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The transmembrane protein Perdido interacts with Grip and integrins to mediate myotube projection and attachment in the *Drosophila* embryo

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The molecular mechanisms underlying muscle guidance and formation of myotendinous junctions are poorly understood both in vertebrates and in *Drosophila*. We have identified a novel gene that is essential for *Drosophila* embryonic muscles to form proper projections and stable attachments to epidermal tendon cells. Loss-of-function of this gene – which we named *perdido* (*perd*) – results in rounded, unattached muscles. *perd* is expressed prior to myoblast fusion in a subset of muscle founder cells, and it encodes a conserved single-pass transmembrane cell adhesion protein that contains laminin globular extracellular domains and a small intracellular domain with a C-terminal PDZ-binding consensus sequence. Biochemical experiments revealed that the Perd intracellular domain interacts directly with one of the PDZ domains of the Glutamate receptor interacting protein (Grip), another factor required for formation of proper muscle projections. In addition, Perd is necessary to localize Grip to the plasma membrane of developing myofibers. Using a newly developed, whole-embryo RNA interference assay to analyze genetic interactions, *perd* was shown to interact not only with *Grip* but also with *multiple edematous wings*, which encodes one subunit of the αPS1-βPS integrin expressed in tendon cells. These experiments uncovered a previously unrecognized role for the αPS1-βPS integrin in the formation of muscle projections during early stages of myotendinous junction development. We propose that Perd regulates projection of myotube processes toward and subsequent differentiation of the myotendinous junction by priming formation of a protein complex through its intracellular interaction with Grip and its transient engagement with the tendon cell-expressed laminin-binding αPS1-βPS integrin.

KEY WORDS: Muscle attachment, Myotendinous junction, Myogenesis, NG2, MCSP, Integrins, *Drosophila*

INTRODUCTION

The development of many tissues, such as muscles and neurons, requires that cells communicate, migrate, recognize and adhere to each other. Both in vertebrates and in *Drosophila*, muscles and tendons form stable attachments via the myotendinous junction which maintains a strong association between these cells during muscle contraction. Development of the myotendinous junction in the *Drosophila* embryo provides an excellent in vivo model for studying the molecular mechanisms underlying cellular interactions and tissue morphogenesis.

The embryonic body wall muscles of *Drosophila* have a stereotypic pattern that is readily investigated. Here, myofiber development starts with the specification of two types of myoblasts, founder cells and fusion-competent myoblasts, which fuse with each other to form nascent multinucleated myotubes. Founder cells contain all of the information each muscle needs for its development, including its size, position, orientation, innervation and specific attachment to the epidermis (Bate, 1990; Baylies and Michelson, 2001). These growing myotubes then migrate underneath the ectoderm, and eventually they stably adhere to specific epidermis-derived tendon cells to form apodemes or muscle attachment sites (Baylies et al., 1998; Schnorrer and Dickson, 2004). During the migration phase, myotubes elongate and multiple radiating projections or filopodia – similar to neuronal growth cones – are observed at the leading edge of the muscle. By stages 15-16, most muscles have a smooth surface with both poles stably associated with separate tendon cells (Bate, 1990; Bate, 1993; Schnorrer and Dickson, 2004). In both *Drosophila* and vertebrates, the correct development of the myotendinous junction involves cross-talk between these two cell types (Volk, 1999). Where tendon cells not only serve as attachment sites but also provide guiding cues for the migrating myotube, the muscle is essential for tendon-specific gene expression and terminal differentiation (Bate, 1990; Frommer et al., 1996; Volk, 1999; Vorbruggen and Jackle, 1997).

Despite this detailed morphological view of myogenesis, the molecular cues that guide myotube migration, muscle-epidermal recognition and apodeme formation remain largely unknown. Interestingly, all of the proteins that are currently known to be involved in muscle migration have been shown to participate in axon guidance and/or synaptic junction signaling, raising the possibility that both neurons and muscles utilize similar molecular mechanisms for target recognition (Schnorrer and Dickson, 2004). One of the few known non autonomous signals provided by tendon cells is the secreted protein Slit – the ligand for the Roundabout (Robo) receptors – which is necessary for migration of the embryonic ventral longitudinal muscles (VLMs) (Kidd et al., 1998; Kramer et al., 2001). Similarly, the receptor tyrosine kinase, Derailed, controls lateral transverse muscle (LTM) guidance (Callahan et al., 1996).

In addition to the slit-Robo signaling pathway, the multi-PDZ domain-containing protein, Glutamate receptor interacting protein (Grip), is required for VLM guidance through its involvement in the

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formation of cellular extensions (Swan et al., 2004). PDZ domains facilitate protein-protein interactions and are capable of recruiting multiple proteins to form a molecular complex that mediates specific and localized signaling (Bilder, 2001; Sheng and Sala, 2001). Indeed, PDZ domain 7 of Grip is essential for its function by binding to the cell adhesion protein, Echinoid (Ed). This interaction suggests that these two proteins form a signaling complex during muscle morphogenesis (Swan et al., 2006).

Once a myotube has reached its attachment site, cell surface adhesion molecules become localized toward the tendon cell to form a stable connection that is capable of withstanding muscle contraction (Brown et al., 2000). Integrins, which are major cellular and extracellular matrix transmembrane receptors, are localized at the myotendinous junction where they play an important role in stabilizing this intercellular connection. Thus, integrin mutant embryos contain spherical myotubes secondary to detachment following muscle contraction (Bokel and Brown, 2002; Brown et al., 2000). Each integrin consists of a heterodimer composed of one α and one β subunit. In *Drosophila*, there are two integrins relevant to somatic muscle development, αPS1-βPS, which is expressed in tendon cells, and αPS2-βPS, which is expressed in the muscle. The integrin subunits are encoded by the genes, *multiple edematous wing* (*mew*, αPS1), *lethal myospheroid* (*mys*, βPS), and *inflated* (*if*, αPS2). It is thought that the attachment of muscles to the epidermis is a sequential process, in which the muscle first transiently comes in close contact with the epidermal cell, thereby initiating assembly of a hemiadherens-type junction. Integrin activation follows, which, in turn, triggers strong attachment of the integrins to the extracellular matrix and stabilization of the attachment prior to muscle contraction (Brown et al., 2000; Prokop et al., 1998; Tepass and Hartenstein, 1994).

Here, we report the identification of a novel gene that is essential for development of a subset of muscle attachments in the *Drosophila* embryo, a gene which we named *perdido* (*perd*; 'lost' in Spanish). *perd* encodes a single-pass transmembrane cell adhesion protein that contains two extracellular globular-laminin domains and a small intracellular domain with a PDZ-binding consensus sequence. In vivo visualization of muscle development both in *perd* ethane methyl sulphonate (EMS)-induced mutants and in wild-type embryos injected with *perd* double-stranded RNA (dsRNA), revealed that specific ventral muscles fail to form proper guidance projections and do not attach stably to their tendon cells. Consequently, affected muscles detach upon contraction, and rounded myotubes appear in place of elongated myofibers. This phenotype resembles that observed with loss-of-function of the PDZ domain-containing Grip protein and each of the three integrin subunits. Using a newly developed, sensitive, RNA interference (RNAi)-based method to rapidly and efficiently test genetic interactions, we demonstrate that *perd* interacts with *Grip*, *mys* and *mew*. In addition, we have found that Perd protein binds directly to PDZ domain 7 of Grip, and that this interaction is required to localize Grip to the muscle plasma membrane. We hypothesize that *perd* primes formation of a protein complex at the myotendinous junction by transient binding of its extracellular domain to Mew – the integrin subunit expressed by the tendon cell – and by localizing Grip to the internal surface of the muscle membrane. Together, this Perd-dependent mechanism activates a signaling pathway within the muscle that is essential for myotube guidance, recognition and attachment. Of note, while this manuscript was in preparation, a related paper was published in which equivalent findings were reported for *Grip* and *perd* (referred to there as *kon-tiki*). However, an additional connection between *perd* (*kon-tiki*) and integrin function was not established by that work (Schnorrer et al., 2007).

MATERIALS AND METHODS

Drosophila strains

The deficiency Df(2L)M36-S5/CTG from the Bloomington *Drosophila* Stock Center was used to test the lethality of the newly generated EMS *perd* alleles. The following *perd* alleles were isolated and sequenced in an EMS mutagenesis: $perd^{F1\text{-}3}$/CTG, $perd^{F2\text{-}5}$/CTG, $perd^{H2\text{-}5}$/CTG, $perd^{187(C2)}$/CTG, $perd^{H1\text{-}4}$/CTG. The CTG (CyO, twi-Gal4 UAS-2EGFP) balancer chromosome was used to identify homozygous mutants for *perd* (Halfon et al., 2002). Flies carrying the 5053Gal4 line (Swan et al., 2004) (J. Lopez, personal communication to FlyBase 1998) and UAS-*lacZ* were used to visualize the morphology of the VL1 muscle in *perd* ($perd^{F1\text{-}3}$UAS-*lacZ*/$perd^{F1\text{-}3}$; 5053Gal4/+), and $Grip^{ex36}$ (Swan et al., 2004) mutants ($Grip^{ex36}$/Y; UAS-*lacZ*/+; 5053Gal4/+). Myosin heavy chain (MHC)-tau-GFP (Chen and Olson, 2001) and UAS-srcEGFP; 5053Gal4/+ (encoding a membrane-bound form of GFP) embryos were used to visualize the muscle pattern upon dsRNA injections.

EMS mutagenesis

yw flies isogenic for chromosome II were starved for 12 hours and fed for 18 hours with 25 mM EMS in 5% sucrose. Crosses between 3090 single male progeny of mutagenized males and Df(2L)M36-S5/CTG females were tested for lethality. Lethal chromosomes were collected over the CTG balancer and fly lines were established for subsequent phenotypic analysis.

In situ hybridizations and immunohistochemistry

Histochemical in situ hybridization was done as described by Estrada et al. (Estrada et al., 2006). RNA-labeled probes for *perd* and *Grip* were obtained from *Drosophila* Gene Collection (DGC) clones LD31354 and RE14068, respectively. Fluorescent in situ hybridizations followed standard protocols (Kosman et al., 2004). Antibody stainings were carried out as described previously (Carmena et al., 1998). The following primary antibodies were used: rabbit and mouse anti-MHC (D. Kiehart, Duke University, Durham, NC), rabbit anti-Grip (S. Sigrist, Max-Planck-Society, Göttingen, Germany), guinea pig anti-Kruppel (D. Kosman, UCSD, San Diego, CA), rabbit and mouse anti-β-galactosidase (Cappel, Promega), rabbit and mouse anti-GFP (Invitrogen, Clontech), and anti-digoxigenin-alkaline phosphatase (Roche).

RNA interference assays and in vivo microscopy

RNAi assays were carried out as described by Estrada et al. (Estrada et al., 2006). Any gene segment matching any other predicted gene at 18 consecutive nucleotides was excluded to avoid off-target effects (Kulkarni et al., 2006). To test for synergistic interactions, each individual dsRNA to be tested was first injected at high concentration (2 mg/ml) and the embryos assessed for a robust and penetrant phenotype. Dilutions were then tested until a critical concentration was ascertained at which a majority of embryos appeared completely wild-type and the remainder were only mildly affected. Images of all GFP-expressing embryos were scored into four phenotypic classes: wild type, marginally affected (effects were equivocal or limited to a single hemisegment), clearly affected (improperly attached muscles visible in multiple hemisegments), or unscorable (embryos exhibiting the severely aberrant morphology observed at low frequency as a result of all injections, including *lacZ* and buffer controls). For examples of each class, see Fig. S1 in the supplementary material. For the quantification of various titration experiments, see Fig. S2 in the supplementary material. The critical concentration varied widely for different dsRNAs – compare, for example, Fig. S2A,D and G in the supplementary material. Titrations were performed in the presence of a constant dsRNA concentration, achieved by inclusion of an appropriate concentration of inactive control (*lacZ*) dsRNA. This proved to be crucial, as the concentration of inactive dsRNA could profoundly influence the effect of an identical dose of test dsRNA; 20 μg/ml of *perd* dsRNA has a very strong effect in the absence of any inactive dsRNA (Fig. S2H in the supplementary material; total dsRNA concentration in this experiment was 20 μg/ml), but little effect when *lacZ* dsRNA was added to a final concentration of 2 mg/ml (Fig. S2G in the supplementary material).

For each interaction experiment, tubes containing negative control (500 μg/ml *lacZ* dsRNA), dsRNA 1 at its critical concentration, dsRNA 2 at its critical concentration, or both tested dsRNAs together, each at its critical concentration (plus *lacZ* dsRNA to 500 μg/ml in all cases) were coded by a different individual so that the identity of the dsRNAs was not known to the

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operator at the time of either the injection or the scoring of the embryos. dsRNAs were injected, alone and in combination, into at least 30 MHC-tau-GFP embryos per sample. We estimate the injected volume to be 10-100 pl per embryo. Late stage 17 embryos were photographed for GFP fluorescence, and the resulting image files were computationally scrambled using a simple Perl program (see Fig. S3 in the supplementary material).

Encoded images were scored into four phenotypic classes as described. Unscorable embryos were excluded from further analysis. Each interaction experiment was performed four times, with separately prepared dsRNA dilutions, and scorable embryo counts from all four experiments were pooled for analysis. The distribution of phenotypes expected from an additive effect of individual dsRNAs was predicted based on the following conservative assumptions: that all effects observed in single-dsRNA-injected embryos are specific RNAi effects, that each dsRNA will affect a fraction of all embryos independently, that any embryo clearly affected by either dsRNA independently will remain clearly affected in the additive condition, and that any embryo marginally affected by both dsRNAs will be clearly affected. Thus, if a, b and c are the fractions of embryos injected with dsRNA 1 scored wild type, marginally affected and clearly affected, respectively, and x, y and z are the corresponding fractions for dsRNA 2, the predicted fraction of wild-type embryos=ax, the predicted fraction of marginally affected embryos=$ay+bx$, and the predicted fraction of clearly affected embryos=$cx+cy+cz+bz+az+by$. This predicted distribution is compared to the observed distribution with a χ^2 test. The resulting P value can be interpreted as the probability of obtaining the observed distribution of phenotypes if the effects of each dsRNA were independent and additive. Any interaction that we considered reliable evidence for synergy was characterized by a clear shift toward more severely affected embryos and a P value less than 0.001. A more detailed version of this protocol is available from the authors by request.

Biochemical interaction assays

The intracellular domain of Perd was cloned by PCR, inserted into pET30a(+) (Novagen), and expressed as a fusion protein with an amino-terminal 6xHis tag and an S-Tag, then purified by nickel affinity chromatography and eluted in binding buffer (Kumar and Shieh, 2001) supplemented with 0.5 M imidazole. Isolated PDZ domains from Grip [PDZ1: aa71-171, PDZ2 (not shown): 172-274, PDZ3: 267-408, PDZ4: 468-559, PDZ5: 559-669, PDZ6: 809-964, PDZ7: 972-1058] were cloned by PCR, inserted into pGEX-5X-1, and expressed as GST fusion proteins. Bacteria expressing soluble protein (including GST only) were resuspended in binding buffer, lysed by sonication, cleared by centrifugation, applied to GST-Bind resin (Novagen) at saturating concentrations, and incubated on ice for 1 hour. Bound resin was washed with excess binding buffer, then purified Perd fusion protein was applied and incubated on ice for 1 hour. Resin was washed twice with binding buffer at room temperature, and bound proteins were eluted in reducing gel sample buffer and western blotted in duplicate according to standard protocols. GST fusion proteins were detected with rabbit anti-GST and anti-rabbit HRP conjugate; Perd fusion protein was detected with S-protein HRP conjugate (Novagen) and visualized by chemiluminescence (Pierce).

RESULTS

The gene CG10275 is expressed in a subset of myoblasts and myotubes

In a previous study, we used an integrated genetic, genomic and computational strategy to comprehensively determine the molecular identities of distinct myoblast subpopulations within the *Drosophila* embryonic mesoderm (Estrada et al., 2006). We identified hundreds of genes specifically expressed in founder cells or fusion-competent myoblasts, one of which is the gene CG10275. Embryonic in situ hybridization experiments in wild-type and informative mutant backgrounds showed that CG10275 is specifically expressed in founder cells at stage 11, when cell fate specification occurs (Estrada et al., 2006). Detailed analysis of CG10275 RNA expression revealed that it is present in only a subset of Kruppel-positive founder cells (Fig. 1A). Later in embryonic development, CG10275 RNA is restricted to myotubes at the site of attachment with the epidermal tendon cells (arrows in Fig. 1B). Other tissues also

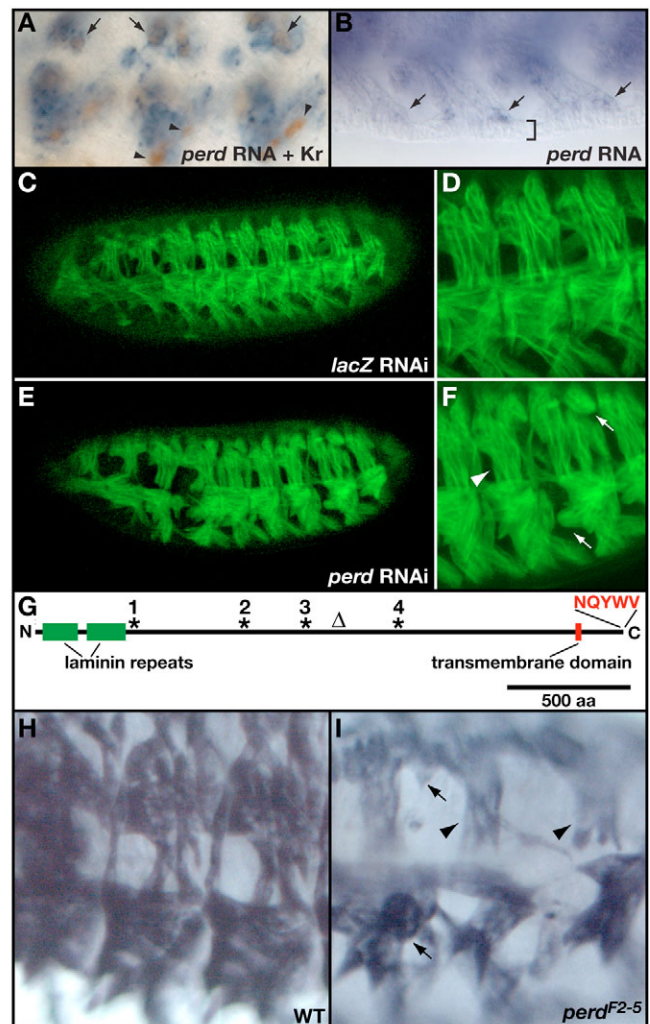

Fig. 1. *perd* is expressed in a subset of muscle founder cells (FCs) and is required for proper muscle development. (**A**) Double-labeling of a stage 12 wild-type embryo for *perd* RNA (blue) and Kruppel (Kr; brown) protein (which marks a subset of FCs) shows coexpression in some (arrows) but not all (arrowheads) Kr-positive FCs. (**B**) A high-magnification view of a stage 15 embryo shows that *perd* RNA is present in the muscle (arrows) and not in the tendon cell (bracket). (**C-F**) In contrast to injection of inactive control *lacZ* dsRNA (C,D), injection of double-stranded RNA corresponding to a portion of the *perd* gene (E,F) into embryos expressing tau-GFP in the somatic musculature causes specific muscles to develop with a rounded morphology and incorrect attachments (arrows), while other muscle groups are unaffected (arrowhead). (**G**) *perd* encodes a single-pass transmembrane protein of 2355 amino acids with two laminin G domains near its amino terminus and a carboxyl-terminal class II PDZ-binding motif (NQYWV). Asterisks indicate the positions of nonsense mutations in the EMS-induced alleles H2-5 (1), F1-3 (2), F2-5 (3) and 187(C2) (4). Δ indicates the position of a four-nucleotide deletion in H1-4, resulting in a frameshift and early termination. (**H**,**I**) Immunostaining for myosin heavy chain shows that genetic loss of *perd* function causes the same muscle phenotype as RNAi, in which ventral longitudinal and segment border muscles have a rounded or teardrop shape (arrows), whereas lateral transverse muscles are normal (arrowhead).

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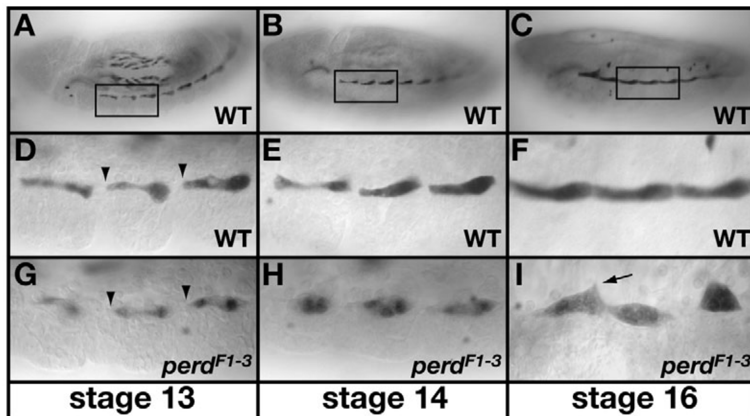

Fig. 2. *perd* mutant muscles fail to form correct attachments. In wild-type embryos expressing β-galactosidase in muscle VL1 (with the 5053 Gal4 line) (**A-F**), this muscle sends out blunt-ended processes anteriorly during stage 13 (arrowheads in D), which continue to extend during stage 14 (E) and form mature-appearing connections by stage 16 (F). In a *perd* mutant (**G-I**), at stage 13 myotube projections appear thinner and less well-spread but are correctly oriented (arrowheads in G), then fail to extend through stage 14 (H). By stage 16, muscles are rounded up and new projections extend in inappropriate directions (arrow, I).

express this gene, including the visceral mesoderm, the heart, the gonadal mesoderm, the endoderm and the central nervous system. This pattern of expression suggests that CG10275 could be playing important roles in the development of the *Drosophila* embryo.

CG10275 encodes Perdido, a single pass transmembrane cell adhesion protein that is essential for myotube projection, recognition and stable attachment to epidermal tendon cells

To determine if CG10275 functions in muscle development, we carried out CG10275-specific dsRNA injections into live embryos that expressed the tau-GFP fusion protein under control of the myosin heavy chain (MHC) promoter, which facilitates visualization of muscle development in real time (Chen and Olson, 2001; Estrada et al., 2006). In such an RNAi experiment, the band of ventral longitudinal muscles was abnormal, presenting globular-shaped muscles instead of elongated myofibers that span the entire intersegmental space (Fig. 1E,F, arrows; compare control *lacZ* dsRNA in Fig. 1C,D). This phenotype suggests that loss of CG10275 function causes defects in muscle attachment, but does not reveal when in development the abnormality initially occurs.

CG10275 encodes a single pass type I transmembrane protein of 2355 amino acids and molecular weight of 267 kDa, with two predicted laminin G domains at the extracellular amino terminus. The intracellular domain is small and ends in a class II PDZ binding motif (Fig. 1G), suggesting that it may form part of a protein complex with one or more PDZ domain-containing proteins. It appears (based on the bidirectional best BLAST hit) to be orthologous to the mammalian NG2/AN2/MCSP proteoglycan; however, the predicted open reading frame lacks the signal sequence for that gene. As the translation initiation site in the predicted open reading frame is not supported by available experimental evidence, we performed 5′ RACE to determine the true N terminus of the protein and discovered an additional exon ~5 kb upstream of the predicted initiation site, with a canonical TATA box and high-quality splice donor site (data not shown). The predicted protein product of this transcript begins with a classic signal sequence.

The expression pattern of CG10275, its RNAi phenotype and the molecular nature of the encoded protein motivated us to undertake an EMS mutagenesis screen to isolate mutant alleles for this gene. We screened 3090 mutagenized second chromosomes for embryonic lethality over a deficiency that uncovered the gene CG10275 (Df(2L)M36-S5), and found nine chromosomes that failed to complement the deficiency and each other. These alleles showed abnormalities in the ventral band of muscles, as ascertained by MHC inmunostainings. After sequencing all 12 annotated exons of CG10275 from five independent mutant alleles, we found that four of them created early stop codons in the protein (H2-5=Q404*, F1-3=K868*, F2-5=Q1095* and 187(C2)=L1462*; asterisks in Fig. 1G), and one of them contained a four-nucleotide deletion, resulting in a frameshift and early termination of the protein (after T1216, with an additional 50 amino acids translated out of frame; Fig. 1G). Phenotypic analysis of these mutants showed that ventral longitudinal muscles were rounded and teardrop-shaped, whereas dorsal and lateral transverse muscles were normal (Fig. 1H,I). The fact that homozygous mutant embryos for each of these alleles shows a phenotype identical to that of the same allele placed over the Df(2L)M36-S5 deficiency suggests that these are null alleles. The molecular lesions associated with these mutations (translational terminations prior to the transmembrane and intracellular domains) are consistent with this conclusion. Since the morphology of the mutant muscles indicates that they are not attached to the epidermal tendon cells, we named the gene CG10275, *perdido* (*perd*), which means ‘lost’ in Spanish. Of note, the previously identified RNAi phenotype for CG10275 is the same – albeit less severe – as that exhibited by the EMS alleles of this same gene.

To better understand the phenotype of *perd* mutant embryos, we focused on the development of a single ventral longitudinal muscle (VL1). This muscle can be visualized using the VL1-specific 5053Gal4 line (Swan 04; J. Lopez, personal communication to FlyBase 1998). At stage 13, wild-type VL1 muscles have an elongated, rectangular shape, and both ends of the myotube are already attached to the segment border tendon cells (Fig. 2A,D). Later in development, this muscle maintains its elongated shape, even at stages 16 and 17 when muscle contraction has started and the myotendinous junction is under tension (Fig. 2C,F). By contrast, *perd* mutant VL1 muscles begin, at stage 13, to exhibit an abnormal teardrop shape, ending in thin projections oriented toward the appropriate tendon cells, instead of having a wide rectangular surface of attachment (Fig. 2G). As development proceeds, these muscles become more and more dysmorphic, assuming rounded and irregular shapes (Fig. 2H,I). This phenotype suggests abnormal differentiation of the attachment site. Occasionally, in later stages, we observe random projections originating from the mutant myotubes, as if they were searching for a new attachment after failing to find one in the normal location (arrow in Fig. 2I).

These results indicate that the adhesion transmembrane protein Perd selectively affects the development of a subset of muscles by regulating myotube projections and subsequent muscle attachment during formation of the myotendinous junction.

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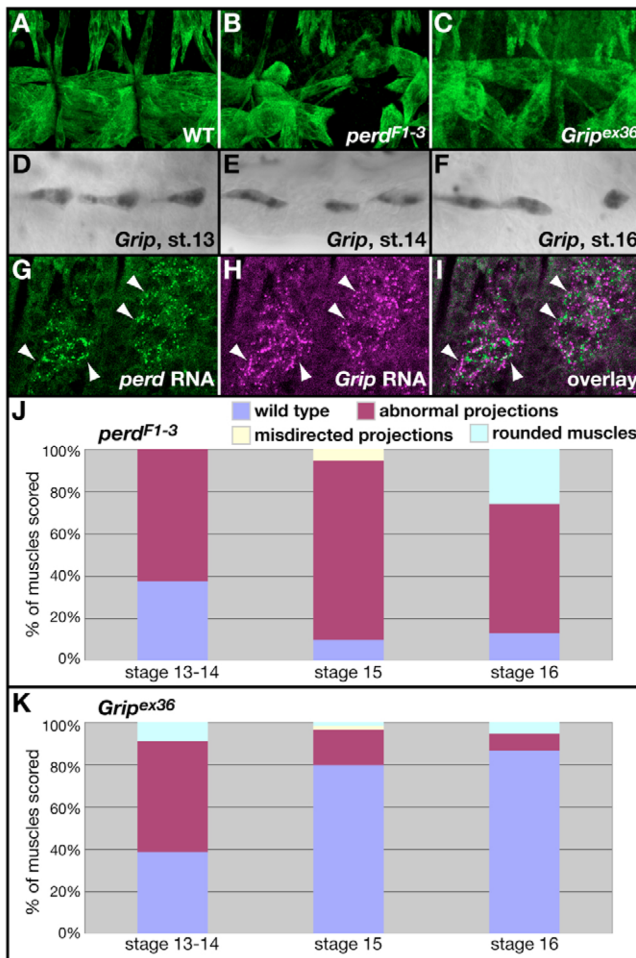

Fig. 3. *perd* and *Grip* have similar phenotypes and are coexpressed. (**A-C**) The terminal muscle phenotype in *Grip* mutant embryos (C) appears similar to, although less severe than, the *perd* phenotype (B). (**D-F**) The time course of development of the *Grip* phenotype is very similar to that of *perd* (compare with Fig. 2). (**G-I**) Simultaneous detection of *perd* RNA (G) and *Grip* RNA (H) by fluorescence in situ hybridization shows that many cells coexpress both genes (arrowheads). (**J**,**K**) Quantification of muscle VL1 phenotypes in *perd* (J) and *Grip* (K) mutant embryos at three different stages of development. Each mutation causes a majority of muscles to appear abnormal in the earliest phases of process extension, but most muscles recover and achieve an appropriate morphology at later stages in the *Grip* mutant, whereas the *perd* mutant phenotype is increasingly severe. In addition, a small number of muscles with misdirected projections can be observed at stage 15 in both genotypes (yellow).

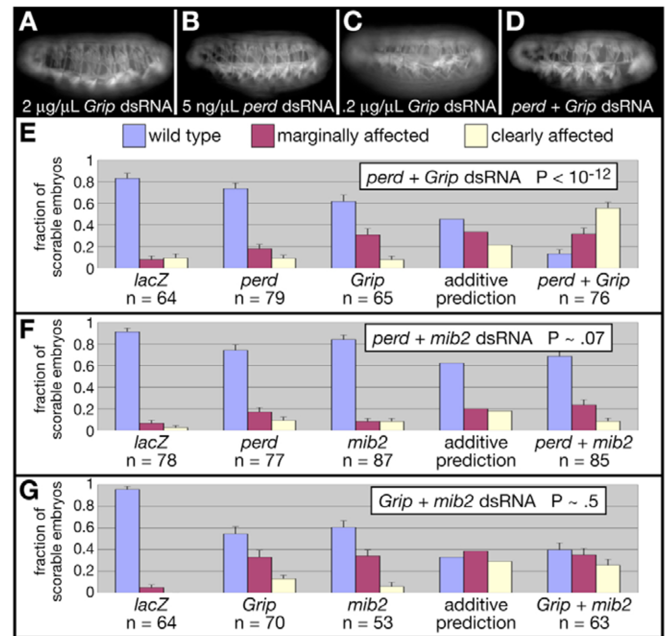

Fig. 4. *perd* and *Grip* interact specifically in a novel RNAi interaction assay. (**A**) Injection of *Grip* dsRNA at high concentration phenocopies a loss-of-function mutation. (**B-D**) Representative embryos injected with *perd* dsRNA (B) or *Grip* dsRNA (C) at concentrations titrated to give minimal effect, and the stronger effect observed when they are combined (D). (**E**) Quantification of pooled data from four independent experiments, comparing the frequency with which effects are seen from each dsRNA individually or from both together, along with the distribution predicted if effects were additive (see Materials and methods for details). *n*=the number of informative embryos scored for each condition. The probability of obtaining the observed frequencies from the additive distribution was $<10^{-12}$ (χ^2 test). (**F**,**G**) In identical experiments, *mib2* dsRNA does not exhibit synergy with *perd* or *Grip* dsRNA, despite causing detachment and rounding of a similar subset of muscles when injected at high concentration.

A synergistic genetic interaction between *perdido* and the *Glutamate receptor interacting protein* is revealed by a sensitized RNAi interaction assay

Having analyzed the aberrant muscle phenotype of *perd* mutant embryos, we noticed a striking similarity to the abnormalities associated with mutations in *Grip* (Swan et al., 2004). Grip is required for proper formation of cellular extensions during myotube guidance (Swan et al., 2004). At the end of embryogenesis, both *Grip* and *perd* mutants contain rounded, detached muscles that span half a hemisegment (Swan et al., 2004) (Fig. 3A-C). Also, in both mutants the ventral longitudinal and segment border muscles are affected, but the lateral transverse muscles are spared (Fig. 3A-C). Early in myogenesis, the morphology of affected muscles is similar in *perd* and *Grip* mutants, where myotubes start extending thin projections to the proper tendon cell, but the apodeme does not differentiate properly and the muscles detach and round up as development proceeds (Fig. 3D-F). These observations led us to conclude that *perd* and *Grip* affect myogenesis in a qualitatively similar manner. However, a quantitative analysis of null alleles of both genes indicates that the expressivity of the phenotype is lower in Grip mutants (Fig. 3J,K). These phenotypes, together with the fact that *perd* and *Grip* transcripts are coexpressed in the same myoblasts (Fig. 3G-I), suggest that these two genes could be functioning in a common developmental pathway within the same cells. To test this hypothesis, we developed a method to rapidly detect genetic interactions using RNAi in living embryos.

To accomplish this goal, we first injected high concentrations of Grip dsRNA into live MHC-tau-GFP embryos to determine if the *Grip* mutant could be phenocopied by RNAi. These embryos contained numerous spherical muscles in the ventral region (Fig. 4A). Next, we titrated down the concentration of both *Grip* and *perd* dsRNAs to a level where the muscle phenotype was barely detectable in a minority of embryos (Fig. 4B,C). Finally, with the expectation that the individual weak RNAi effects represent sensitized backgrounds that could be enhanced when combined – provided that the genes are

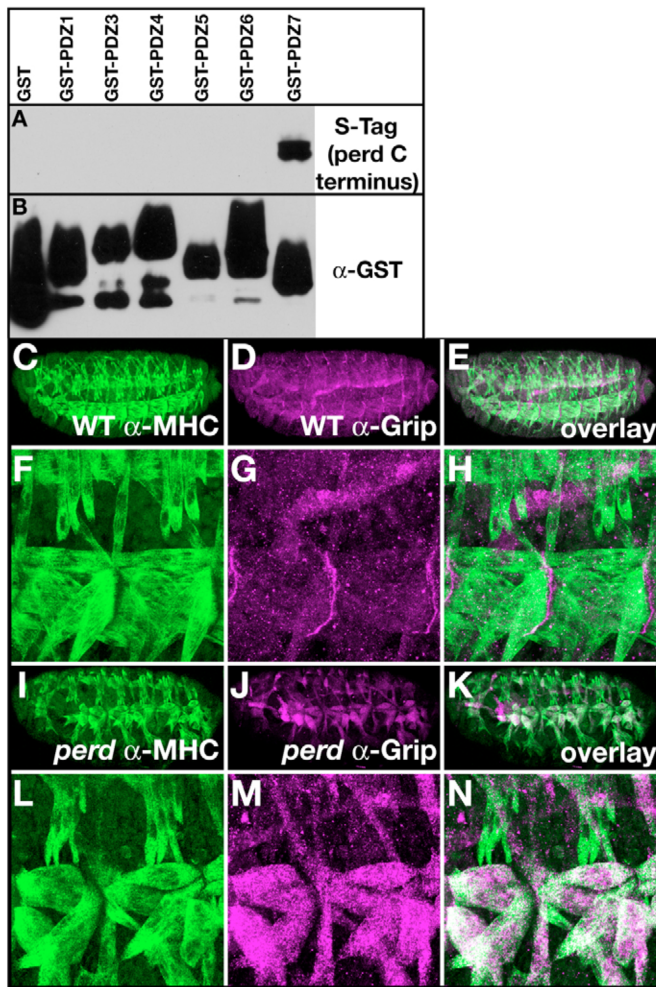

Fig. 5. Perd protein physically interacts with Grip and localizes it to muscle attachment sites. (**A**,**B**) Individual PDZ domains from Grip were prepared as GST fusion proteins, incubated with epitope-tagged Perd intracellular domain, and purified by glutathione affinity (PDZ2 was not successfully assessed in this experiment). Detection of the epitope tag (A) shows that PerdIC is present only when Grip PDZ7 is present, whereas anti-GST antibody (B) reveals that fusion protein was present in all lanes. (**C-H**) Double-labeling of wild-type embryos for myosin heavy chain and Grip protein shows that Grip is localized to sites of muscle attachment. In *perd* mutant embryos (**I-N**), Grip protein fails to localize and is visible over the entire periphery of Grip-expressing muscles. F-H and I-N are higher magnification images of part of the embryos shown in C-E and I-K, respectively.

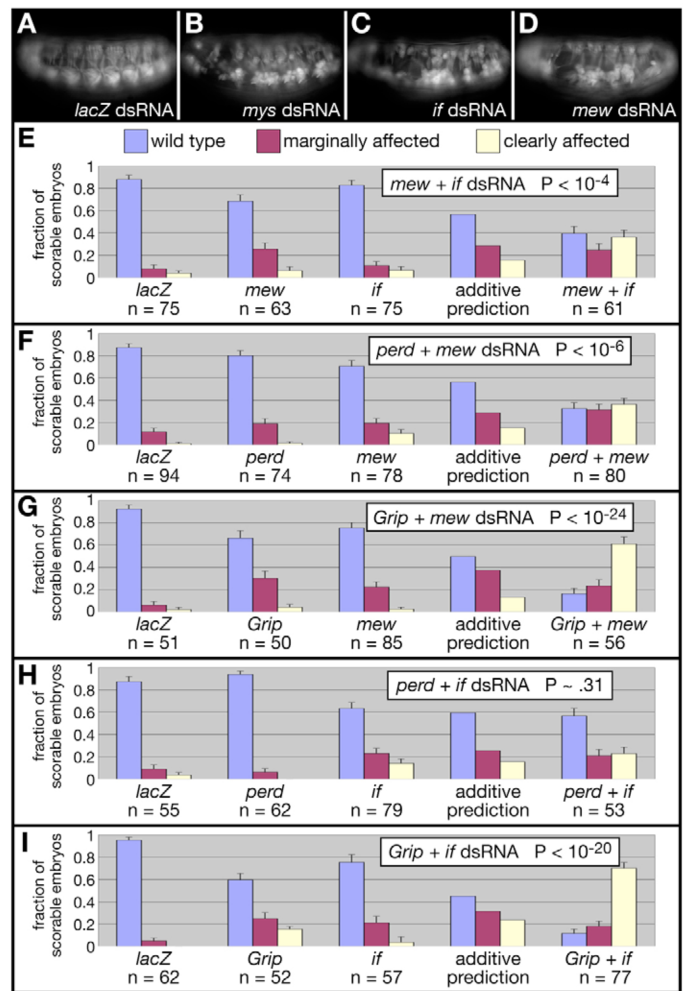

Fig. 6. RNAi directed against integrin subunits causes similar phenotypes to, and interactions with, *perd* and *Grip*. (**A-D**) High concentrations of dsRNA for the βPS integrin gene *mys* (B), the αPS2 gene *if* (C), or the αPS1 gene *mew* (D) cause muscle rounding, particularly in the ventral region. (Compare with negative control dsRNA in A.) (**E-I**) Pooled data from quadruplicate experiments combining low-concentration dsRNAs shows that *mew* exhibits synergistic interactions with *if* (E; $P<10^{-4}$ by χ^2 test against predicted additive distribution), *perd* (F; $P<10^{-6}$), and *Grip* (G; $P<10^{-24}$), whereas *if* interacts significantly with *Grip* (I; $P<10^{-20}$) but not with *perd* (H).

functionally related – we mixed the low concentrations of each dsRNA in a single injection. The observed phenotypes confirmed a synergistic interaction (Fig. 4D): embryos injected with both dsRNAs showed a significantly stronger distribution of phenotypes ($P<10^{-12}$, χ^2 test) than either dsRNA alone or than the distribution predicted to result from an additive effect of the individual dsRNAs (see Materials and methods for details of the additive prediction and statistical approach; Fig. 4E). We verified the specificity of the strategy by testing for a genetic interaction between *perd* or *Grip* and the gene *mind bomb 2* (*mib2*), mutations of which produce a similar muscle morphology phenotype and which affects a similar and overlapping group of muscles (Estrada et al., 2006). In the experiments involving *mib2*, no statistically significant interaction occurred (Fig. 4F,G). This RNAi-based method suggests that *perd* and *Grip* interact specifically in the formation of muscle attachments. It also represents a new, rapid and generalizable assay to test for genetic interactions in living embryos, one that is potentially more sensitive than classical genetic methods which are limited by the strengths of the alleles that are available to be combined.

Perdido binds biochemically to a Grip PDZ domain and recruits Grip to the muscle plasma membrane

The genetic interaction between *perd* and *Grip*, combined with the nature of their encoded protein domains, prompted us to test if the proteins interact biochemically. The Grip protein contains seven PDZ domains, whereas the Perd protein has a consensus PDZ

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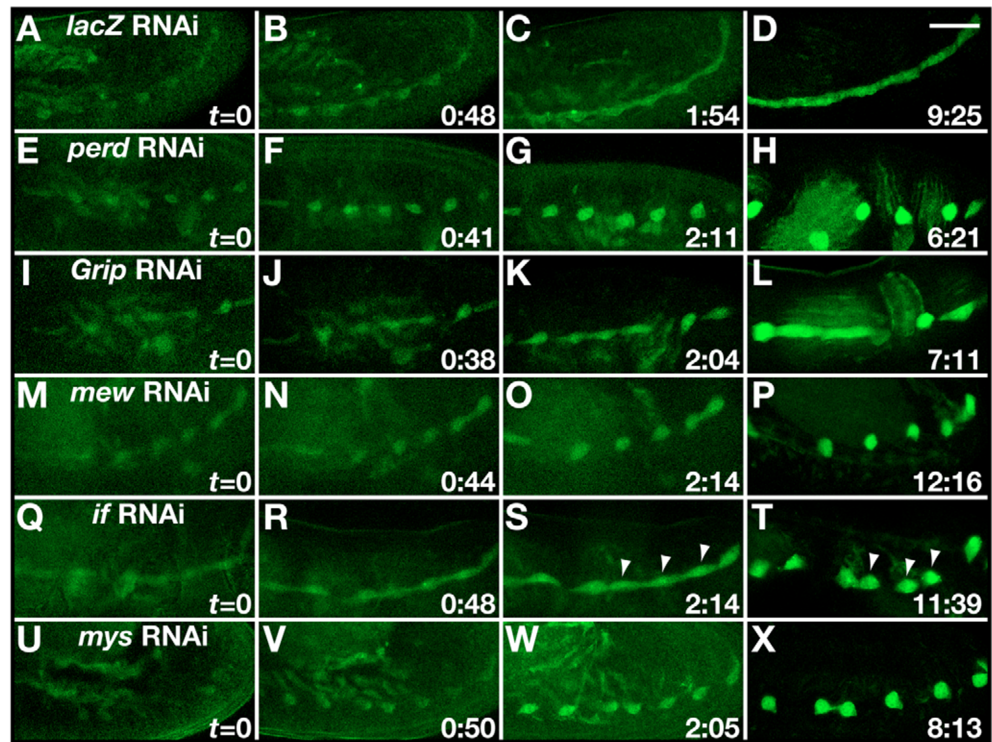

Fig. 7. Loss of PS1, but not PS2, integrin function phenocopies *perd* loss-of-function in muscles. Each row shows the development of a single embryo injected with dsRNA from the time GFP expression in muscle VL1 first becomes detectable (*t*=0, approximately stage 14) through late stages, when muscles are contracting and embryos are moving. Additional GFP expression comes from visceral muscles that also express Gal4, internally to the VL1 muscle. Scale bar: 50 μm. (**A-D**) In control *lacZ* dsRNA-injected embryos, muscles are initially unattached, but rapidly achieve their mature attachment sites (B) and elongated morphology (C). (**E-H**) RNAi directed against *perd* prevents muscles from ever forming proper attachments. (**I-L**) *Grip* dsRNA affects fewer muscles, with a timecourse similar to that of *perd*. (**M-P**) RNAi for the αPS1 integrin subunit *mew*, which affects both maternal and zygotic transcripts, causes a severe phenotype identical to that of *perd*. By contrast, in embryos injected with dsRNA for the αPS2 subunit *if* (**Q-T**), many muscles develop apparently normal attachments and elongated morphology at stage 16 (arrowheads in S) before assuming a rounded-up appearance after muscle contraction begins (arrowheads in T). Removal of both PS integrins by RNAi directed against the common β subunit *mys* (**U-X**) gives the more severe early phenotype.

binding sequence at its C terminus. In a GST pulldown assay (Fig. 5A,B), PDZ domain 7 from Grip interacts with an epitope-tagged version of the Perd C-terminal intracellular domain.

Grip protein is normally localized to the attachment sites of elongated myotubes that contact segment borders (Swan et al., 2004) (Fig. 5C-H). Since *perd* encodes a muscle transmembrane protein, and it interacts genetically and biochemically with Grip, we hypothesized that it might be responsible for the translocation of Grip to the plasma membrane. Indeed, in *perd* mutant myotubes, Grip protein is distributed throughout the cytoplasm and fails to localize to the muscle membrane (Fig. 5J,K,M,N). This finding suggests that Perd is the protein responsible for establishing the normal subcellular distribution of Grip at muscle attachment sites where the two proteins may form a signaling complex.

The integrin genes *mys* and *mew* are required to form proper muscle guidance projections and interact genetically with *perdido*

Phenotypic analysis of *perd* loss-of-function embryos indicates that this gene encodes an important muscle transmembrane protein regulating muscle projections during the early phases of myotendinous junction formation. The spherical muscles observed in *perd* mutant embryos are reminiscent of the phenotypes of the integrin mutants, *mys* and *if* (Fig. 6B-D) (Brabant and Brower, 1993; Brown, 1994; Leptin et al., 1989; Prokop et al., 1998; Wright, 1960). This phenotype – together with the fact that Perd contains laminin domains, to which integrins are known to bind (Gotwals et al., 1994) – led us to investigate whether there is a genetic interaction between integrin genes and *perd*.

To address this question, we first studied the phenotypes of embryos separately injected with dsRNA specific for the genes *mys*, *mew* and *if*. RNAi for each gene resulted in embryos with many rounded muscles. This result is consistent with the phenotype reported for *mys* and *if* null zygotic alleles. However, the *mew* RNAi result contrasts with the minor effects that have been described for *mew* null alleles (Roote and Zusman, 1995). Although our dsRNAs are routinely designed to minimize the likelihood of off-target effects (Estrada et al., 2006) (also, see Materials and methods), we tested an additional dsRNA corresponding to a non-overlapping region of the *mew* gene and obtained identical results (data not shown). We therefore proceeded with titration and co-injection experiments to assay potential interaction of each integrin α subunit with *perd* and with *Grip*, as well as with each other. *mys* was not included in this analysis because its requirement for both PS1 and PS2 function would make the result uninformative. The genetic interaction between *mew* and *if* – previously observed at the level of muscle ultrastructure (Prokop et al., 1998) – is clearly detected in this assay (Fig. 6E). As predicted, both *perd* and *Grip* interact significantly with *mew* (Fig. 6F,G). Strikingly, the distribution of muscle phenotypes observed when *perd* and *if* dsRNA are combined is indistinguishable from the additive effects of each individual dsRNA (Fig. 6H), whereas *Grip* and *if* interact strongly (Fig. 6I). This result suggests that the PS2 integrin (comprising the *if* and *mys* gene products) may participate in muscle attachment in a manner different from the inferred PS1-Perd complex, possibly by stabilizing myotendinous junctions after they have formed.

To test this possibility, we visualized the development of muscle VL1 in vivo with RNAi directed against each of the integrin subunit genes. These results, along with those for *perd*, *Grip*, and *lacZ*

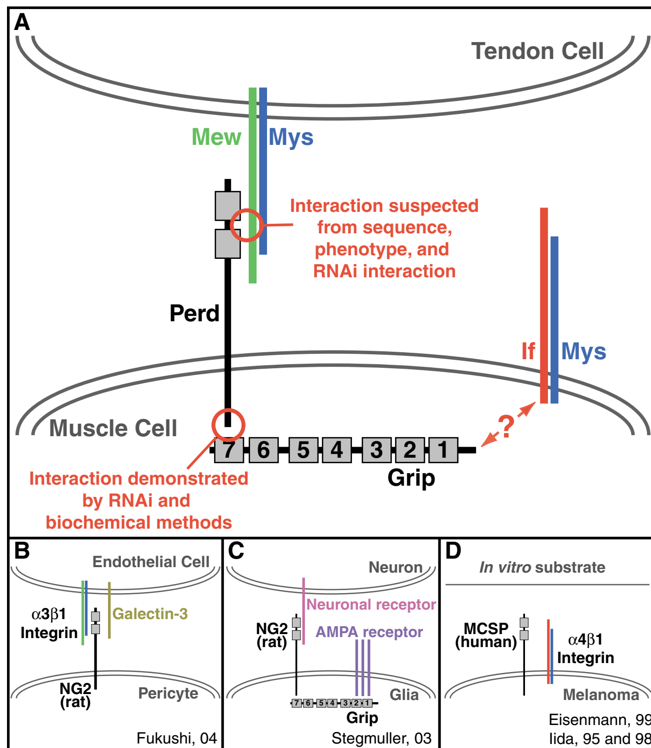

Fig. 8. A model for Perd function in formation of muscle attachments. (**A**) Muscle-expressed Perd protein forms a complex with Grip, directing it to sites of tendon contact. We hypothesize that PS1 integrin heterodimers (the product of the *mew* and *mys* genes) expressed on tendon cells may serve as the ligand for Perd binding and thus mediate target recognition. See Discussion for details. The additional PDZ domains of Grip can then recruit other proteins required for the maturation of the myotendinous junction; the PS2 integrin expressed on the muscle and known to be required for stable muscle attachment is an attractive candidate, but no direct interaction with Grip has been demonstrated. (**B-D**) Elements of this model have previously been reported (references, bottom right and in Discussion) in other systems where Perd orthologues are expressed, including interaction with an integrin of the laminin-binding class (B), interaction with Grip (C), and acting as an adhesion coreceptor for an integrin in cis (D).

dsRNAs, are shown in Fig. 7. Each row shows four time points after injection of the same embryo; *t*=0 represents the time at which we were first able to visualize the muscle by GFP fluorescence (that is, during stage 14), and the last panel shows stage 17 embryos, at which point the muscles are clearly contracting when directly visualized by fluorescence microscopy. In the *lacZ* dsRNA-injected embryos, the muscles are clearly elongating at the earliest time at which they are visible (Fig. 7A,B). Embryos injected with *mew* or *mys* dsRNA exhibit an aberrant phenotype, appearing more rounded and with less robust projections than in age-matched control embryos, as early as stage 14-15 (Fig. 7N,V). In this regard they resemble embryos injected with *perd* dsRNA, where the muscles have a rounded shape early in development, suggesting that PS1 integrin is necessary for the formation of proper projections and muscle attachment. By contrast, *if* dsRNA-injected embryos have numerous muscles that extend normally and achieve an apparently mature morphology (arrowheads in Fig. 7R,S), but which then round up late in development, after muscle contraction starts (arrowheads in Fig. 7T). This finding is consistent with the established role of integrins in the stabilization of the attachment between muscle and tendon cells (Brown et al., 2000).

DISCUSSION

The development of the myotendinous junction in *Drosophila* provides an excellent in vivo system to better understand the molecular mechanisms underlying cell migration and adhesion. These cellular processes are essential for normal organogenesis, and, when dysregulated, lead to pathological behaviors such as tumor cell metastasis. In this study, we have identified a new gene, *perd*, that is required for the development of the myotendinous junction. *perd* is expressed in a subset of muscle founder cells and in growing myotubes, and it encodes a single-pass transmembrane protein with two laminin domains in the extracellular portion and a class II PDZ binding sequence at the intracellular C terminus. Loss of *perd* function is associated with failure to extend proper myofilopodial projections and to form stable attachments between expressing muscles and the overlying epidermis.

Very little is known about how individual muscles acquire their specific morphologies. It is thought that the restricted expression of certain transcription factor genes, known as 'muscle identity genes', and their regulation of downstream targets play an important role in this process (Baylies et al., 1998; Frasch, 1999). *perd* is both expressed and required in a subset of muscle founder cells, although there are more cells expressing *perd* than muscles with a detectable mutant phenotype. These observations suggest that *perd* encodes some of the specific information that founder cells provide to a given muscle so that it can acquire its particular characteristics, in this case related to the formation of the attachment to the tendon cell. It remains unkown if there are similar transmembrane proteins with an equivalent role in the muscles that do not express *perd*.

Inactivation of *perd* function, either by RNAi or by chemically induced null alleles, causes embryonic muscle projections to appear dysmorphic at early stages of embryogenesis and prevents them from forming stable attachments to the appropriate tendon cells, resulting in rounded, unattached muscles. There could be several reasons for such a phenotype, including failure of muscle guidance, of migration, and/or of attachment to the tendon cell. To better understand the function of *perd*, we carried out a series of genetic interaction experiments with genes having similar phenotypes. To this end, we developed a new method to assess genetic interactions by testing for phenotypic synergy between low doses of different gene-specific dsRNAs injected into live embryos. Our results suggest that RNAi-based interaction experiments are more sensitive than allelic genetic interaction because we can achieve greater reductions in mRNA levels than is possible in a heterozygous condition (even with null alleles), and we can simultaneously reduce both maternal and zygotic mRNA contributions in the embryo (Kennerdell and Carthew, 1998). This experimental protocol is readily adapted to explore any pairwise genetic interaction when a suitable phenotype can be scored.

One gene with a similar phenotype to *perd* is *Grip*, which is required in muscles for proper formation of cellular extensions during guidance (Swan et al., 2004); thus, mutations in this gene result in rounded muscles. Using our RNAi assay, we have found a genetic interaction between these two genes, supporting the idea that they could be playing related roles during the formation of the myotendinous junction. In addition to this similarity, we noticed that the C terminus of Perd resembles a PDZ binding consensus, leading us to test a possible biochemical interaction between the intracellular

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domain of Perd and isolated PDZ domains from Grip. The finding of a biochemical interaction between Perd and a functionally essential Grip PDZ domain, PDZ7 (Swan et al., 2006), together with the observation that Grip fails to localize to the attachment site in *perd* mutants, suggests that Perd is physically recruiting Grip to the muscle membrane. We hypothesize that Perd may be priming a molecular complex at the muscle membrane attachment site by localizing Grip and other proteins through its multiple PDZ protein interaction domains.

Quantification of the affected muscles in *Grip* and *perd* null mutant embryos demonstrates that the *Grip* phenotype is of lower expressivity, suggesting that *perd* has additional functions independent of *Grip*. In order to investigate other possible *perd* interactors, we focused on the study of *Drosophila* integrins, for three reasons. First, loss-of-function mutations in *mys* and *if* result in spherical unattached embryonic muscles (Brown et al., 2000), similar to the terminal phenotype of *perd* mutants. Second, Perd contains two laminin domains, which are known integrin ligands (Gotwals et al., 1994). Third, integrins can bind both to extracellular matrix molecules and to transmembrane receptors (Brown et al., 2000). We found that interfering with the function of the *mys*, *mew* and *if* genes by injection of gene-specific dsRNAs into live embryos generates unattached, rounded muscles. Interestingly, these results are comparable with loss-of-function mutations in *mys* and *if*, but are more severe than previously described for *mew*; only 10% of *mew* mutant embryos were reported to have muscle abnormalities (Roote and Zusman, 1995). Inactivating both the maternal and zygotic mRNAs by RNAi might account for the higher number of affected muscles in embryos injected with *mew* dsRNA. In addition, double mutants for *mew* and *if* show muscle detachments at an earlier stage than *if* single mutants, providing another line of evidence that *mew* has a role in muscle attachment (Roote and Zusman, 1995). We carried out a detailed analysis of these phenotypes using single myotube imaging in live embryos for which integrin function was blocked by RNAi, where we indeed uncovered an early role for both *mys* and *mew* in myogenesis, that is, in the formation of proper projections and attachments to the tendon cells, similar to *perd* and *Grip* dsRNA-injected embryos. By contrast, embryos injected with *if* dsRNA exhibited normal muscle projections at early stages.

There are several possible molecular functions for *perd*. Perd could be a receptor for guidance cues, although the fact that muscles initially migrate toward and make contact with the correct tendon cell in *perd* mutants suggests that it is not involved in directed migration. Alternatively, Perd could serve as a muscle receptor for an anchoring molecule in the tendon cell; the rounding up of muscles and the rare misdirected projections in later stages suggest that it is the differentiation of the cell contacts between the muscle and the tendon cell that is impaired in *perd* mutants. In fact, it has been described that, as development progresses, the cellular interactions mediated by integrins change from small transient contacts, involved in cell recognition and migration, to the larger, more stable adhesive contacts known as hemiadherens junctions (Brown et al., 2000; Prokop et al., 1998). Indeed, in the careful ultrastructural analysis of Prokop et al. the authors point out that although the distance between cell membranes at mature myotendinous junctions is too great for direct contact between integral membrane proteins of the muscle and tendon cells, "At early muscle attachments, closer cell contacts with an extracellular gap of only about 15 nm are also seen." Thus, we hypothesize that Perd serves as a receptor for the tendon-expressed PS1 integrin, encoded by *mew* and *mys*, and that Perd also recruits the PDZ protein, Grip, to regulate muscle projection and differentiation of the myotendinous junction (Fig. 8A). A series of results supports this hypothesis: the biochemical and genetic interaction between *Grip* and *perd*; the fact that localization of Grip to the membrane is dependent on *perd*; the fact that Perd contains laminin domains and that PS1 is an integrin of the laminin-binding class (Gotwals et al., 1994); and the occurrence of rounded muscles before muscle contraction in embryos injected with *mys* or *mew* dsRNA, phenotypes similar to those observed in *perd* mutants and in embryos injected with *perd* dsRNA.

During preparation of this manuscript, Schnorrer and colleagues (Schnorrer et al., 2007), described the phenotypic characterization of loss-of-function mutations in CG10275 (which they named *kon-tiki*), and physical and functional interaction of the corresponding protein with Grip. The present work not only corroborates but also extends their findings by demonstrating a requirement for Perd in localizing Grip protein to developing myotendinous junctions, and by proposing a mechanism by which this localization occurs: recognition of tendon cell-expressed PS1 integrin heterodimers by Perd.

Interestingly, the vertebrate orthologues of Perd (NG2 in rats, AN2 in mice and MCSP in humans) are chondroitin sulphate proteoglycans required for cell migration and communication during the normal development of certain cell types, such as glia and mesenchyme progenitors. MCSP has also been implicated in tumor cell metastasis in which cellular adhesion and migration are abnormally regulated (Stallcup, 2002). Similar to Perd, these mammalian proteins also interact with integrins or Grip proteins in different cellular contexts that promote cell migration and signaling, although the underlying molecular mechanisms are largely unkown (Eisenmann et al., 1999; Fukushi et al., 2004; Iida et al., 1998; Iida et al., 1995; Stegmuller et al., 2003) (Fig. 8B-D). Our findings for the function of Perd provide a new model in another cellular and in vivo system, the myotendinous junction, in which it may be possible to better understand the function of the vertebrate orthologues in the regulation of cell adhesion and metastasis. In addition, our novel dsRNA interaction method offers a fast, generalizable and directed way to test hypothetical interactions in an experimentally tractable model system.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/24/4469/DC1

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