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, multiplet edematous wing (mew, αPS1), lethal myosporoid (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 hemiatherens-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: perdF1-3/CTG, perdF2-2/CTG, perdH1-4/CTG, perdH1-4/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-lacz2 were used to visualize the morphology of the VL1 muscle in perd (PS1-1/US-lacZ, perdF1-3/5053Gal4/4), and Gripex36 (Swan et al., 2004) mutants (Gripy0/y; US-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), mildly 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 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, tubules 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
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 scorables scored for each experiment. 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 α, β, γ and δ 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 = αx, the predicted fraction of marginally affected embryos = αy + βx, and the predicted fraction of clearly affected embryos = αz + cγ + cβ + cδ + cxy + cyz. 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 6×His 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, 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 with twice with buffer at room temperature, and bound proteins were eluted in 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 (NQYVV). 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).
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-SS), 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 immunostainings. 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. 1H1). 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.
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 4473

**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 (x^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.
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 ($P < 10^{-12}$, $\chi^2$ 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...
of Grip to the plasma membrane. Indeed, in we hypothesized that it might be responsible for the translocation protein, and it interacts genetically and biochemically with Grip,
2004) (Fig. 5C-H). Since elongated myotubes that contact segment borders (Swan et al.,
version of the Perd C-terminal intracellular domain.
5A,B), PDZ domain 7 from Grip interacts with an epitope-tagged binding sequence at its C terminus. In a GST pulldown assay (Fig. 4A-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 (Estrad 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
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 underlying 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 unknown 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

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. By contrast, 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).
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 mew 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 (Gottwals 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.

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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 hemiattachment contacts (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 (Gottwals 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 unknown (Eisenmann et al., 1999; Fukushima 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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