGrip species that could efficiently interfere with muscle morphogenesis.

The question arose whether DGripΔ1–3 overexpression executed its effects via interfering with proper guidance of nascent LTMs, similar to the manner that VLM guidance suffers from a loss of DGrip function. In fact, when driven with *twist-gal4*, DGripΔ1–3 provoked the formation of ectopic, integrin-positive attachment sites, obvious in late, muscle myosin-positive embryos (Figure 2E–F). However, the fact that *twist-gal4* drives expression in all muscles, interfered with directly scoring guidance behavior in individual muscles. Thus, we decided to use *S59-gal4*—labeling only muscles 5, 8, 25, 27 and 29 from stage 11 until the end of embryogenesis (Brennan *et al*, 1999)—to drive lacZ marker protein. When expressing DGripΔ1–3 with this driver, the full set of muscles expressing *S59* could be observed (Figure 2G), indicating proper determination of muscle cell fate in the presence of DGripΔ1–3 (Figure 2H). Of the LacZ labeled muscles, nascent muscle 5 (Figure 2G–L, green label) was best suited to score guidance behavior. In fact, muscle 5 overexpressing DGripΔ1–3 nearly always formed a third, ectopic projection attaching at the segment border (Figure 2G–L, arrowheads). These additional projections of *S59-gal4*::DGripΔ1–3 expressing muscles were still obvious in larval stages (Figure 2L). We conclude that loss of the first three PDZDs of DGrip renders the protein overactive, ectopically stabilizing projections during embryonic muscle guidance, which were fully propagated into larval muscle morphology.

PDZDs 1 and 2 mediate repression, PDZD 3 de-repression of DGrip activity

As DGripΔ1–3 is obviously an overactive species, we suspected PDZDs 1–3 conferred repression on DGrip activity. Thereby, dominant activity of DGripΔ1–3 may be either mediated by a loss of repressive interactions mediated via PDZDs 1–3 or by a more general structural defect of the protein. To discriminate between these possibilities, we tested if DGripΔ1–3's dominant activity could be mimicked by destroying ligand binding via PDZDs 1, 2 or 3. When the first three PDZD ligand binding surfaces (DGripx1, 2, 3) were mutated together, dominant defects on LTM morphology comparable to DGripΔ1–3 were observed in both embryos (Figure 2C) and larvae (Figure 3C). Dominant LTM defects were also present when only PDZD 1 (DGripx1, Figure 3D) and to a slightly lesser extent when only PDZD 2 (DGripx2, Figure 3E) ligand-binding was abolished. Both DGripx1 and DGripx2 rescued the

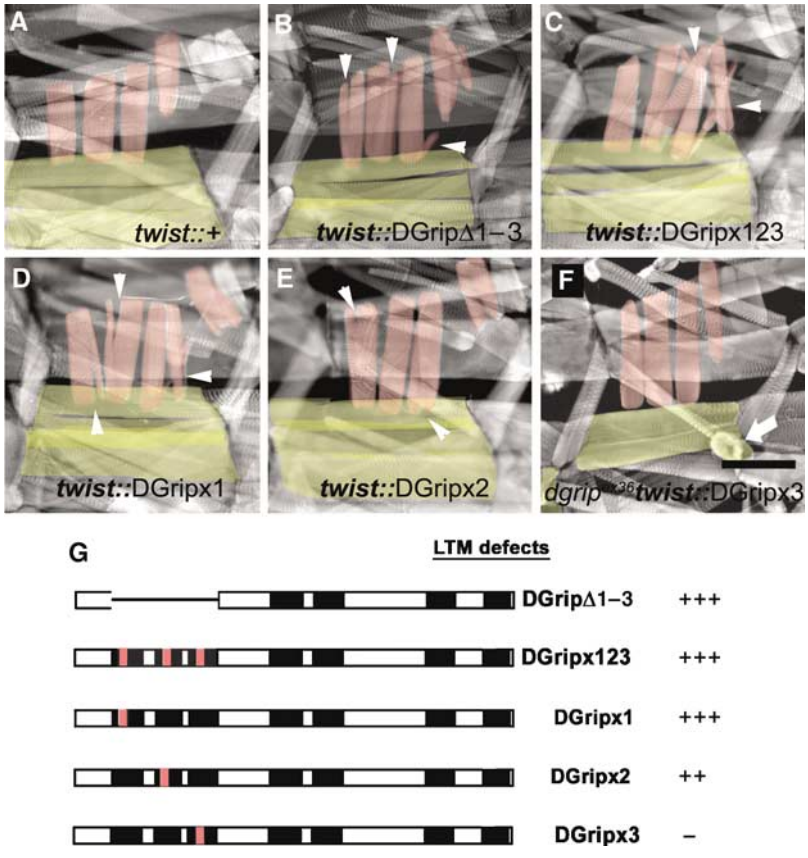


Figure 3 Removal of PDZDs 1–3, or of ligand binding surfaces of PDZD 1 or 2, results in dominantly active Dgrip. The dominant DGripΔ1–3 phenotype is recapitulated by specifically point mutating the ligand binding sites of individual PDZDs. (**A–F**) Phalloidin labeling of 3rd instar larvae, VLMs in yellow, LTMs in red. (A) Typical bar-shaped LTMs and VLMs in control larvae. (B) Ectopic LTM projections (arrowheads) and mild morphological defects of VLMs in DGripΔ1–3 expressing animal. This phenotype is fully recapitulated (arrowheads) in DGripx123-expressing larvae, where the ligand binding capability of the PDZDs 1–3 is disturbed by point mutations. Mutation of PDZD 1 only (D) results in a similar phenotype, expression of DGrip point-mutated at PDZD 2 only (E) produces slightly weaker dominant defects. (F) Mutation of PDZD 3 does not cause dominant defects, but does not allow proper rescue of *dgrip*^{ex36} VLMs (arrowhead). (**G**) Summary of LTM defects caused by *twist-gal4* driven expression of the indicated *dgrip* constructs. Scale bar in (F): 150 μm.

dgrip^{ex36} VLMs, again indicating that these are overactive DGrip species (not shown).

Unlike DGripx1 and DGripx2, DGripx3 did not allow a complete rescue of VLM defects of *dgrip*^{ex36} and showed only negligible dominant defects in LTM guidance (Figure 3F, VLM marked by arrow). Accordingly, DGripx3 appeared to have reduced DGrip activity. However, DGripΔ1–3 and DGripx1, 2, 3 had excessive activity. Thus, ligand binding via PDZD 3 is apparently relevant only in the presence of ligands binding PDZD 1 and 2. PDZDs 1 and 2 in return mediate repression of DGrip function. These findings are easiest explained by postulating that DGrip activity is normally repressed by ligand binding to PDZD 1 and 2, while ligand binding to PDZD 3 is needed to allow efficient de-repression of DGrip activity.

Non-PDZD regions of DGrip are dispensable for muscle guidance function

We had found that PDZDs 4, 5 and 6 when singly mutated (x6) or deleted (Δ4–5) did not affect DGrip guidance function. However, deletion or point mutation of PDZDs 3 and 7 (x3: Figure 3F, x7: Figure 1H, Δ1–3: Figure 1F) only partially compromised DGrip function *in vivo*. We thus investigated if critical functions of DGrip reside in regions between PDZDs. However, deleting the large regions between PDZD 3 and 4 (in DGripΔ4–5L) and between PDZD 5 and 6 (DGripΔint) did not compromise rescue function (Figure 1B).

Moreover, smaller clusters of PDZDs contributing to DGrip function show no activity in isolation. Neither the first three (DGripΔ4–7) nor the last two (DGripΔ1–5) PDZDs show rescue of VLM misguidance (Figure 1B) or any dominant activity.

DGrip interacts with the cell adhesion molecule Ed via a C-terminal PDZD-binding motif

To identify PDZD-interactors mediating DGrip function *in vivo*, we performed a yeast-two-hybrid (Y2H) screen using the first three PDZDs of DGrip as baits. Four independent clones encoding fragments of the cell adhesion molecule Ed were retrieved (Figure 4A). All fragments included the C-terminus of the molecule, which contains a type II PDZD-interaction motif (EIIV). Interaction of Ed with DGrip in Y2H was dependent on the EIIV motif (Figure 4C). Moreover, recombinant DGrip expressed in Sf.9 cells (Figure 4B) efficiently interacted with a matrix-bound peptide representing the C-terminal 10 amino acids of Ed (Ed1/2), but not with a scrambled version of it (control).

We mapped Ed binding versus individual PDZDs in the Y2H assay (Figure 4C). Ed binding to DGrip was dependent on an intact PDZD 2 and greatly weakened by point mutation of PDZD 1. Binding was unaffected by point-mutating PDZD 3. No interaction was found between Ed and PDZDs 4 and 5. Thus, Ed binds to PDZD 1 and 2 and might well be involved in the repressive function of these domains. Surprisingly, Ed also interacted with PDZD 7 in a manner that was dependent on the PDZD 7 ligand-binding surface and EIIV motif of Ed (Figure 4C).

Loss of Ed provokes defects in both LTMs and VLMs

Ed is an L1-CAM-like molecule, known as a regulator of both the EGF receptor (Bai *et al*, 2001; Escudero *et al*, 2003; Islam *et al*, 2003; Rawlins *et al*, 2003a, b; Spencer and Cagan, 2003)

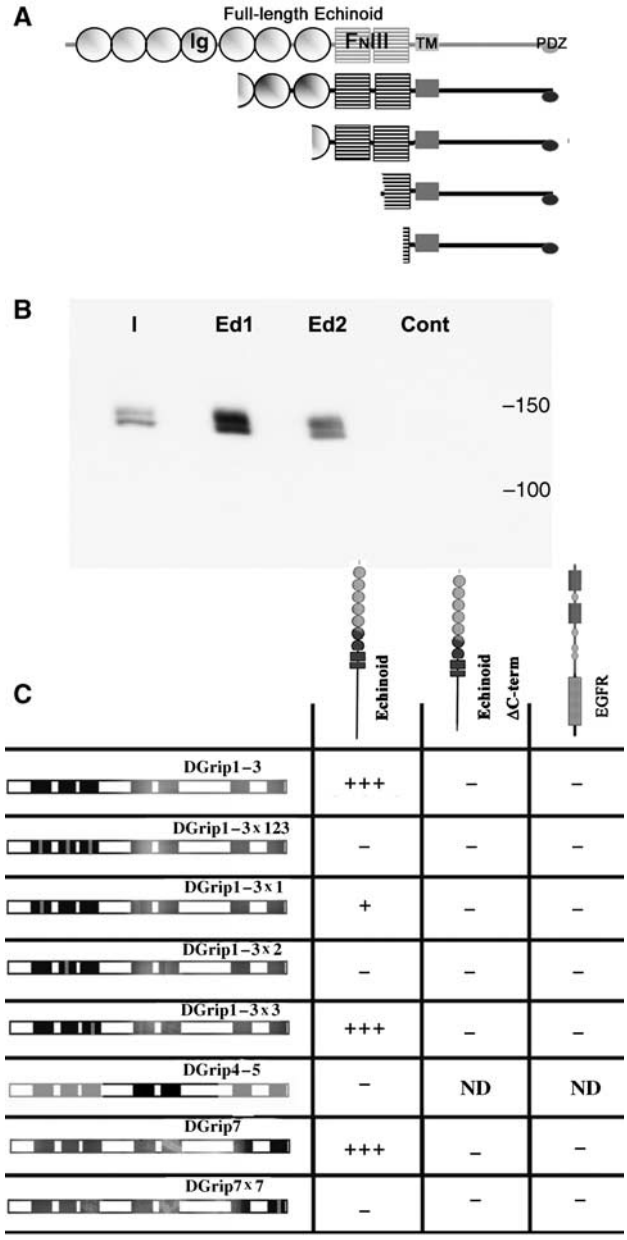


Figure 4 The cell-adhesion molecule Echinoid physically interacts with Dgrip. (**A**) Y2H screen performed using the first three PDZDs of DGrip as bait returned four independent isolates of the Immunoglobulin (Ig) and Fibronectin type III (FNIII)-domain containing cell adhesion molecule Echinoid (Ed). All isolates contained the transmembrane (TM) region and the entire cytosolic tail, including the EIIV PDZ-ligand motif. (**B**) Full-length, C-terminally myc-tagged DGrip expressed in Sf.9 cells specifically binds to a 10aa peptide representing the C-terminus of Ed. Shown is the input (I), binding of DGrip-myc to the Ed peptide (Ed1/2, two independent experiments), and binding to a 10aa scrambled control peptide (cont). (**C**) Y2H experiments reveal a specific pattern of Ed binding to DGrip PDZDs. The Ed cytosolic tail strongly interacts with a construct containing the first three PDZDs of DGrip, or containing PDZD 7 only (+++). This interaction was abolished by point mutation of PDZDs 2 or 7 (−), strongly reduced by point mutation of PDZD 1 (+) and unaffected by point mutation of PDZD 3 (+++). This interaction depended on the EIIV motif at the C-terminal of Ed, as Ed-ΔC-term did not interact with DGrip constructs. DGrip did not interact with the EGFR C-terminus, used here as a control, which has a C-terminal type I PDZ-ligand motif (ETRV).

and Notch (Ahmed *et al*, 2003; Escudero *et al*, 2003) signaling pathways. Ed has not previously been reported as having a phenotype related to muscle development, although both EGFR and Notch signaling are critical for the specification of muscle precursors (Artero *et al*, 2003). Using the $P(lacZ)ed^{k01102}$ line (lacZ is inserted in the first intron of *ed*) to mark *ed* expressing tissues, we found *ed* expression in nascent muscles (identified by the muscle precursor marker Vg (Ruiz-Gomez, 1998; Figure 5A)) as well as in many other cells. Moreover, antibodies against the intracellular tail of Ed also showed that Ed protein is in fact concentrated at the ends of embryonic VLMs, where these muscles contact the extracellular matrix (Figure 5B), colocalizing here with muscle expressed DGRIP-GFP (Figure 5C). To test for a role of Ed in muscle formation, muscle morphology was examined in several independent *ed* alleles. The ed^{1x5} allele (Bai *et al*, 2001) is predominantly embryonic lethal under our raising conditions, with some animals developing into second instar larvae. Consistent and prominent defects in muscle morphology could be found in both ed^{1x5} embryos (Figure 5E and E′) and larvae (Figure 5G). The same muscle defects were also observed in strong hypomorph ed^{SlH8} homozygotes (Figure 6I) or ed^{1x5}/ed^{SlH8} animals (Figure 5H). Loss of Ed function resulted in defects of both LTMs and VLMs. VLM defects were reminiscent of partial loss of DGrip function. As such, they showed 'fasciculated' VLMs also forming ectopic muscle–muscle adhesion within the segment (compare VLMs indicated by arrowheads in *echinoid* mutants in Figure 5H with partially rescued *dgrip* mutant in Figure 7G). Muscle-specific overexpression of Ed produced rather mild defects in LTM morphology (Figure 5I).

The *ed* muscle defects could, in principle, reflect a requirement for Ed in forming epidermal tendon cells. However, tendon cells are positioned correctly in ed^{1x5} zygotic mutants in stainings for tendon cell-specific markers (not shown). It appears very likely that additional removal of maternal Ed could reveal even stronger defects in muscle morphology. However, maternal *ed* is present in the epidermis in high amounts, making it unlikely that such a function could be demonstrated in the light of epidermal defects to be expected upon removal of maternal Ed.

Genetic interactions between DGrip and Ed signaling in muscles

Given that Ed physically interacts with DGrip, we asked whether DGrip and Ed functionally interacted *in vivo*. In fact, heterozygosity for ed^{1x5} strongly enhanced the VLM defects in $dgrip^{ex36}$ hemizygous embryos (Figure 6D). In severe cases, a complete disruption of the muscle field, in milder cases a strong enhancement of *dgrip* muscle defects (compare VLMs labeled by arrows in Figure 6D with B) was observed. LTMs were also defective in $dgrip^{ex36}/Y$; $ed^{1x5}/+$ (Figure 6D, asterisks), while they appeared normal in both $ed^{1x5}/+$ (Figure 6C) and $dgrip^{ex36}$ (Figure 6B). Thus, the reduction of Ed protein levels in embryonic muscles apparently uncovered a subcritical requirement for DGrip in LTM morphogenesis, leading to LTM defects. Therefore, Ed operates in LTM formation even in the absence of DGrip, and its requirement there becomes more obvious in the absence of DGrip. We conclude that DGrip and Ed functionally interact for VLM and, surprisingly, also LTM guidance. $dgrip^{ex36}$ muscles, including LTMs, were sensitive to *twist-gal4*-driven overexpression of Ed (Figure 6H, asterisks LTMs, arrows VLMs), whereas Ed expression in wild-type background produced only very minor defects (Figure 6G, asterisk and arrow). This again suggested that DGrip was functionally linked to Ed. In this context, however, DGrip acted as an inhibitor of excessive Ed-mediated signaling.

Mutations in **echinoid** *suppress dominant activity of DGripΔ1–3*

Our data indicated that Ed and DGrip interact physically, and that this complex is involved in controlling muscle morphology. Whether this interaction promoted or inhibited DGrip associated signaling was context-dependent, suggesting that their interaction with one another may be complex. Ed bound PDZDs 1–3 of DGrip as well as PDZD 7. Loss of Ed function should not be able to suppress DGripΔ1–3 activity if Ed binding to DGrip was in fact restricted to PDZDs 1–3. However, the LTM phenotype induced by *twist-gal4* mediated expression of DGripΔ1–3 protein was greatly diminished by homozygosity for the ed^{SlH8} chromosome. ed^{SlH8}*;twist-gal4*::*UAS-dgripΔ1–3* animals (Figure 6K) showed LTM phenotypes more closely resembling pure ed^{SlH8} homozygotes rather than identically processed and simultaneously raised *twist-gal4*::*UAS-dgripΔ1–3* controls (Figure 6J). These interactions suggest that Ed is involved in mediating the unrepressed activity of the DGripΔ1–3 protein.

Ed binds to the PDZDs controlling muscle guidance activity

In our Y2H assay, Ed specifically interacted with PDZD7, as well as with the 'repressive' PDZDs 1 and 2. As DGripΔ1–3 activity was repressed by reduction in Ed protein, we asked whether the activity of DGripΔ1–3 might be regulated by interactions via PDZDs 6 or 7. To this end, transgenic lines expressing DGripΔ1–3x6 and DGripΔ1–3x7 were constructed and expressed with *twist-gal4* in $dgrip^{ex36}$ background. The PDZD 6 point mutation did not suppress the dominant action of DGripΔ1–3 but still allowed VLM rescue. DGripΔ1–3x6 behaved identically to DGripΔ1–3, suggesting that PDZD 6 does not mediate DGrip guidance activity after its de-repression in DGripΔ1–3 (not shown). In contrast, DGripΔ1–3x7 showed a severely impaired ability to rescue $dgrip^{ex36}$ VLMs in embryos and larvae (Figure 7F, arrowheads) when compared to DGripΔ1–3 (Figure 7E). Consistently, expression of DGripΔ1–3x7 (Figure 7B, embryo, D larva) produced only mild defects in LTMs comparable to the mild defects obtained by expressing DGripx7 protein alone (Figure 7G, arrows), but not to the severe LTM defects observed upon DGripΔ1–3 expression (Figure 7A and E). Thus, ligand binding to PDZD 7 but not to PDZD 6 appeared to be a significant mediator of DGripΔ1–3 activity.

We thus propose that DGrip and Ed functionally interact during muscle guidance. Reduction of Ed protein or defective binding to PDZD 7 of DGrip interfered with the overactivity of DGripΔ1–3, thus suggesting a model (Figure 7H) where DGrip is responsible for the equilibrium between 'repressive' and 'active' Ed signaling.

Discussion

We have used genetics to develop a mechanistic model concerning a well-defined function mediated by *Drosophila*

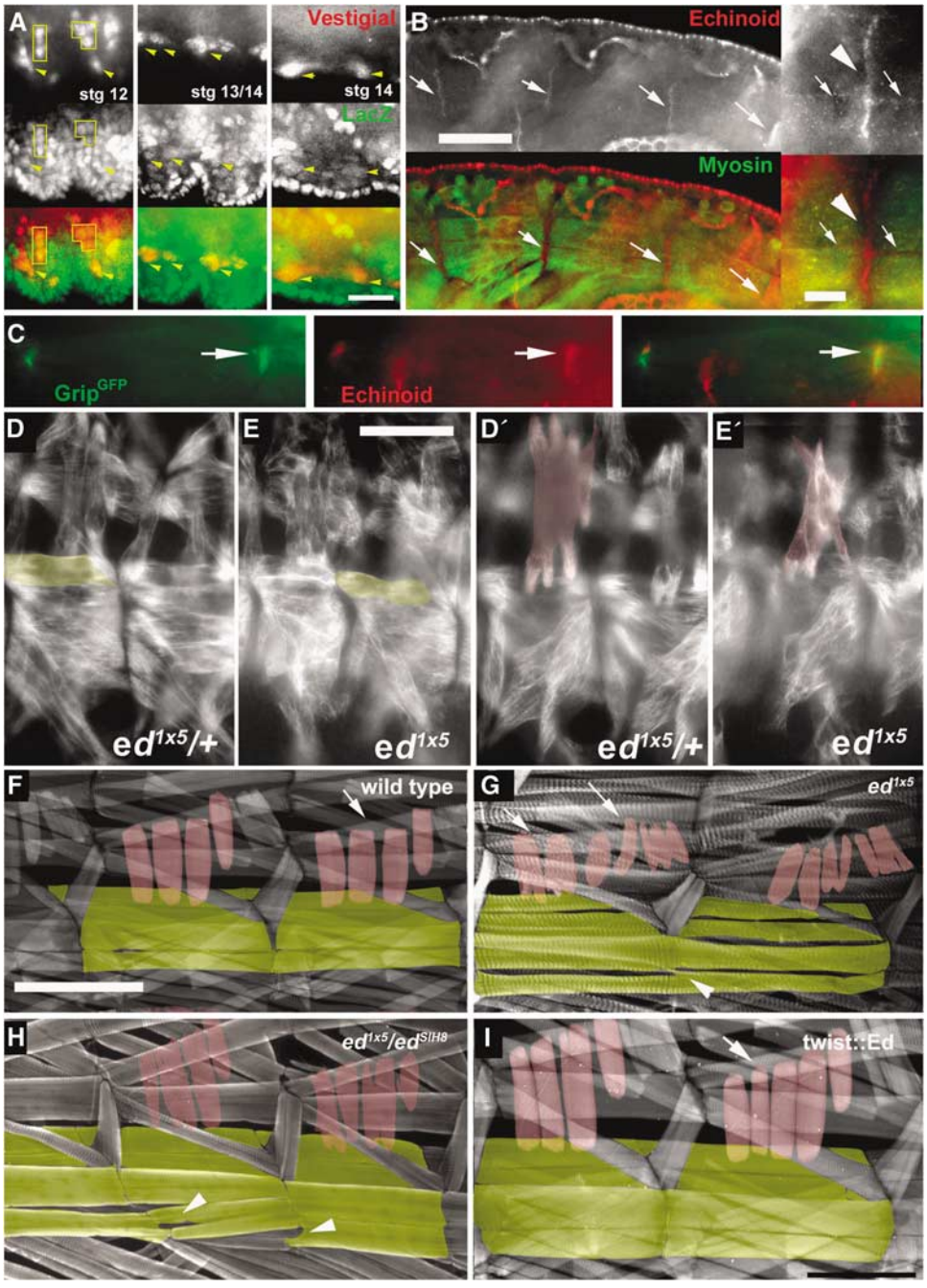


Figure 5 Echinoid in muscle morphogenesis of the *Drosophila* embryo. (**A**) Expression of lacZ (green) from the *ed* locus in *P(lacZ)ed*^{k01120} combined with labeling of muscle precursors with Vestigial (red). Scale bar in (A): 10 μm. (**B**) Anti-Ed antibodies (red) stain the ends of morphologically mature VLMs (arrows) visualized by antibodies against muscle myosin (green). Scale bar in (B): 30 μm; Inset: magnified view of Ed accumulation at muscle ends (arrowhead). Some Ed protein is also found at other parts of the muscle membrane (arrows). Scale bar in inset: 5 μm. (**C**) Ed protein colocalizes with muscle expressed *twist-gal4*::*DGrip-GFP* at muscle ends (arrows). (**D–H**) *ed* mutants displaying morphological defects of both VLMs (yellow) and LTMs (red) in embryos (D–E′) and larvae (F–H). (D, **D′**) *ed*^{1x5}/+ control embryo showing normal VLMs and LTMs, respectively. (E, **E′**) *ed*^{1x5} embryo shows defects in both VLM and LTM morphology. The same muscle field is shown in two focal planes. (F) Control larva. (G) The strong *ed* allele *ed*^{1x5} produces few homozygous larvae, which survive to 2nd instar. In these larvae, defects in VLMs (arrowhead) and LTMs (arrows) are evident, with both muscle groups forming aberrant projections and ectopic adhesion points. (H) Similar muscle phenotypes are also observed in 3rd instar larvae of the genotype *ed*^{1x5} over the strong hypomorphic allele *ed*^{SIH8} (arrows and arrowheads indicate VLMs and LTMs respectively). (**I**) Only minor defects of LTMs are evoked by pan-muscular expression of Ed. Scale bar in (E): 35 μm; Scale bar in (F): 200 μm.

Grip—embryonic muscle guidance. Functional requirements were not transmitted by single domains, but were found to be distributed over the whole length of this 7 PDZD protein in an unexpectedly complex manner. Binding ligands via PDZDs 1 and 2 repressed the activity of the protein, binding to PDZD 3 was involved in de-repression, and PDZ-ligand binding via PDZD 7-mediated DGrip function after its de-repression. Despite the fact that there was no critical dependence on PDZDs 4–5 or interdomains for function, we cannot exclude that interactions over these domains play a subthreshold role.

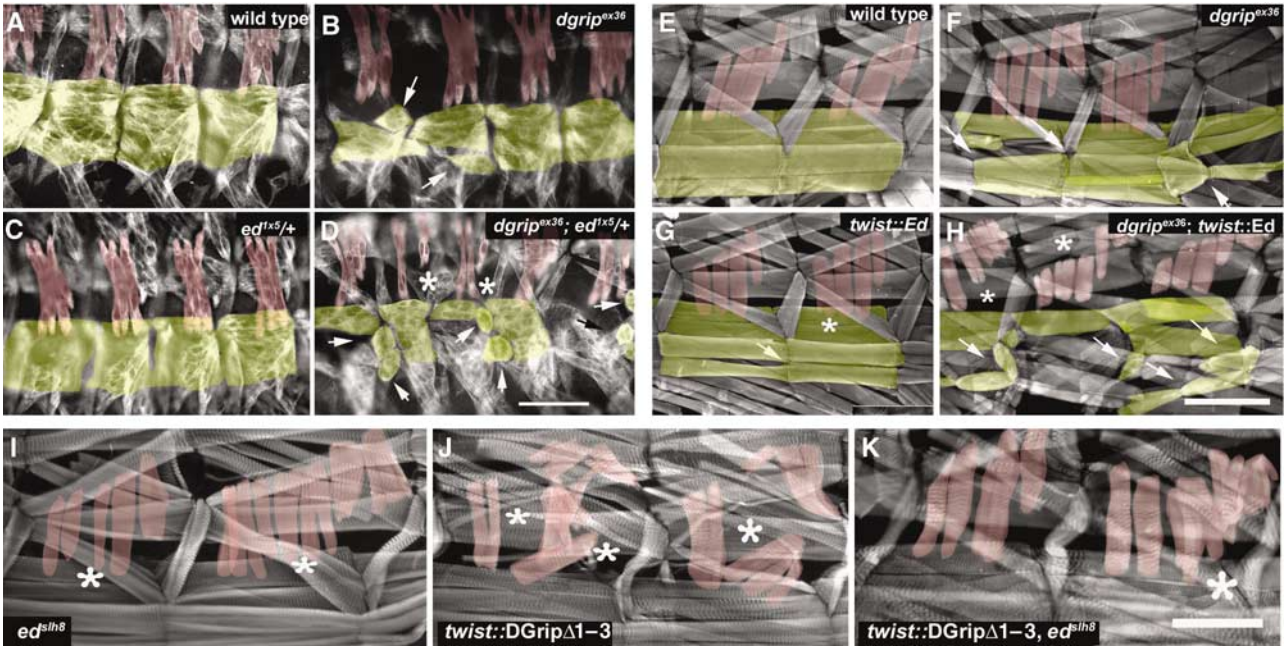


Figure 6 Functional interactions between Echinoid and DGrip in muscle morphogenesis. (A–D) Heterozygosity for *ed* mutant allele enhances defects in VLM and provokes LTM defects as shown by muscle myosin stainings of stage 16 embryos (**A**) wild type; (**B**) $dgrip^{ex36}$ mutants with characteristic defects in VLM morphology (arrow); (**C**) $ed^{1x5}/+$ embryos show no defects in LTMs or VLMs. (**D**) $dgrip^{ex36}$; $ed^{1x5}/+$ embryos exhibit more severe VLM defects (arrows) than $dgrip^{ex36}$ embryos, where sometimes LTMs might be missing (asterisks). More severe examples of $dgrip^{ex36}$; $ed^{1x5}/+$ embryos (not shown) exhibit completely deranged somatic musculature, where muscle identification is no longer possible. Scale bar in (D): 50 μm. (E–H) Muscle-specific overexpression of Ed enhances defects in $dgrip^{ex36}$ mutants. (**E**) Control larvae, showing bar-like morphology in VLM (yellow) and LTM (red). (**F**) $dgrip^{ex36}$ larvae; (**G**) *twist-gal4*::*UAS-ed* larvae exhibit few, mild defects in LTM and VLM morphology. Arrow indicates VLM with weakly distorted morphology, asterisks mark slightly split LTMs. (**H**) *twist*-mediated expression of Echinoid in the $dgrip^{ex36}$ background greatly enhances defects; VLMs are more severely deranged than in $dgrip^{ex36}$, and often appear to adhere to other muscles (arrows), whereas LTMs split (asterisks). Scale bar in (H): 300 μm. (I–K) Homozgosity for ed^{SIH8} suppresses DGripΔ1–3 activity. (**I**) ed^{SIH8} larvae show defects typical for *ed* zygotic alleles with slight LTM splitting (asterisks) and some malformation of VLMs. (**J**) *twist-gal4*::*UAS-dgripΔ1–3* controls with severe malformation of LTMs (asteriks). (**K**) ed^{SIH8}; *twist-gal4*::*UAS-dgripΔ1–3* larvae consistently showed far milder LTM (asteriks) defects than *twist-gal4*::*UAS-dgripΔ1–3* processed in parallel ($n > 30$ hemisegments per genotype). Scale bar in (K) 150 μm.

In fact, the DGripΔ1–3x7 construct showed some residual functionality in terms of muscle rescue. Thus, the *whole* protein might be used as an 'intelligent frame' designed to execute fine controls such as thresholding functions or coincidence detections. In fact, all attempts to provide DGrip activity or to repress DGrip activities with only partial fragments (DGripΔ4–7, DGripΔ1–5) failed (Figure 1B, our data), leading us to believe that DGrip is responsible for the organization of a macromolecular complex, of which the transmembrane protein Ed is part.

PDZDs are not functionally isolated

Our analysis suggests that a critical number of PDZDs are utilized for DGrip function, with both negative and positive interactions occurring. Such dependence between PDZDs may be due to structural chaperoning (Feng *et al*, 2003). Alternatively, a fixed orientation might be required for high-affinity binding to its targets as found for tandem PDZDs 1 and 2 in PSD-95 (Long *et al*, 2003), with a complex of two PDZDs having higher binding affinity than either PDZD alone. Moreover, allosteric changes upon PDZD-ligand binding could change binding affinities of neighboring domains (Fuentes *et al*, 2004; Peterson *et al*, 2004) or via bridging interactions where one molecule contacts multiple sites on a PDZ protein to effect conformational change (van Huizen *et al*, 1998; Schlieker *et al*, 2004; Wilken *et al*, 2004). Such mechanisms might be the substrate for integrating ligand binding and functional output over a large 'multivalent' PDZD protein.

Point mutations of PDZD 1 and PDZD 2 recapitulated the DGripΔ1–3 phenotype in the LTM group of muscles (Figure 3), indicating that the repressive function of the PDZDs 1–3 region is not 'structural' (i.e. by covering other PDZDs on the protein). Instead, we suggest that ligand interactions are communicated over the whole protein to steer equilibrium between two different functional modes of DGrip signaling.

DGrip interacts with Ed

Ed was identified as a novel DGrip interactor. Ed is cell adhesion protein with 7 Ig and 2 FNIII domains, described to have both adherence and signaling roles in *Drosophila* tissues (Islam *et al*, 2003; Rawlins *et al*, 2003a, b; Wei *et al*, 2005). It is highly conserved among invertebrates and its closest vertebrate homologues are Nectins, which exhibit 3 Ig domains and end in the PDZ-binding motif E/A-X-Y-V. Functionally, both protein families are similar: although not functionally redundant with Ed (Wei *et al*, 2005), Nectins are present at mammalian adherens junctions (AJs) along with l-afadin (Tachibana *et al*, 2000) and, like Ed, regulate Cadherin-based adherence at AJs (Sato *et al*, 2006). Several lines of evidence link Ed to DGrip:

(1) Ed interacted with DGrip in a yeast two-hybrid screen, dependent on the C-terminal EIIV motif, mediated via

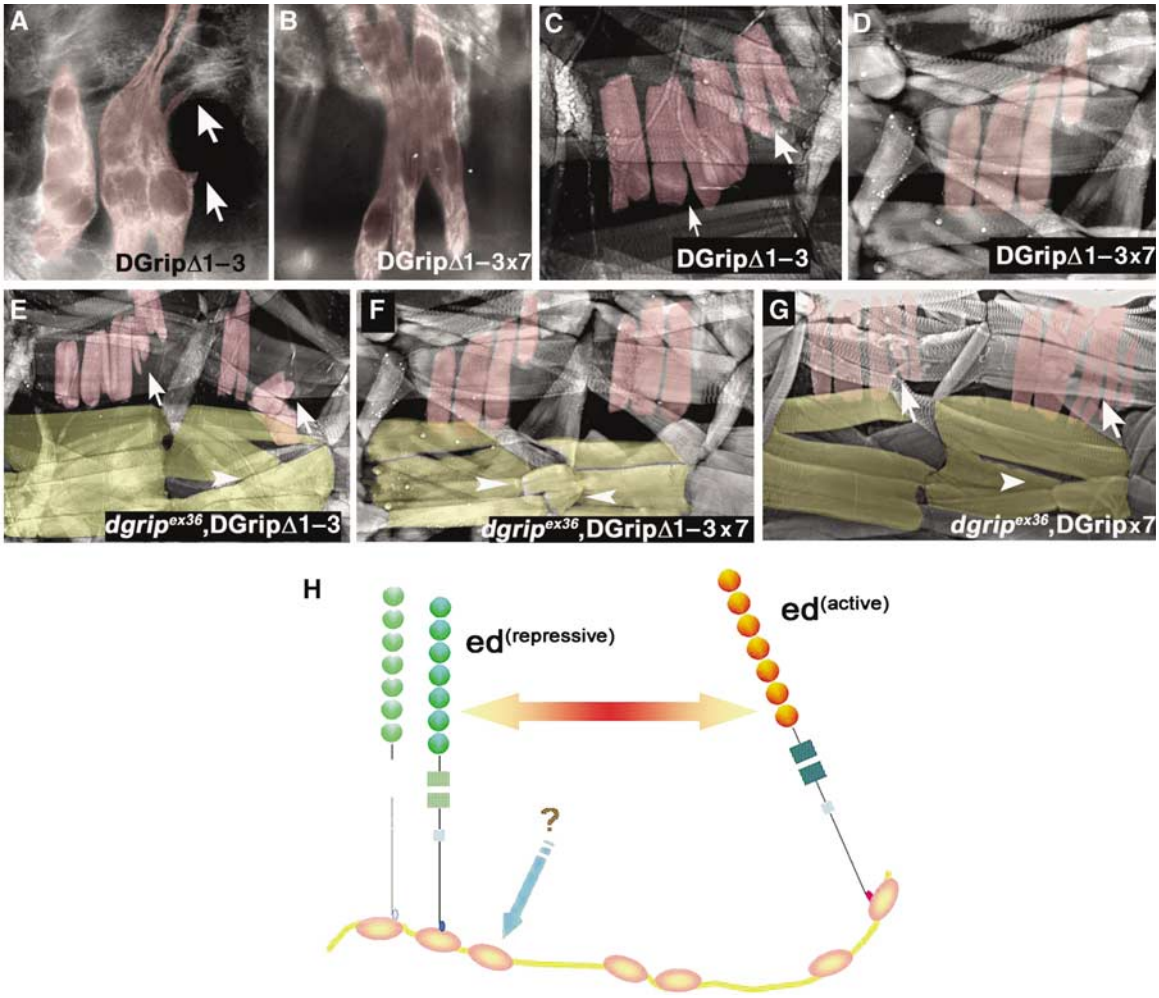


Figure 7 Mutation of the Echinoid-binding DGrip PDZD 7 represses DGripΔ1–3 activity. This figure depicts muscle myosin stainings in embryos (**A, B**) and phalloidin labeling in larvae (**C, D**). Point mutation of PDZD 7 reduces DGripΔ1–3 activity. (A–D) The dominant activity of the DGripΔ1–3 protein ((A) embryo; (C) larva), which causes abnormal muscle projections (arrows) is reduced by point mutation of the PDZD 7 ligand binding surface, producing DGripΔ1–3x7, which shows only slight defects in LTM morphology in embryo (B) or larva (D). (**E**, **F**) DGripΔ1–3x7 shows only limited rescue ability in *dgrip*^{ex36} VLMs (F, arrowheads) when compared to DGripΔ1–3 (E, arrowheads). (**G**) DGripx7 produces mild dominant defects of LTMs (arrows) and impaired rescue of VLMs (arrowheads). (**H**) Model of DGrip–Echinoid functional interaction during muscle morphogenesis. DGrip may act by maintaining the equilibrium between active and repressive Echinoid signaling. Ed binds DGrip at PDZD 2 (and possibly 1), where it is repressed. Interaction of an unknown protein with PDZD 3 relieves this repression, allowing Ed to bind PDZD 7 and activating the complex.

PDZDs 1, 2 or 7 (Figure 4). Myc-tagged DGrip specifically interacts with a peptide representing the last 10 amino acids of the Ed protein, including the EIIV PDZ-binding motif.

(2) *ed* zygotic mutants have defects in the morphogenesis of embryonic muscles qualitatively similar to DGripΔ1–3 overexpression.

(3) the *dgrip*^{ex36} muscle phenotype in embryos is enhanced by heterozygosity for *ed*^{1x5}. Here, LTMs (unaffected in pure *dgrip*^{ex36}) are affected as well (Figure 6D).

(4) *dgrip*^{ex36} mutant muscles (both VLMs and LTMs) are sensitive to Ed overexpression (Figure 6H). These synthetic defects suggest that DGrip, while itself not essential for LTM morphogenesis, regulates Ed in this group of muscles.

(5) homozygosity for hypomorphic *ed*^{slH8} chromosome strongly reduced the severity of the phenotype evoked by pan-muscular expression of DGripΔ1–3 (Figure 6K), indicating that Ed acts downstream of activated DGrip.

Notably, the pattern of Ed-PDZD binding correlates with the DGrip-dependent LTM phenotype. Expression of DGrip missing PDZDs 1, 2 and 3 together, or ligand binding in PDZD 1 and PDZD 2 only, showed a strong dominant active phenotype (Figures 2 and 3). Mutation of PDZD 2 caused a dominant phenotype in LTMs (Figure 3E). In a yeast-two hybrid test, Ed interacted strongly with PDZD 2 with and PDZD 7 (Figure 4C), and more weakly with PDZD 1.

In imaginal discs, Ed binds two different PDZD proteins via its EIIV motif: Canoe, an F-actin interacting protein and PAR-3/Bazooka. This interaction is mutually exclusive, thereby influencing cell adhesion and the remodeling of subcortical actin at AJs (Wei *et al*, 2005). Here, we propose a similar mechanism, in that both functional states of Ed are established via binding to the same protein (DGrip) at different sites. In this model, DGrip may assist in maintaining equilibrium between active and inactive signaling states of Ed, which in its inactive state binds to PDZDs 1 and 2, and in its active form to PDZD 7 of DGrip. This interaction appears

tissue specific in nature, as DGrip mutants do not display the full spectrum of defects of *ed* mutants (such as neurogenic phenotypes (Ahmed *et al*, 2003), our data) and that there are as yet unknown members of the DGrip–Ed complex, such as that which binds to the 'de-repressing' PDZD 3.

Both Ed loss of function and overexpression can produce similar phenotypes in muscles (Figures 5E, G–I and 6G, I), which are strongly enhanced by the absence of DGrip. Ed is described as a homophilic cell adhesion molecule (Islam *et al*, 2003; Rawlins *et al*, 2003a; Spencer and Cagan, 2003), and is maternally expressed in the epidermis, over which nascent muscles 'crawl' during the muscle guidance process to reach their target apodeme. *ed* clones in wing discs show cell sorting behavior, causing aggregation and adhesion of only those cells expressing the same complement of cell adhesion molecules (Wei *et al*, 2005). Thus, both reduction and excess of Ed on the 'muscle side' of transient muscle–epidermal adhesions could lead to significant changes in the cell adhesion properties of the developing muscle. The experiments shown in this study for DGripΔ1–3 overexpression in muscle 5 (Figure 2G–L) and for VLMs in *dgrip* mutants (Swan *et al*, 2004, Figure 5) imply that a tight balance of DGrip activity might particularly be needed to keep navigating muscle projections motile and to avoid their premature stabilization at ectopic epidermis contacts during the 'steering' process—ultimately instructed by Slit/Robo or other guidance systems. It is likely that Ed and DGrip form complexes enriched at muscle projection membranes to locally control adhesiveness. Ectopic adhesions among muscles cells with aberrant DGrip activity are in fact indicative of changes in muscle adhesiveness (e.g. see arrow in Figure 2L).

Natural variants of mGRIP missing PDZDs 1–3 have been localized to mammalian synapses (Charych *et al*, 2004), and it has recently been found that the type 5 metalloproteinase MT5-MMP is recruited by GRIP1/2 to growth-cone filopodia and to both mature and developing synapses, where it proteolyses N-cadherins (Monea *et al*, 2006). GRIP2 was also observed to be a member of a δ-catenin containing complex (Monea *et al*, 2006). *Drosophila* Echinoid is known to regulate DE-Cadherin in homeotypic cell–cell junctions (Wei *et al*, 2005). Given these promising indications, it will prove interesting to see whether in the context Grip proteins became famous for—synapse assembly—similar molecular strategies are used by the GRIP protein as those we describe here in the context of muscle morphogenesis.

Materials and methods

Immunostaining

Staining of embryos and larvae as well as most antibodies used were described recently (Swan *et al*, 2004). In addition were used: anti-Ed (rabbit, used at 1:250 (Rawlins *et al*, 2003a)), GFP (mouse, used at 1:200; MolProbes) and β-PS integrin (rabbit, used at 1:50; Nick Brown).

Biochemistry

The detailed procedure is described (Soltau *et al*, 2004). In brief, a synthetic peptide representing the C-terminus of Ed (NRRVIREIIV) and a scrambled control (RIVRIRIEVN) were generated by peptides&elephants GmbH, Nuthetal, Germany. These were coupled to NHS-activated sepharose at a concentration of 3 mg/ml matrix. Transfected Sf.9 cells were lysed in NTEP-buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 10 mM iodacetamide, 1 mM PMSF and 0.5% (v/v) Nonidet NP40, pH 7.9) on ice. Sf.9 cell extracts were 'precleared' 3 h with 400 μl NHS-sepharose-slurry to prevent unspecific binding to the NHS-sepharose. Precleared supernatant was applied to the peptide/NHS-matrix for 1 h at 4°C, the matrix washed five times, eluted by boiling in SDS sample buffer and analyzed by SDS–polyacrylamide gel electrophoresis followed by Western blotting. Anti-Myc-Ab (mouse, 1:500, Santa Cruz) was used for detection.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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