Slowdown promotes muscle integrity by modulating integrin-mediated adhesion at the myotendinous junction

Eliezer Gilsohn and Talila Volk*

SUMMARY
The correct assembly of the myotendinous junction (MTJ) is crucial for proper muscle function. In Drosophila, this junction comprises hemi-adherens junctions that are formed upon arrival of muscles at their corresponding tendon cells. The MTJ mainly comprises muscle-specific αPS2βPS integrin receptors and their tendon-derived extracellular matrix ligand Thrombospondin (Tsp). We report the identification and functional analysis of a novel tendon-derived secreted protein named Slowdown (Slow). Homozygous slow mutant larvae exhibit muscle or tendon rupture, sluggish larval movement, partial lethality, and the surviving adult flies are unable to fly. These defects result from improper assembly of the embryonic MTJ. In slow mutants, Tsp prematurely accumulates at muscle ends, the morphology of the muscle leading edge changes and the MTJ architecture is aberrant. Slow was found to form a protein complex with Tsp. This complex is biologically active and capable of altering the morphology and directionality of muscle ends. Our analysis implicates Slow as an essential component of the MTJ, crucial for ensuring muscle and tendon integrity during larval locomotion.

KEY WORDS: Drosophila, Egl7, Thrombospondin, Integrin, Muscle, Tendon

INTRODUCTION
Adhesion between distinct cell types forms the basis for tissue assembly during embryonic development. The establishment of the myotendinous junction (MTJ) in the Drosophila embryo has been used as a model system in which to study how the interplay between distinct cell types results in a complex, functioning contractile tissue.

Initially, muscle founder cells are specified at distinct locations in the embryo, fuse with surrounding myoblasts, and migrate towards their corresponding tendon cells in the ectoderm (Bate, 1990; Bronner and Dickson, 2004). These tendon cells are specified by the early growth response (EGR)-like transcription factor Stripe. Stripe has been shown to be both necessary and sufficient for the induction of most of the tendon-specific genes, including gene products that provide the correct guidance and adhesion cues to direct muscle migration towards tendon cells (Becker et al., 1997; Frommer et al., 1996; Lee et al., 1995; Volk and VijayRaghavan, 1994).

Once muscles reach their corresponding tendon cells their migration is arrested; only after migration arrest do integrin-mediated hemi-adherens junctions form between the muscles and tendon cells to establish the MTJ. The timing of hemi-adherens junction formation is crucial, as premature junction formation, prior to migration arrest, may result in an aberrant MTJ. At these junctions, the integrin receptors on the muscle cell membrane (αPS2βPS) bind to the extracellular matrix (ECM) protein Thrombospondin (Tsp), which is deposited on the tendon cell surface. The integrin receptors on the tendon cell membrane (αPS1βPS) bind to distinct ECM proteins (e.g. Laminin) that are deposited at the same site (Bokel and Brown, 2002; Brower, 2003; Brown, 2000). Tiggrin, an additional ECM component, mediates intercellular adhesion between muscle cells at some of the MTJ sites. Importantly, mutant embryos lacking either integrin receptors or their ECM ligands display aberrant muscle function due to dissociation of muscles from their respective tendon cells.

Time-lapse microscopy performed on individual migrating muscles indicated significant alterations in the morphology of the muscle leading edge during the establishment of the MTJ. Prior to muscle arrival at the tendon cell, numerous filopodia are detected at the muscle leading edge, whereas during the establishment of the MTJ these filopodia disappear and the surfaces of the muscle ends become wider and smoother. It is not clear whether these morphological changes are linked to the distribution and ligand occupation of the αPS2βPS integrin receptors at the muscle ends.

Drosophila MTJs must be strong enough to withstand the massive mechanical forces produced by muscle contraction during larval locomotion. This is achieved by a gradual accumulation of the αPS2βPS integrin heterodimers at the muscle ends, as well as by rearrangement and further deposition of the ECM proteins Tsp, Tiggrin and Laminin (Bunch et al., 1998; Chanana et al., 2007; Fogerty et al., 1994; Subramanian et al., 2007). By the end of embryogenesis, the MTJ is a complex structure with symmetrically arranged integrin receptors and large amounts of ECM proteins located in between the plasma membranes of both cell types.

In the Drosophila embryo, integrin receptors are detected at the muscle ends only upon the arrival of the muscles at their target tendon cells, presumably following integrin-ligand interaction. This suggests that the integrin-ligand interaction at the muscle leading edge must be regulated. Subsequent to the integrin-ECM interaction, many of the cytoplasmic proteins that are characteristic of focal adhesions accumulate at the cytoplasmic surfaces of the attachment site, including Talin (Rhea – FlyBase), Integrin linked kinase, Pinch (Steamer duck – FlyBase) and others (Bokel and Brown, 2002; Brower, 2003; Brown et al., 2002).

The main αPS2βPS integrin ligand expressed on muscle, Tsp, is secreted by tendon cells prior to the arrival of the muscles at the tendons (Subramanian et al., 2007). However, it does not trigger...
αPS2βPS integrin receptor accumulation at the surface of the migrating muscle prior to the encounter of the muscle with the tendon cell. Premature accumulation of integrin receptors at the leading edge of the migrating muscle might interfere with both its migration capability and leading edge morphology and, consequently, with the fine architecture of the MTJ. Thus, temporal and spatial regulation of the association between integrin receptors and their ECM ligand Tsp is essential to promote proper assembly of the MTJ.

In this study, we describe a novel Drosophila gene product, Slowdown (Slow), which is secreted by tendon cells and is essential for proper assembly of the MTJ. Embryos mutant for slow exhibit premature accumulation of integrin and Tsp at the muscle ends, aberrant morphology of the muscle ends, and subsequent abnormal architecture of the MTJ. This leads to muscle and/or tendon rupture upon muscle contraction during larval locomotion and to partial larval lethality. Further analysis showed that Slow forms a protein complex with Tsp and that this complex is capable of changing muscle leading edge morphology and directionality when ectopically expressed. Thus, our analysis elucidates a novel mechanism for the proper assembly of the MTJ that is essential for correct muscle function.

MATERIALS AND METHODS

Expression constructs for cell lines, bacteria and flies
UAS-Slow-HA was produced by PCR on the LD16414 EST [Drosophila Genomics Resource Center (DGRC)] and cloned into the pTWH destination vector (DGRC). The UAS-TspAD construct was prepared as described (Subramanian et al., 2007). The TspAD-GFP construct was produced by PCR on UAS-Tsp AD and subcloned into the pTWG destination vector (DGRC). The TspN-TD-GFP (GFP-tagged N-terminal part of Tsp, amino acids 1-390) was produced by PCR and cloned into the pTWG destination vector. The SP-Tsp-CTD-GFP construct was produced by PCR and cloned into the pTWG destination vector. The SP-TspCTD<sup>GGD-1<sup><sup>GRELP</sup> GFP construct was produced by two rounds of site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene, USA). For primer sequences, see Table S1 in the supplementary material.

Fly strains
engrailed-gal4, repo-gal4, 5053-gal4 and stripe-gal4 were obtained from the Bloomington Drosophila Stock Center (BDSC), MHC-tauGFP from Elizabeth Chen (Johns Hopkins University, Baltimore, MD, USA), UAS-StrikeA, Tsp<sup>SR</sup> and UAS-TspAD were produced in our laboratory (Voloshynsky et al., 2007; Subramanian et al., 2007), f03584 and d01019 were obtained from the Exelixis Collection at Harvard Medical School, stock #18495 and P{hsFLP}1, y[1] w[1118]; Dr[Mio]/TM3, ry[*] Sb[1] were obtained from the Exelixis Collection at Harvard Medical School, (Volohonsky et al., 2007; Subramanian et al., 2007); (Gilsohn and Volk, in press); (Wayburn and Volk, 2009), we identified the previously uncharacterized gene slowdown (slow, CG7447). Slow contains a signal peptide, an EMI domain [protein-protein interaction domain present in proteins involved in adhesion, phagocytosis and elasticity (Callebaut et al., 2003; Doliana et al., 2000; Kurant et al., 2008)], and EGF and EGF Ca-binding domains, named NIM repeats; these sequences differ from the typical EGF domains and are present in proteins involved in phagocytosis and adhesion (Kurucz et al., 2007) (Fig. 1). RNA in situ staining using a slow-specific probe showed that slow mRNA is expressed in a pattern that is very similar to that of Stripe and Tsp, indicating expression in tendons (Fig. 1A). To verify that slow is indeed under Stripe control, embryos overexpressing Stripe under the engrailed-gal4 driver were stained using a slow-specific probe. This revealed engrailed-like stripes, indicating that slow mRNA is expressed wherever Stripe is overexpressed (Fig. 1B). According to the Inparanoid web tool (O’Brien et al., 2005), Slow is an ortholog of the recently discovered Egfl7 protein. Egfl7 is a conserved protein present in mouse, zebrafish and human, and is highly expressed and secreted by vascular endothelial cells. Egfl7 has been shown to be essential for angiogenic processes such as tubulogenesis, cell migration and cell adhesion (De Maziere et al., 2008; Fitch et al., 2004; Parker et al., 2004; Schmidt et al., 2007). Its mechanism of action, however, remains unclear.

Staining embryos with a polyclonal antibody raised against the full-length Slow protein showed that it is mainly concentrated at the muscle-tendon junction (Fig. 1C;D; see also Fig. 8D). Antibody specificity was confirmed by a western blot against an extract of S2 cells expressing Slow-HA (see Fig. S1 in the supplementary material).
Generation of a slow-null mutant

To characterize Slow function we generated a null mutation for slow. We used a fly strain with an FRT-containing PBac element insertion upstream to the slow locus (P_{XP}d01019) and a fly strain harboring an FRT-containing PBac element insertion in the 5’/H11032 UTR of the Syntaxin 17 (Syx17) gene, downstream of slow (PBac{WH}Syx17f01971) (Fig. 2A). Crossing these flies in trans on the background of heat-shock-FLP and heat shocking the larval progeny resulted in trans-recombination between the FRT sites and deletion of the intervening genomic region (Parks et al., 2004). In situ hybridization performed on the homozygous slow mutant embryos using a slow-specific RNA probe showed no visible staining, verifying excision of the entire gene (Fig. 2B). In addition, several PCR reactions performed on genomic DNA extracted from slow mutant flies, followed by sequencing, verified the gene deletion and determined its boundaries to chromosome 3L: 4383909-4403698 (data not shown).

The mutation deletes the entire slow locus, as well as ~200 bp from the 5’ UTR of the downstream Syx17 gene. Several lines of evidence demonstrated that the deleted ~200 bp of the Syx17 5’ UTR did not contribute to the phenotypes described below. First, unlike the slow deletion, a fly strain containing the downstream PBac element inserted within the 5’ UTR of Syx17 was homozygous viable. Moreover, crossing the mutant fly line to a fly line containing a P element within a coding exon of Syx17 (line f03584 from the Exelixis Collection) resulted in viable flies (even though the f03584 line is homozygous lethal). Finally, ectopic expression of Slow in tendon cells (using the stripe-gal4 driver) fully rescued the slow mutant phenotype (see below).

Mutation in slow is semi-lethal

Upon generation of the fly strain deleted for slow, the viability of homozygous mutant adult flies was examined. We found that ~80% of the homozygous mutant flies were non-viable (n=200). To examine at which stage the lethality occurred, the development of heterozygous and homozygous embryos was examined. We found that 75-80% (n=210) of the homozygous mutant embryos hatched. However, ~68% of the resulting homozygous mutant first instar larvae died either immediately after hatching or within 1-2 hours.
The larvae that lived for 1-2 hours showed a significantly lower rate of body contractions per minute and their locomotion speed was reduced; hence, the gene was named slow. Lethality occurred throughout the course of development, including the period before, as well as during, pupation. Importantly, most of the homozygous escaper flies were unable to fly and died within 1-2 days of eclosion. When the mutant line was crossed to a deficiency uncovering slow, a similar mortality rate was observed, suggesting that the mutant allele is a complete loss-of-function allele.

**Mutation in slow results in the rupture of larval muscles and tendons**
The behavior of the homozygous mutant larvae and escaper adult flies suggested that the mutant animals have impaired muscle function. Examining the muscle pattern of homozygous mutant embryos, however, did not show a noticeable muscle pattern defect (data not shown). To visualize the muscles in living escaper larvae, the mutation was recombined with an MHC-tauGFP reporter, resulting in GFP-labeled microtubules inside the muscles. Homozygous mutant live third instar larvae exhibited torn somatic muscles in most of the segments. Larvae fillets stained for either GFP or for rhodamine-phalloidin, which labels actin fibers within the muscles, indicated that the somatic muscles were torn and/or detached from their insertion sites (Fig. 2C-F). Similar to the phenotype observed in third instar larvae, torn thoracic muscles were observed in surviving slow mutant flies (not shown), explaining their inability to fly.

Next, tendons were examined by staining third instar larvae fillets with an antibody against Shortstop (Shot), a microtubule-binding protein that is highly enriched in tendon cells (Gregory and Brown, 1998; Strumpf and Volk, 1998) (Fig. 2G,H). In wild-type larvae, the three lateral transverse muscles were stretched between two intact Shot-positive tendons (Fig. 2G, arrows). In slow mutant larvae, the muscles were rounded up and still associated with portions of Shot-positive torn tendons (Fig. 2H, arrowheads). Importantly, muscle/tendon rupture was rescued by driving Slow expression from tendon cells using the stripe-gal4 driver (Fig. 2I,J), indicating that the phenotype was caused by the lack of Slow expression.

In summary, lack of Slow affects both muscle and tendon integrity. The rupture of muscles and tendons detected in the mutant larvae would explain both the aberrant larval locomotion and the inability of adult homozygous mutant flies to fly.

**Absence of Slow prevents proper spreading of muscle edges prior to muscle attachment**
The impaired muscle function of the hatched mutant first instar larvae, coupled with the effect on both muscles and tendons, suggested that the lack of Slow influences an early process of MTJ establishment. To address this possibility, we followed muscle migration in living embryos. A Moesin-GFP reporter driven by 5053-gal4, which drives expression only in ventral longitudinal muscle 1 (also known as muscle 12), was used to follow muscle migration in the slow mutant embryos.

In order to observe how Slow affects muscle attachment, we followed the last stages of muscle migration by selecting embryos at stages ~12-13 (when muscle migration has already begun, but muscles have not yet reached their respective tendons). Muscles were photographed every ~5-7 minutes over a 3-hour period at room temperature.

We first noticed that the posterior edges of the wild-type muscles were wider than the anterior migrating muscle ends, presumably owing to an earlier association with the tendon cells [also seen by Schnorrer et al. (Schnorrer et al., 2007)]. In addition, wild-type muscles send filopodia towards the anterior segmental border from a single pointed leading edge, which spreads out upon arrival at the tendon cell at the segment border (Fig. 3A, arrowheads and schematic). In slow mutant embryos, however, the muscle anterior migrating edge, as well as the posterior edge, did not appear to spread upon arrival at the tendon at the segmental border (Fig. 3B, arrowheads and schematic). Similar observations were obtained using CD8-GFP as a marker for the muscle membrane, thereby excluding a possible indirect effect of moesin-GFP on muscle leading edge morphology. The abnormal morphology of the muscle ends in slow mutants led us to further examine the possibility that Slow is essential for MTJ architecture by observing the distribution of integrin and Tsp in slow mutant embryos during the arrival of muscles at their target tendon cells.
Premature accumulation of Tsp and integrin at the muscle ends precedes changes in the architecture of the MTJ detected at later developmental stages in slow mutants

To elucidate potential changes in the assembly of the MTJ in slow mutant embryos, we followed the distribution of integrin and its main ligand, Tsp, at the leading edge of muscle 12 in stage 13-14 embryos. At this stage, the muscle leading edge has not yet arrived at its corresponding tendon. A significantly higher accumulation of Tsp was detected in slow mutant embryos at the muscle leading edge (Fig. 4G,H). Similarly, integrin staining was detected as a fine line overlapping the Tsp deposit at the muscle anterior end (Fig. 4D, arrowhead), in contrast to a more scattered, dot-like pattern of both Tsp and integrin at the muscle anterior end of wild-type embryos (Fig. 4C, arrowhead). Consistently, premature accumulation of Talin was detected at these sites in slow mutants (Fig. 4L, arrow), in line with a premature functional integrin-Tsp association. We concluded that the accumulation of Tsp and integrin, as well as their association, are more intense and presumably established earlier in slow mutant embryos. The changes in Tsp and integrin distribution occur prior to any morphological changes detected at the muscle edges (as described in Fig. 3), indicating that they are not secondary to these changes.

Importantly, analysis of MTJ architecture at stage 16 revealed abnormal MTJ morphology, in which the surface area of the muscle-tendon junction was smaller than in wild type (Fig. 5, arrows in A,B,D,F,H,J). Consistently, premature accumulation of Talin was detected in slow mutant embryos (Fig. 4L, arrow), in line with a premature functional integrin-Tsp association. We concluded that the accumulation of Tsp and integrin, as well as their association, are more intense and presumably established earlier in slow mutant embryos. The changes in Tsp and integrin distribution occur prior to any morphological changes detected at the muscle edges (as described in Fig. 3), indicating that they are not secondary to these changes.

Irregular muscle ends are observed at the muscle-tendon junction of slow mutant larvae

To examine whether the abnormal morphology and aberrant embryonic MTJ are also observed at later developmental stages, we examined slow homozygous escaper third instar larvae. In wild-type larvae, βPS integrin was detected as a thick continuous line between two opposing muscles and the tendon cell (located below the focal plane, Fig. 6A, arrowhead). By contrast, in slow mutant larvae, β integrin staining was irregular at the muscle edges (Fig. 6B,C-C'), presumably reflecting the abnormal architecture of the MTJ seen during embryonic development (Figs 4, 5). The localization of Talin, which serves as a readout of ligand-bound integrin (Brown et al., 2002), appeared similarly aberrant (Fig. 6D,E, arrowheads), suggesting that the integrin detected is bound to the ECM, and the major defect stems from its aberrant distribution at the MTJ. No significant changes in the distribution of actin and myosin were detected in the slow mutant muscles.

These results indicate that the aberrant morphology of the MTJ in slow mutant embryos, as reflected by integrin staining, was preserved during larval stages and could explain the rupture of the muscle/tendon cells as a result of uneven distribution of the contractile forces taking place during larval locomotion.

Fig. 4. Tsp and βPS integrin accumulate prematurely at the muscle ends in slow mutant Drosophila embryos. (A-J) Wild-type (A,C,E,G,I) and slow mutant (B,D,F,H,J) embryos at stage 13-14 expressing UAS-CD8::GFP under the 5053-gal4 driver (driving expression in muscle 12) stained for βPS integrin (red; C,D,E,F,H,J), GFP (green; A,B,E,F,I,J) and Tsp (white; G,H,I,J). Arrowheads indicate the anterior edge of muscle 12, and arrows mark the posterior edge of muscle 12 in a more anterior segment. Note the accumulation of Tsp in slow mutants and the corresponding accumulation of integrin at the same sites. (K,L) Wild-type (K) or slow mutant (L) embryos at stages 13-14 expressing UAS-CD8::GFP under the 5053-gal4 driver, stained for GFP (green) and Talin (red, arrows). Note the accumulation of Talin in slow mutant embryos. Scale bar: 20 μm.

Genetic interaction between slow and inflated

Our results so far suggested that Slow modulates the interaction between the muscle-specific integrin receptors and their ligand Tsp. To further address the functional link between Slow and muscle-specific integrin, we compared the viability of slow homozygous mutant female flies to that of slow mutant females that lack, in addition, one allele of the muscle-specific cPS2 integrin subunit [encoded by inflated (if)]. The inflated mutation is on the X chromosome and thus only heterozygous females are viable.
Significantly, the viability of the slow mutant flies carrying one allele of inflated was reduced by half compared with that of the slow mutant alone (see Fig. 7D). Moreover, all surviving slow+;if276/FM7 flies exhibited a held-out wing phenotype (Fig. 7), which is characteristic of aberrant function of the flight muscles. Such a phenotype was not observed in homozygous slow mutant flies, nor in heterozygous inflated females.

These results indicate a functional link between Slow and αPS2 integrin in adult flight muscles, and suggest that the lack of Slow reduces the activity of the muscle-specific integrin receptors.

**Slow forms a protein complex with Tsp**

As seen from the live imaging, Slow affects the way that muscles attach to their respective tendon cells. Tsp is the crucial ECM component that mediates the binding of muscle-specific integrin heterodimers to the myotendinous ECM (Chanana et al., 2007; Subramanian et al., 2007). As shown in Fig. 4, in the absence of Slow, Tsp prematurely concentrates at the MTJ. We therefore reasoned that Slow might interact with Tsp and thus affect integrin organization and MTJ architecture. Both Tsp and Slow are secreted from tendon cells and colocalize at the muscle-tendon junction (Fig. 8D). To examine the possible interaction of Tsp and Slow, we performed a co-immunoprecipitation assay in Drosophila S2 cells expressing HA-tagged Slow and full-length GFP-tagged Tsp. Slow-HA co-precipitated with Tsp-GFP immunoprecipitated on immobilized anti-GFP beads (Fig. 8B). To dissect which of the domains of Tsp is associated with Slow – the non-conserved N-terminal domain or the evolutionarily conserved C-terminal domain (Fig. 8A) – each was separately fused with GFP and tested for its ability to co-precipitate HA-tagged Slow (the Tsp signal peptide was attached to the C-terminal domain construct to ensure its extracellular localization). We detected a specific complex between Slow and the conserved C-terminal domain of Tsp, but not the N-terminal domain. Importantly, mutating the KGD integrin-binding domain (see below) in the conserved C-terminal
domain of Tsp to LGE resulted in significantly reduced Slow association, demonstrating that the Slow-Tsp interaction is dependent on the intact KGD integrin-binding sequence (Fig. 8C).

This suggested that the interaction between Slow and Tsp might affect the Tsp-integrin interaction through hindrance of the KGD integrin-binding site on the Tsp protein.

Ectopic overexpression of both Slow and Tsp affects the directionality of somatic muscles and the morphology of muscle ends

To address the functional significance of the formation of the Tsp-Slow complex, we ectopically expressed both proteins and examined their effect on muscle behavior. We used the repo-gal4 driver because it drives expression in the glia that cover all peripheral nerves, including the intersegmental nerve, which passes in between segments. The intersegmental nerve elongates dorsally on a time course parallel to the extension of the ventral longitudinal muscles towards the next segment and prior to the full extension of the lateral transverse muscles located close to the path of this nerve. Expression of Slow tagged with HA from the repo-gal4 driver resulted in its accumulation at muscle-tendon junctions (Fig. 9C, arrowheads), as well as along the peripheral nerves (Fig. 9C, arrows) and glial cells of the CNS. This did not, however, lead to any muscle pattern defect (Fig. 9D). Similarly, overexpression of Tsp from the repo-gal4 driver did not cause any muscle pattern defect (Fig. 9A,B, arrows). The latter finding is somewhat surprising considering that Tsp has been shown

Fig. 7. Genetic interaction between slow and inflated. (A-C) Adult female flies heterozygous for inflated (A), homozygous for slow (B), or homozygous for slow and carrying one allele of inflated (C). Note the ‘held-out’ wing phenotype in C. (D) The viability rates of slow mutants and of slow mutants that are also heterozygous for the inflated mutation. Flies that are heterozygous for inflated in addition to the slow mutation display a twofold lower viability.

Fig. 8. Slow forms a protein complex with Tsp. (A) Domain structure of Drosophila Tsp showing the non-conserved N-terminal domain (NTD), which contains putative RGD and KGD integrin-binding sequences, and the conserved C-terminal domain (CTD), which contains the KGD integrin-binding sequence. (B) Co-immunoprecipitation between Slow-HA and full-length Tsp fused to GFP. Tsp-GFP was pulled down by immobilized anti-GFP antibodies and a western blot was performed using anti-HA or anti-GFP antibodies. The levels of Slow-HA in the crude extract are shown by a western with anti-HA. (C) Co-immunoprecipitation between Slow and Tsp, using anti-GFP to capture one of the Tsp constructs: TspNTD-GFP, SP-TspCTD-GFP or SP-TspCTDLGE-GFP (SP, signal peptide; see A). Anti-HA was used in the western blot to assay the association of Slow-HA with the Tsp constructs. A representative western blot is depicted, demonstrating a stronger association of Slow with the Tsp conserved C-terminal domain than with the N-terminal domain or with a mutated C-terminal domain in which the KGD integrin-binding domain is mutated to LGE. The lower panel shows quantification of three co-immunoprecipitation experiments, using ImageJ software (NIH, USA), demonstrating that the KGD>LGE mutation reduces Slow binding approximately threefold. (D) A stage 16 embryo stained with anti-Slow (red) and anti-Tsp (green) indicating the colocalization of Tsp and Slow at the MTJ. Scale bar: 20 μm.
Significantly, ectopic overexpression of both Tsp and Slow together resulted in a clear change in the directionality of somatic muscles; in addition, the morphology of the muscle ends was affected, and the edge of the muscles appeared pointed (Fig. 9E-H). In some cases, the pointed muscle end was directed towards the ectopic site of Tsp and Slow (Fig. 9E,F, arrows), whereas other muscles were associated with the correct attachment site at the segmental border (Fig. 9E,F, arrowheads), but their morphology was abnormally pointed. At a slightly earlier stage (stage 15), the pointed muscle edges were associated with the ectopic site of Tsp and Slow expression (Fig. 9G,H, arrows). β integrin staining was rarely observed at the muscle edges (data not shown).

The variability in phenotypes may be attributed to the timing of expression of both Tsp and Slow relative to the location of the migrating muscle and to the distance of a particular muscle from the tendon cells, which continue to provide attractive cues, as well as expressing endogenous Tsp and Slow.

The morphological alteration of the muscle ends following co-expression of Tsp and Slow was observed not only when expressed ectopically on the motor axon glia, but also when expressed from muscle 12 during its migration using the 5053-gal4 driver (Fig. 10A,B). In this case, aberrant accumulation of integrin was observed, in addition to a change in muscle end morphology. Overexpression of Tsp or Slow alone did not affect muscle morphology. Interestingly, third instar larvae overexpressing both Tsp and Slow driven by the Mef2-gal4 driver exhibited torn muscles (Fig. 10E), supporting the idea that the premature concentration of Tsp and integrin at the muscle ends eventually leads to a muscle tearing phenotype.

In line with this idea, overexpression of the muscle-specific integrin heterodimer (αPS2βPS) in the somatic muscles using the Mef2-gal4 driver (which drives integrin expression during muscle migration) led to a pointed morphology of the muscle ends, reminiscent of that observed in slow mutant embryos (Fig. 10C,D). Examination of third instar larvae overexpressing αPS2βPS using the Mef2-gal4 driver revealed torn muscles, similar to slow mutant larvae (Fig. 10F).

These results suggest that the Tsp-Slow complex is biologically active in vivo and that it is capable of altering the morphology of muscle ends. Importantly, these overexpression experiments suggest that the premature accumulation of αPS2βPS and Tsp at muscle ends of slow mutant embryos might explain the muscle tearing phenotype observed at larval stages.

**DISCUSSION**

When migrating muscles reach their target tendons they reorganize their leading edge in order to arrest migration and form the integrin-mediated MTJ. The possible molecular link between the arrest of muscle migration and the formation of the MTJ has not yet been characterized. In the present study, we analyzed these processes, revealing the function of the novel gene product Slow, a Drosophila ortholog of vertebrate Egfl7, as an important modulator of integrin-mediated adhesion.

The phenotype of muscle/tendon rupture observed in slow mutant larvae is unique and does not resemble that of mutants for the muscle- or tendon-specific integrins, or for their specific ligands Tsp, Laminin or Tiggrin, in which the typical phenotype is muscle detachment from tendon cells and muscle cell rounding following initial muscle contraction in the embryo (Bokel and Brown, 2002; Brown, 1994; Subramanian et al., 2007). Despite the localization of Slow at the MTJ, its deletion leads to phenotypes similar to those caused by mutations affecting either the cytoskeletal arrangement of mature muscles (Naimi et al., 2001) or mature tendons (Subramanian et al., 2003). These unique phenotypes enabled us to identify a novel and crucial aspect of MTJ construction, namely the correct assembly of the integrin receptors and their ECM ligand Tsp at the surfaces of the muscle ends.

We suggest that the defect in slow mutants, manifested by the rupture of both muscles and tendons, might be explained by two mechanisms, which are not mutually exclusive. First, the lack of...
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Fig. 10. Early muscle-specific expression of either PS2βPS integrin or Tsp and Slow results in altered muscle end morphology and larval muscle tears. (A,B) A stage 16 wild-type Drosophila embryo overexpressing UAS-CDB:.GFP, Slow-HA and Tsp from muscle 12 using the 5053-gal4 driver, and stained for GFP (green), βPS integrin (red) and Tsp (white). Arrow indicates the narrowing of the muscle end and the altered integrin distribution. (C,D) A stage 16 wild-type embryo overexpressing the muscle-specific integrin heterodimer βPSαPS2 in all the muscles using the Mef2-gal4 driver, stained for Myosin heavy chain (green) and βPS integrin (red). Arrow indicates the narrowing of muscle ends. (E,F) Wild-type third instar larvae overexpressing either Tsp and Slow-HA (E) or the muscle-specific integrin heterodimer βPSαPS2 (F) in all the muscles using the Mef2-gal4 driver and stained with phalloidin (red). Arrows point to muscle tears that are visible in both types of larvae. Scale bars: 20 μm in A–D; 100 μm in E,F.

Slow may result in an aberrant arrangement of the ECM material deposited between the muscle and the tendon, which becomes too rigid and compact; therefore, the mechanical stress imposed by muscle contraction would lead to muscle or tendon rupture. Second, the fine architecture of the muscle-tendon hemi-adherens junction is aberrant, leading to an unequal distribution of mechanical forces upon muscle contraction, resulting in sporadic muscle or tendon rupture. Ultrastructure electron microscopy analysis of the larval muscle-tendon junction did not reveal significant changes in the arrangement of the electron-dense material deposited between the two cell types (not shown). Thus, we favor the possibility that muscle/tendon rupture occurs due to aberrant formation of the MTJ and the unequal distribution of mechanical forces occurring following muscle contraction.

When the muscle leading edge reaches the tendon cell, it must undergo morphological changes prior to the establishment of the hemi-adherens junction with the ECM ligand(s) provided by the tendon cell. Should junction formation precede the smoothening and widening of the muscle leading edge, it might lead to the formation of an MTJ with aberrant morphology, in which the integrin receptors are not homogenously distributed and the muscle surfaces are rough. Such a scenario is consistent with our observations. We demonstrated that in slow mutant embryos, Tsp and muscle-specific integrins prematurely accumulate at the muscle leading edge prior to its arrival at the tendon cell. We also showed, both in live embryos and fixed material, that at a later developmental stage the muscle leading edge in slow mutant embryos does not spread, resulting in a narrow contact area. These two processes may be interconnected so that the premature accumulation of Tsp and integrin leads to the abnormal pointed morphology of the muscle leading edge. These changes then lead to abnormal MTJ architecture and eventually to defective muscle function, resulting in muscle/tendon rupture and lethality. Additional support for this model comes from the observation that the sole overexpression of αPS2βPS integrin at an early stage of muscle migration leads to a muscle phenotype that is reminiscent of slow, i.e. an altered morphology of the muscle ends and torn muscles at the larval stage.

We therefore suggest that Slow activity allows the muscle leading edges to correctly spread along the surfaces of the tendon cells in order to maximize the contact surface area and to enable the gradual and homogenous distribution of integrins along the entire surfaces of the contact area.

Whether the formation of the Slow-Tsp complex (observed in S2 cells) is directly linked to the premature accumulation of Tsp and integrin at the muscle leading edge is not clear at this stage. It is possible that the KGD domain in Tsp, which is required both for integrin binding and for association with Slow, is masked by Slow, attenuating the Tsp-integrin association with this site; thus, in the absence of Slow, Tsp-integrin interaction occurs prematurely by the association of the muscle integrin with this site. Alternatively, Slow-Tsp association at the KGD site may facilitate the association of the integrin receptors with the alternative RGD site located at the N-terminal region of Tsp, and this might be important for proper Tsp-integrin interaction. Our experiments cannot distinguish between these possibilities. In vitro spreading assays of αPS2βPS-expressing S2 cells showed that Slow reduces integrin-dependent cell spreading on a Tsp matrix (see Fig. S2 in the supplementary material). This favors the possibility that Slow attenuates integrin-Tsp binding.

Importantly, the genetic interaction found between slow and inflated indicates that in the absence of Slow, the muscle-specific integrin functions less efficiently in mediating proper muscle function. This supports the possibility that Slow regulates integrin-dependent MTJ formation by allowing gradual accumulation of αPS2βPS integrin at the MTJ, and that reducing integrin levels further worsens MTJ construction.

The ectopic expression of Tsp and Slow led to clear changes in the somatic muscle pattern, as well as to altered morphology of the muscle leading edge. Upon arrival at the ectopic expression site of Tsp and Slow, the leading edge of several muscles displayed a pointed morphology directed towards the ectopic expression site. In other cases, muscles arrived at their respective tendons, although their leading edge conformation was also altered to a narrower edge. This result further suggests a role for Slow in regulating the shape of muscle ends and emphasizes the biological significance of Tsp-Slow complex formation. Ectopic integrin accumulation was barely detected at the muscle edges, which terminated at the ectopic Tsp-Slow sites, supporting the notion that Slow attenuates integrin-Tsp association and providing an explanation for the premature integrin and Tsp accumulation in the absence of Slow.
Taken together, our results demonstrate that Slow modulates the interaction between Tsp and integrin to impose the correct MTJ architecture, although the exact mechanism of Slow action at the molecular level requires further analysis.

Vertebrate Egfl7 is highly expressed in endothelial cells during embryonic stages and after injury, and is downregulated in most fully differentiated blood vessels of adult tissue. Egfl7 knockout in zebrafish and mice leads to aberrant blood vessel formation, resulting in severe hemorrhages throughout the body and potential lethality (De Maziere et al., 2008; Fitch et al., 2004; Parker et al., 2004; Schmidt et al., 2007). Recently, this phenotype was attributed to the deletion of the Mir126 regulatory microRNA, which resides within intronic sequences of Egfl7 (Fish et al., 2008; Kuhnert et al., 2008; Wang et al., 2008). Therefore, the unique role of Egfl7 in blood vessel formation remains to be further clarified. Significantly, Mir126 is not included within intronic sequences of Drosophila slow.

In summary, our results demonstrate a unique and novel function for the Drosophila Egfl7 ortholog Slow in coordinating the morphological changes that occur at the muscle leading edge following its arrival at the tendon cell and in the establishment of the MTJ. The link between Slow and Tsp might be highly relevant to vertebrate development in vertebrates, which, in addition to Egfl7, is also characterized by expression of Tsp1 and Tsp2 (Iruela-Arispe et al., 2003), representing a potential general molecular paradigm for Slow/Egfl7 activity.

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Competing interests statement
The authors declare no competing financial interests.

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