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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*, Egfl7, 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; Schnorrer 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

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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 TspNTD-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$^{KGD>LGE}$-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-StripeA, Tsp^{8R} and UAS-TspAD were produced in our laboratory (Volohonsky 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] from BDSC. The deletion of *slow* was generated as previously described (Parks et al., 2004) and verified using primers targeting the resulting hybrid P-element. Flies mutant for *slow* recombined with MHC-tauGFP; flies carrying both UAS-Slow and UAS-TspAD; *slow*, *5053-gal4*/TM6,YFP,Sb; *slow*, UAS-Moesin-GFP/TM6,YFP,Sb; *slow*, UAS-CD8::GFP/TM6, YFP, Sb; *slow*, *stripe-gal4*/TM6,YFP,Sb; *slow*, MHC-tauGFP/TM6; if^{k27e}/FM7; and *slow*, UAS-Moesin-GFP/TM3 were produced by us. UAS-Moesin-GFP, *5053-gal4*/TM3 and UAS-CD8::GFP, *5053*-gal4/TM6 were from Frank Schnorrer (Max Planck Institute of Biochemistry, Martinsried, Germany). UAS-βPS integrin;UAS-αPS2 integrin was from Guy Tanentzapf (University of British Columbia, Vancouver, BC, Canada).

Antibodies and immunostaining

Antibody staining was performed essentially as described previously (Ashburner et al., 2005) with the following: rat anti-Slow, produced in our laboratory against full-length Slowdown fused to GST; rat anti-Tsp, produced in our laboratory; mouse monoclonal anti-HA (11583816001, Roche, Switzerland); chick anti-HA (ET-HA100, Aves Labs, USA); mouse monoclonal anti-GFP (11814460001, Roche, Switzerland); guinea-pig anti-Shortstop, produced in our laboratory; anti-Myosin heavy chain (P. Fisher, Stony Brook, NY, USA); anti-βPS integrin and anti-Talin (N. Brown, University of Cambridge, Cambridge, UK). Rhodamine-phalloidin (R415, Invitrogen, USA) was used for F-actin staining.

Visualization of stained cells was performed with a Zeiss LSM 510Meta or a Zeiss LSM710 confocal system.

In situ hybridization

An RNA probe for *slow* was produced using the MEGAscript T7 Kit (1334, Ambion, USA) and the DIG RNA labeling mix (11277073910, Roche, Switzerland), and purified using the RNeasy Mini Kit (74106, Qiagen, USA). The in situ reaction was performed essentially according to the protocol of the Berkley *Drosophila* Genome Project (http://www.fruitfly.org/about/methods/RNAinsitu.html). Embryos were mounted and observed using a Zeiss Axioskop microscope coupled with an Olympus DP11 camera.

Tissue culture and immunoprecipitation

Drosophila S2 cells were transfected using the Escort IV transfection reagent (L3287, Sigma-Aldrich, USA). For the immunoprecipitation, cells were transfected, scraped 3 days following transfection, lysed and loaded on Protein A/G PLUS-Agarose beads (sc-2003, Santa Cruz Biotechnology, USA) preloaded with anti-GFP antibody overnight at 4°C.

Live imaging

GFP-positive embryos (9 hours after egg laying) were arranged on an agar slab in a ventral-lateral orientation, glued to the edge of a coverslip, submerged in Halocarbon oil 700 (H8898, Sigma-Aldrich, USA) and visualized using a Zeiss LSM710 confocal system.

RESULTS

Identification of *slowdown* and characterization of its expression pattern

In a microarray screen aimed at finding novel Stripe target genes that might be important for muscle-tendon interaction [as described previously (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).

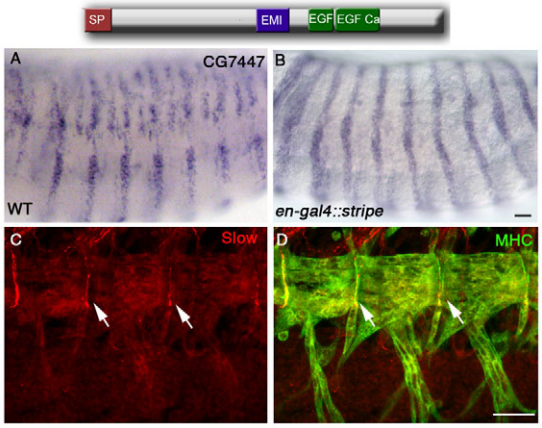


Fig. 1. Slow is produced by tendon cells and concentrates at the muscle-tendon junction. Domain structure of *Drosophila* Slow (top), which contains a signal peptide (SP), an EMI domain, EGF domains and EGF Ca-binding domains. (**A**,**B**) Wild-type embryo (A) and an embryo expressing ectopic StripeA driven by the *engrailed-gal4* driver (B) at stage 15-16 of development, hybridized with a DIG-labeled RNA probe against *slow* mRNA. (**C**,**D**) Stage 16 wild-type embryo stained with antibodies against Slow (red; C,D) and Myosin heavy chain (green; D). Scale bars: 20 μm.

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′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

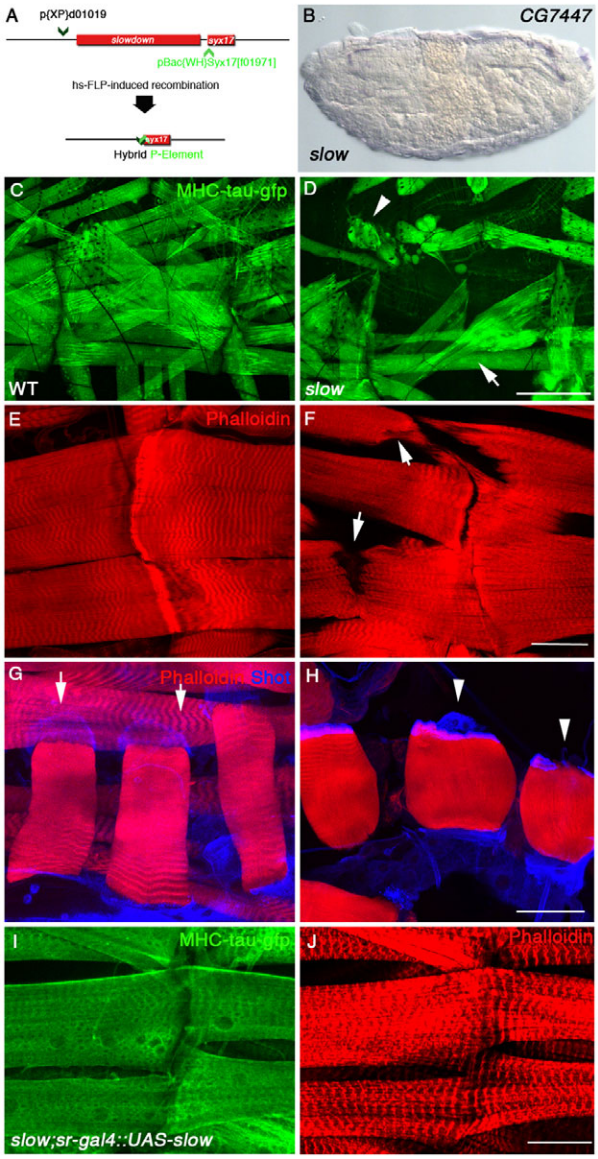


Fig. 2. *slow* mutant third instar *Drosophila* larvae display torn muscles and tendons. (**A**) Generation of the *slow* gene deletion. (**B**) A *slow* homozygous mutant stage 16 embryo hybridized with a DIG-labeled *slow* antisense RNA probe, showing lack of visible staining. (**C**,**D**) Muscles of third instar larvae fillets carrying the MHC-tauGFP reporter. *slow* mutant larvae (D) display torn muscles (arrow, arrowhead) as compared with intact muscles in wild-type larvae (C). (**E**,**F**) Third instar larvae fillets stained with rhodamine-phalloidin (red) to label muscle actin fibers. Mutant larvae (F) display torn muscles (arrows), as compared with wild-type larvae (E). (**G**,**H**) Third instar larvae fillets stained with both rhodamine-phalloidin (red) and anti-Shot antibody (blue, staining tendon cells). Lateral transverse disconnected muscles carrying broken tendons are shown in *slow* mutants (H, arrowheads), as compared with intact tendons in wild-type larvae (G). (**I**,**J**) Rescue of *slow* mutant phenotype in third instar larvae overexpressing UAS-Slow-HA under *stripe-gal4*. Larvae are stained with rhodamine-phalloidin (J, red) to show muscle actin fibers, and with anti-GFP (I) to visualize the MHC-tauGFP-expressing muscles. Scale bars: 200 μm in C,D; 50 μm in E-J.

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

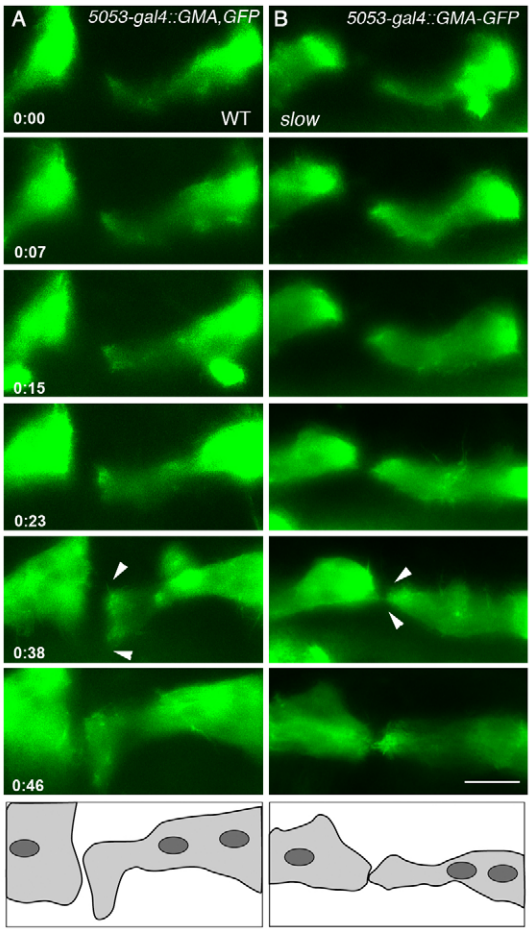


Fig. 3. Live imaging of muscle migration indicates aberrant muscle attachment in *slow* mutant *Drosophila* embryos. UAS-Moesin-GFP was overexpressed in embryos using the *5053-gal4* driver (driving expression in muscle 12). Live embryos were filmed and the final stages of muscle migration were followed every ~5-7 minutes. (**A**) In wild-type embryos, muscle ends widen upon arrival at the tendon cells and attach in a spreadout conformation (arrowheads). (**B**) In *slow* mutant embryos, the muscle ends fail to spread out and attach in a more pointed configuration (arrows). The outlines of the muscles at their final stages of migration are depicted schematically in the bottom panels. Scale bar: 10 μm.

[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 and arrowheads in K,L), leading to an altered overall morphology of the muscle ends as well as to a higher intensity of integrin labeling (Fig. 5C,D, arrows). Tsp labeling was intense in both the wild-type and *slow* mutant embryos (Fig. 5G,H). Three-dimensional reconstruction of the optical sections taken at the *z*-axis of the MTJ revealed that, whereas in wild-type embryos the MTJ appeared as a straight line arranged along the smoothened muscle ends (Fig. 5K), in *slow* mutant embryos the MTJ was smaller and did not appear as a continuous straight line (Fig. 5L), consistent with the pointed morphology observed at the muscle ends.

Taken together, these results suggest that Slow is required for the proper distribution of Tsp and integrin at the muscle leading edge and for the subsequent spreading and smoothening of the muscle ends at the MTJ.

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.

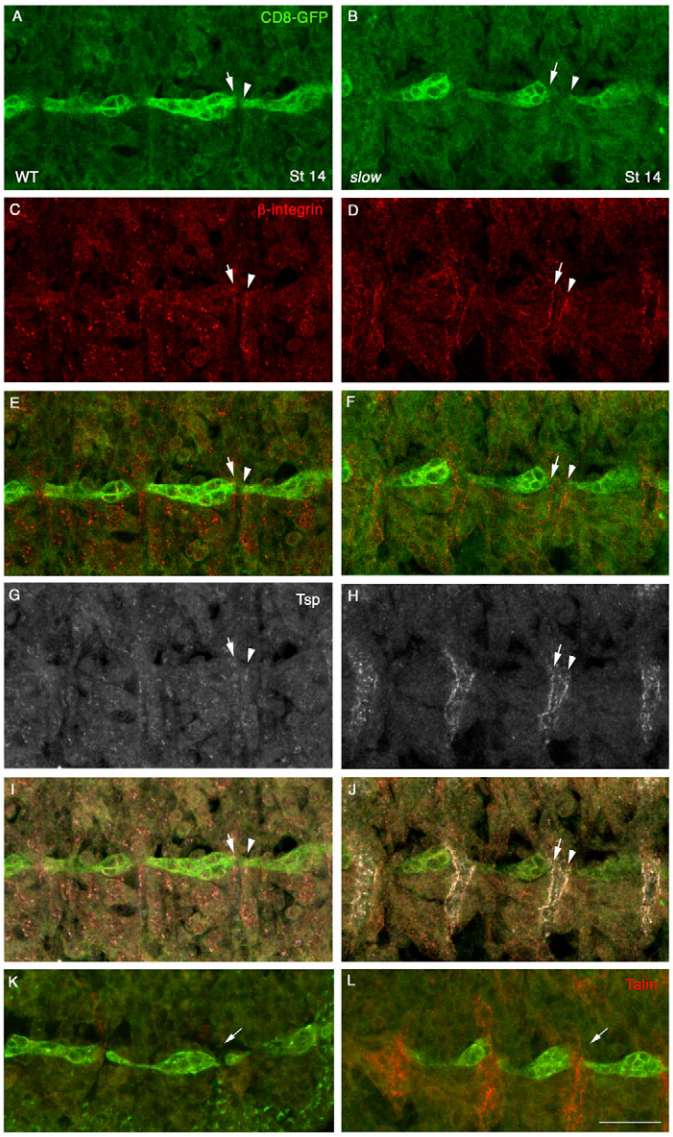


Fig. 4. Tsp and βPS integrin accumulate prematurely at the muscle ends in *slow* mutant *Drosophila* embryos. (**A-J**) Wild-type (A,C,E,G,I) and *slow* mutant (B,D,F,H,J) embryos at stage 13-14 expressing UAS-CD8::GFP under the *5053-gal4* driver (driving expression in muscle 12) stained for βPS integrin (red; C,D,E,F,I,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 αPS2 integrin subunit [encoded by *inflated* (*if*)]. The *inflated* mutation is on the X chromosome and thus only heterozygous females are viable.

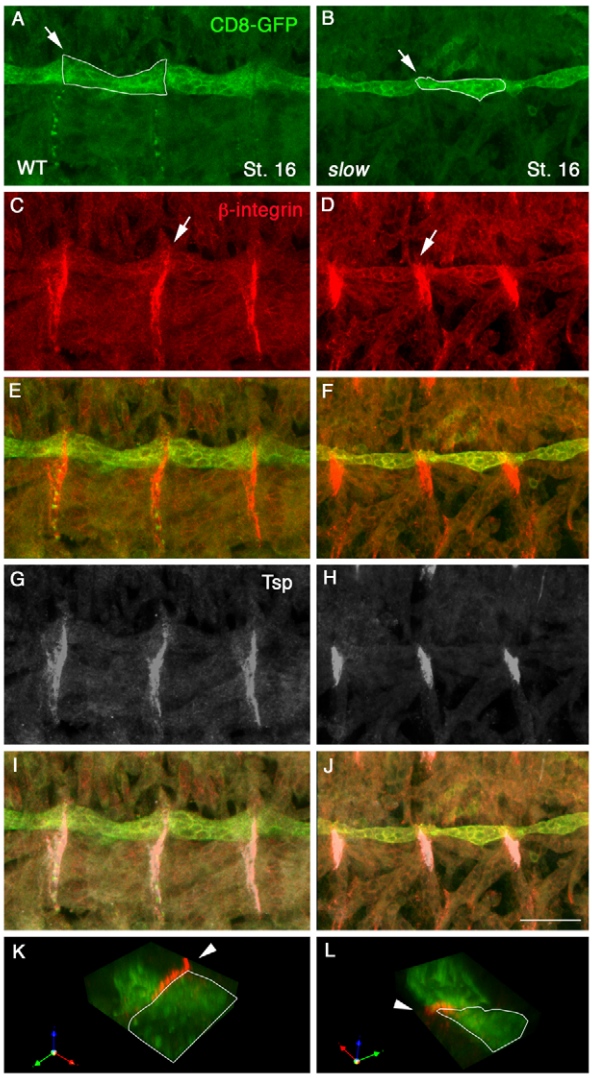


Fig. 5. Aberrant architecture of the myotendinous junction (MTJ) in *slow* mutant *Drosophila* embryos. (**A-L**) Wild-type (A,C,E,G,I,K) and *slow* mutant (B,D,F,H,J,L) embryos at stage 16 expressing UAS-CD8::GFP under the *5053-gal4* driver and stained for βPS integrin (red; C,D,E,F,I,J,K,L), GFP (green; A,B,E,F,I,J,K,L) and Tsp (white; G,H,I,J). (K,L) Three-dimensional reconstruction of an MTJ in wild-type (K) and mutant (L) embryos using the Volocity software package (Improvision, USA). In A,B,K,L a single muscle is outlined to show its aberrant morphology at the leading edge in *slow* mutants. Arrows in A,B,C,D indicate the location of the MTJ. Arrowheads in K,L indicate MTJ morphology. Scale bar: 20 μm.

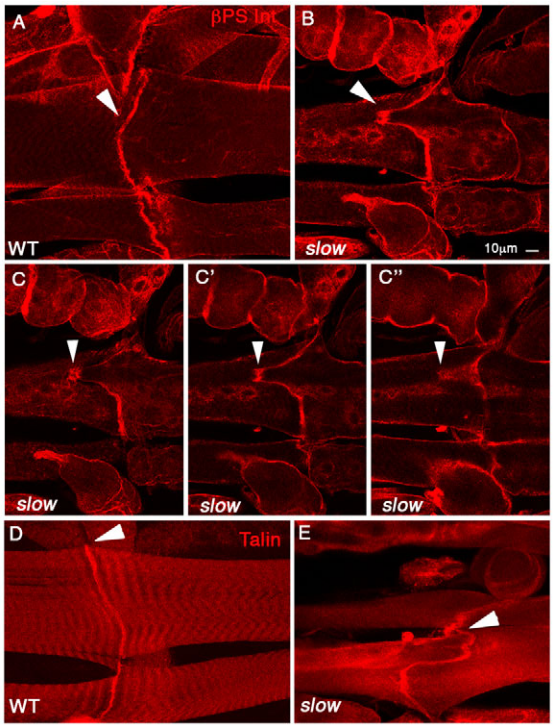


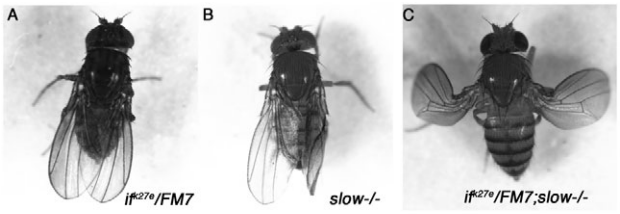
Fig. 6. Abnormal architecture of the MTJ in larvae of homozygous *slow* mutants. (**A-C″**) Third instar wild-type (A) and *slow* homozygous mutant (B-C″) *Drosophila* larvae fillets stained with anti-βPS integrin (red). A and B represent projections of 5 μm depth, and C-C″ are individual optical sections taken at intervals of 1 μm. Arrowheads indicate the MTJ architecture. Note that βPS integrin is seen at multiple sites on muscle ends in distinct focal plains (C-C″). (**D**,**E**) Staining third instar larvae fillets with anti-Talin indicates abnormal membrane extensions in mutant (E) but not wild-type (D) larvae (arrowheads). Scale bar: 10 μm.

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 $slow^{-/-};if^{k27e}/FM7$ 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



D

	slow/+	*slow*/*slow*	Total	Viability percentage
+/+	N=66	N=26	N=92	28.26%
if^{k27e}/+	N=65	N=13	N=78	16.45%

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.

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

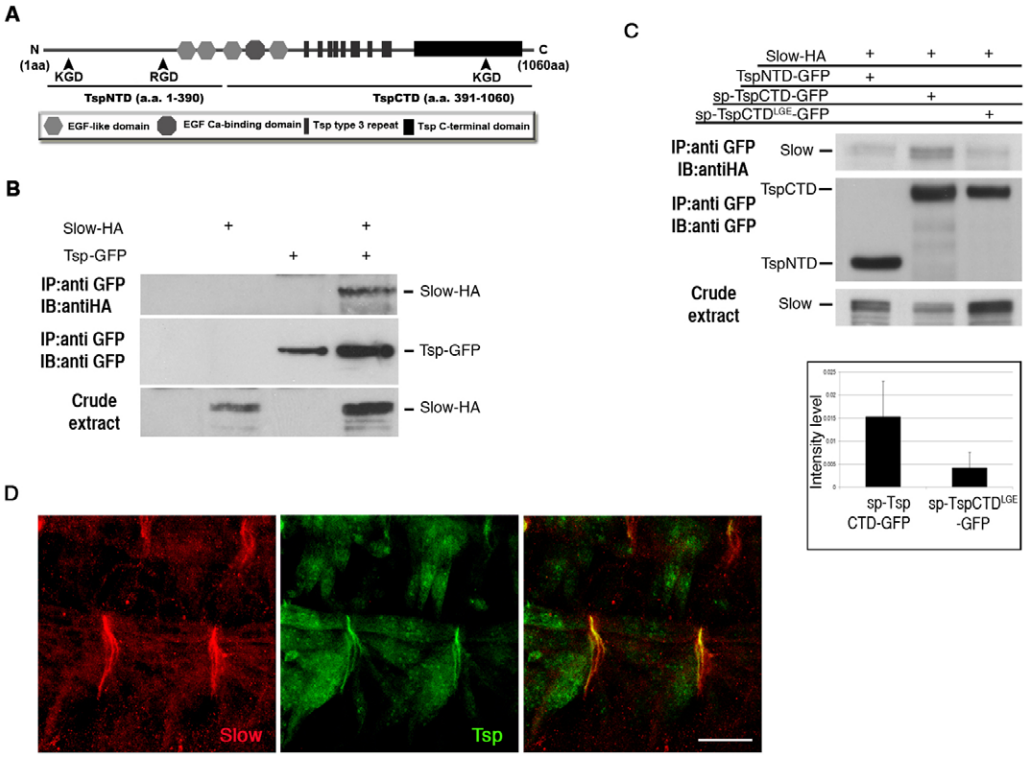


Fig. 8. Slow forms a protein complex with Tsp. (**A**) Domain structure of *Drosophila* Tsp showing the non-conserved N-terminal domain (NTD), which contains putative RGD and KGD integrin-binding sequences, and the conserved C-terminal domain (CTD), which contains the KGD integrin-binding sequence. (**B**) 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-TspCTD^{LGE}-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.

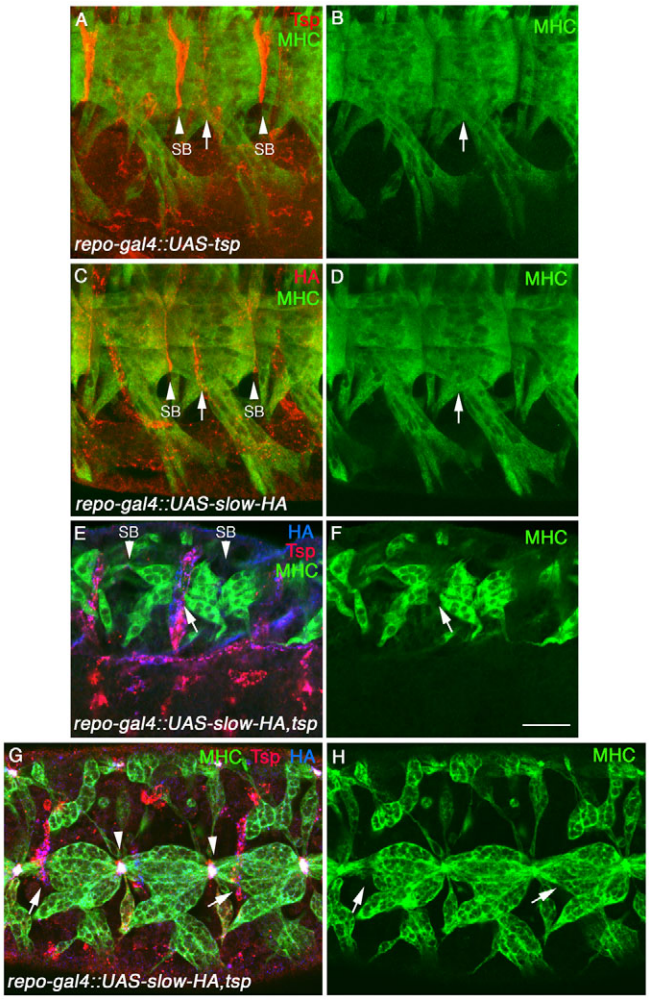


Fig. 9. Ectopic overexpression of both Slow and Tsp promotes aberrant muscle pattern and morphological changes at the muscle leading edges. (**A-H**) Stage 16 *Drosophila* embryos shown in ventrolateral orientation, driven to express (A,B) ectopic Tsp alone, (C,D) Slow alone or (E-H) both Tsp and Slow together, using the *repo-gal4* driver. The sites of ectopic expression at the intersegmental neurons are indicated by arrows. The segmental border sites are indicated by arrowheads. (A) Embryo double labeled for Myosin heavy chain (MHC, green) and Tsp (red), showing normal muscle pattern; MHC staining of this embryo is shown in B. (C) Embryo double labeled for MHC (green) and HA (red, indicating overexpressed Slow tagged with HA), showing normal muscle pattern; MHC staining alone is shown in D. (E) Embryo at stage 16, ectopically expressing both Tsp and Slow, labeled for Tsp (red), HA (blue) and MHC (green), showing aberrant muscle pattern and muscle leading edge morphology; MHC staining is shown in F. (G,H) Embryo at stage 15-16, ectopically expressing both Tsp and Slow, labeled for Tsp (red), HA (blue) and MHC (green); MHC staining is shown in H. Note the pointed edges of the muscles facing the site of ectopic expression (arrows in E-H). Scale bar: 20 μm.

to be a potent adhesion molecule, and suggests that ectopic adhesion cues are insufficient to facilitate muscle migration arrest and ectopic adhesion.

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 accumulation (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

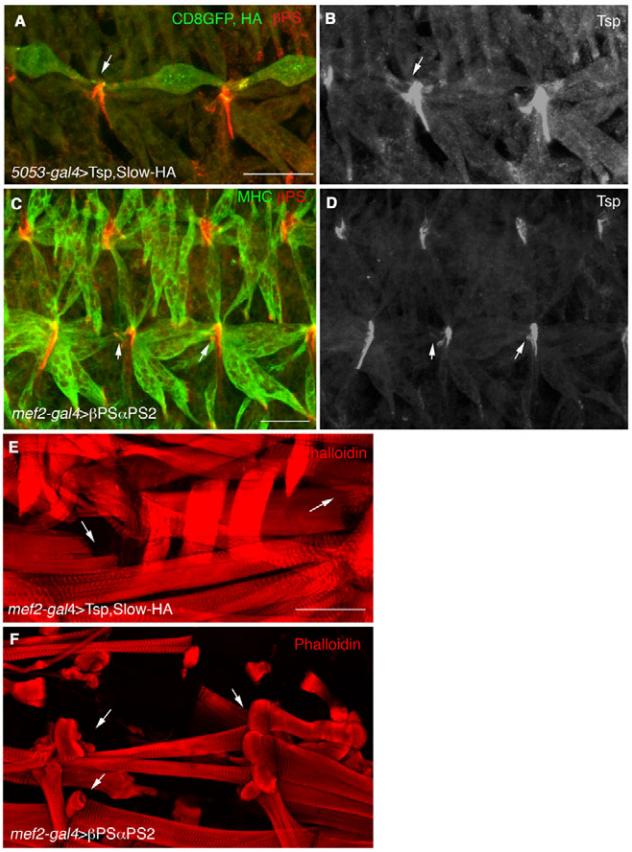


Fig. 10. Early muscle-specific expression of either PS2βPS integrin or Tsp and Slow results in altered muscle end morphology and larval muscle tears. (**A**,**B**) A stage 16 wild-type *Drosophila* embryo overexpressing UAS-CD8::GFP, Slow-HA and Tsp from muscle 12 using the *5053-gal4* driver, and stained 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 blood vessel development in vertebrates, which, in addition to Egfl7, is also characterized by expression of Tsp1 and Tsp2 (Iruela-Arispe et al., 2004), representing a potential general molecular paradigm for Slow/Egfl7 activity.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References
Ashburner, M., Golic, K. G. and Hawley, R. S. (2005). *Drosophila: a Laboratory Handbook*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Bate, M. (1990). The embryonic development of larval muscles in Drosophila. *Development* **110**, 791-804.
Becker, S., Pasca, G., Strumpf, D., Min, L. and Volk, T. (1997). Reciprocal signaling between Drosophila epidermal muscle attachment cells and their corresponding muscles. *Development* **124**, 2615-2622.
Bokel, C. and Brown, N. H. (2002). Integrins in development: moving on, responding to, and sticking to the extracellular matrix. *Dev. Cell* **3**, 311-321.
Brower, D. L. (2003). Platelets with wings: the maturation of Drosophila integrin biology. *Curr. Opin. Cell Biol.* **15**, 607-613.
Brown, N. H. (1994). Null mutations in the alpha PS2 and beta PS integrin subunit genes have distinct phenotypes. *Development* **120**, 1221-1231.
Brown, N. H. (2000). Cell-cell adhesion via the ECM: integrin genetics in fly and worm. *Matrix Biol.* **19**, 191-201.
Brown, N. H., Gregory, S. L., Rickoll, W. L., Fessler, L. I., Prout, M., White, R. A. and Fristrom, J. W. (2002). Talin is essential for integrin function in Drosophila. *Dev. Cell* **3**, 569-579.
Bunch, T. A., Graner, M. W., Fessler, L. I., Fessler, J. H., Schneider, K. D., Kerschen, A., Choy, L. P., Burgess, B. W. and Brower, D. L. (1998). The PS2 integrin ligand tiggrin is required for proper muscle function in Drosophila. *Development* **125**, 1679-1689.
Callebaut, I., Mignotte, V., Souchet, M. and Mornon, J. P. (2003). EMI domains are widespread and reveal the probable orthologs of the Caenorhabditis elegans CED-1 protein. *Biochem. Biophys. Res. Commun.* **300**, 619-623.
Chanana, B., Graf, R., Koledachkina, T., Pflanz, R. and Vorbruggen, G. (2007). AlphaPS2 integrin-mediated muscle attachment in Drosophila requires the ECM protein Thrombospondin. *Mech. Dev.* **124**, 463-475.
De Maziere, A., Parker, L., Van Dijk, S., Ye, W. and Klumperman, J. (2008). Egfl7 knockdown causes defects in the extension and junctional arrangements of endothelial cells during zebrafish vasculogenesis. *Dev. Dyn.* **237**, 580-591.
Doliana, R., Bot, S., Bonaldo, P. and Colombatti, A. (2000). EMI, a novel cysteine-rich domain of EMILINs and other extracellular proteins, interacts with the gC1q domains and participates in multimerization. *FEBS Lett.* **484**, 164-168.
Fish, J. E., Santoro, M. M., Morton, S. U., Yu, S., Yeh, R. F., Wythe, J. D., Ivey, K. N., Bruneau, B. G., Stainier, D. Y. and Srivastava, D. (2008). miR-126 regulates angiogenic signaling and vascular integrity. *Dev. Cell* **15**, 272-284.
Fitch, M. J., Campagnolo, L., Kuhnert, F. and Stuhlmann, H. (2004). Egfl7, a novel epidermal growth factor-domain gene expressed in endothelial cells. *Dev. Dyn.* **230**, 316-324.
Fogerty, F. J., Fessler, L. I., Bunch, T. A., Yaron, Y., Parker, C. G., Nelson, R. E., Brower, D. L., Gullberg, D. and Fessler, J. H. (1994). Tiggrin, a novel Drosophila extracellular matrix protein that functions as a ligand for Drosophila alpha PS2 beta PS integrins. *Development* **120**, 1747-1758.
Frommer, G., Vorbruggen, G., Pasca, G., Jackle, H. and Volk, T. (1996). Epidermal egr-like zinc finger protein of Drosophila participates in myotube guidance. *EMBO J.* **15**, 1642-1649.
Gilsohn, E. and Volk, T. (2010). A screen for tendon-specific genes uncovers new and old components involved in muscle-tendon interaction. *Fly* (in press).
Gregory, S. L. and Brown, N. H. (1998). kakapo, a gene required for adhesion between and within cell layers in Drosophila, encodes a large cytoskeletal linker protein related to plectin and dystrophin. *J. Cell Biol.* **143**, 1271-1282.
Iruela-Arispe, M. L., Luque, A. and Lee, N. (2004). Thrombospondin modules and angiogenesis. *Int. J. Biochem. Cell Biol.* **36**, 1070-1088.
Kuhnert, F., Mancuso, M. R., Hampton, J., Stankunas, K., Asano, T., Chen, C. Z. and Kuo, C. J. (2008). Attribution of vascular phenotypes of the murine Egfl7 locus to the microRNA miR-126. *Development* **135**, 3989-3993.
Kurant, E., Axelrod, S., Leaman, D. and Gaul, U. (2008). Six-microns-under acts upstream of Draper in the glial phagocytosis of apoptotic neurons. *Cell* **133**, 498-509.
Kurucz, E., Markus, R., Zsamboki, J., Folkl-Medzihradszky, K., Darula, Z., Vilmos, P., Udvardy, A., Krausz, I., Lukacsovich, T., Gateff, E. et al. (2007). Nimrod, a putative phagocytosis receptor with EGF repeats in Drosophila plasmatocytes. *Curr. Biol.* **17**, 649-654.
Lee, J. C., VijayRaghavan, K., Celniker, S. E. and Tanouye, M. A. (1995). Identification of a Drosophila muscle development gene with structural homology to mammalian early growth response transcription factors. *Proc. Natl. Acad. Sci. USA* **92**, 10344-10348.
Naimi, B., Harrison, A., Cummins, M., Nongthomba, U., Clark, S., Canal, I., Ferrus, A. and Sparrow, J. C. (2001). A tropomyosin-2 mutation suppresses a troponin I myopathy in Drosophila. *Mol. Biol. Cell* **12**, 1529-1539.
O'Brien, K. P., Remm, M. and Sonnhammer, E. L. (2005). Inparanoid: a comprehensive database of eukaryotic orthologs. *Nucleic Acids Res.* **33**, D476-D480.
Parker, L. H., Schmidt, M., Jin, S. W., Gray, A. M., Beis, D., Pham, T., Frantz, G., Palmieri, S., Hillan, K., Stainier, D. Y. et al. (2004). The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. *Nature* **428**, 754-758.
Parks, A. L., Cook, K. R., Belvin, M., Dompe, N. A., Fawcett, R., Huppert, K., Tan, L. R., Winter, C. G., Bogart, K. P., Deal, J. E. et al. (2004). Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. *Nat. Genet.* **36**, 288-292.
Schmidt, M., Paes, K., De Maziere, A., Smyczek, T., Yang, S., Gray, A., French, D., Kasman, I., Klumperman, J., Rice, D. S. et al. (2007). EGFL7 regulates the collective migration of endothelial cells by restricting their spatial distribution. *Development* **134**, 2913-2923.
Schnorrer, F. and Dickson, B. J. (2004). Muscle building; mechanisms of myotube guidance and attachment site selection. *Dev. Cell* **7**, 9-20.
Schnorrer, F., Kalchhauser, I. and Dickson, B. J. (2007). The transmembrane protein Kon-tiki couples to Dgrip to mediate myotube targeting in Drosophila. *Dev. Cell* **12**, 751-766.
Strumpf, D. and Volk, T. (1998). Kakapo, a novel cytoskeletal-associated protein is essential for the restricted localization of the neuregulin-like factor, vein, at the muscle-tendon junction site. *J. Cell Biol.* **143**, 1259-1270.
Subramanian, A., Prokop, A., Yamamoto, M., Sugimura, K., Uemura, T., Betschinger, J., Knoblich, J. A. and Volk, T. (2003). Shortstop recruits EB1/APC1 and promotes microtubule assembly at the muscle-tendon junction. *Curr. Biol.* **13**, 1086-1095.
Subramanian, A., Wayburn, B., Bunch, T. and Volk, T. (2007). Thrombospondin-mediated adhesion is essential for the formation of the myotendinous junction in Drosophila. *Development* **134**, 1269-1278.
Volk, T. and VijayRaghavan, K. (1994). A central role for epidermal segment border cells in the induction of muscle patterning in the Drosophila embryo. *Development* **120**, 59-70.
Volohonsky, G., Edenfeld, G., Klambt, C. and Volk, T. (2007). Muscle-dependent maturation of tendon cells is induced by post-transcriptional regulation of stripeA. *Development* **134**, 347-356.
Wang, S., Aurora, A. B., Johnson, B. A., Qi, X., McAnally, J., Hill, J. A., Richardson, J. A., Bassel-Duby, R. and Olson, E. N. (2008). The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Dev. Cell* **15**, 261-271.
Wayburn, B. and Volk, T. (2009). LRT, a tendon-specific leucine-rich repeat protein, promotes muscle-tendon targeting through its interaction with Robo. *Development* **136**, 3607-3615.

Table S1. Primers (5′ to 3′) used in this study

Forward	Reverse
Construction of UAS-Slow-HA*	
GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATGCCAGAAATGATTTACTCG	GGGGACCACTTTGTACAAGAAAGCTGGGTCGGGGCAATTGGCATTGGGGCC
Construction of TspAD-GFP†	
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAATTGGACGCGCGTGCTG	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTCCTGCAACTCCACCTTCGTC
Construction of TspNTD-GFP‡	
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAATTGGACGCGCGTGCTG	GGGGACCACTTTGTACAAGAAAGCTGGGTATGAGGGGCACGGTGTGTCCAGGC
Construction of SP-Tsp-CTD-GFP§	
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAATTGGACGCGCGTGCTGTTAATCGGGTTGACCGCCTTGGCGCTGACATTTGTGGATGTTGCATCACTCTCACTCGATCCAGTTGCTTCTGCTGCCCAGTGCCTCCAGGTTGGCTATCCG	GGGGACCACTTTGTACAAGAAAGCTGGGTAGTCCTGCAACTCCACCTTCGTC
Site-directed mutagenesis for construction of SP-TspCTD$^{KGD>LGE}$-GFP¶	
First round: KGD>KGE	
CGGAATGGCAAGGGTGAGTCTTGCGAAGATG	CATCTTCGCAAGACTCACCCTTGCCATTCCG
Second round: KGE>LGE	
GACTTCAATCGGAATGGCTTGGGTGAGTCTTGCGAAG	CTTCGCAAGACTCACCCAAGCCATTCCGATTGAAGTC
PCR verification of the generation of the *slow* mutant**	
Reaction 1	
AATGATTCGCAGTGGAAGGCT	GACGCATGATTATCTTTTACGTGAC
Reaction 2	
GTGCAGAGAGTGCAGAGATGATCC	
RNA probe against *slow*††	
GGGTAACGCCAGGGTTTTCC	ATGACCATGATTACGCCAAGC

*UAS-Slow-HA was produced by PCR on the LD16414 EST (*Drosophila* Genomics Resource Center).
†The TspAD-GFP construct was produced using UAS-TspAD as a PCR template.
‡TspNTD-GFP is GFP-tagged N-terminal part of Tsp (amino acids 1-390).
§SP-Tsp-CTD-GFP is GFP-tagged C-terminal part of Tsp (amino acids 391-1061, including the Tsp signal peptide). The forward primer contains the attB recombination site followed by the Tsp signal peptide sequence and a segment from the beginning of TspCTD.
¶The SP-TspCTD$^{KGD>LGE}$-GFP construct was produced from the non-mutated construct in two rounds of site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene, USA).
**In order to verify the generation of the *slow* mutant, two PCR reactions were performed: reaction 1, using primers representing the resulting hybrid P element; reaction 2, a subsequent PCR using the same reverse primer and a forward primer from genomic sequence.
††PCR reaction was performed on the LD16414 plasmid (*Drosophila* Genomics Resource Center). The PCR fragment was used as a template for a transcription reaction.